Transmembrane Signaling in Chimeras of the *E. coli* Chemotaxis Receptors and Bacterial Class III Adenylyl Cyclases

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Abbreviations

AC	Adenylyl cyclase
CK	Creatine Kinase
CP	Creatine Phosphate
dNTP	deoxy nucleoside triphosphate
GC	Guanylyl cyclase
IPTG	Isopropyl thiogalactoside
MCS	Multiple cloning site
MCPs	Methyl accepting chemotaxis proteins
Ni-NTA	Nickel-nitrilotriacetic acid Agarose
RT	room temperature
TBS	Tris buffered saline
X-Gal	5-bromo 4-chloro3-indolyl β-D- Galactopyranoside
Tsr	Serine chemotaxis receptor
Tar	Aspartate chemotaxis receptor
Trg	Galactose/Ribose chemotaxis receptor
Tap	Dipeptide chemotaxis receptor
Ap	*Arthrospira platensis*
TM	Transmembrane
aa	Amino acids
GBP	Galactose binding protein
MBP	Maltose binding protein
DBP	Dipeptide binding protein
RBP	Ribose binding protein
MalT	DNA binding, transcriptional activator of maltose regulon

SNOC	S-nitrosocysteine
PDEs	Phosphodiesterases
HNOB	Heme-NO binding domain
HNOBA	HNOB associated domain
NCBI	National Center for Biotechnology Information
SMART	Simple Modular Architecture Research Tool

1. INTRODUCTION

1.1 Signal transduction

All living cells must monitor any change in the external environment and adapt accordingly to survive. Unicellular organisms like prokaryotes, archea and some eukaryotes have precise and specific regulatory systems which monitor their living conditions and elicit adaptive responses to the environmental cues (Krell et al., 2010; Parkinson, 1993; Parkinson and Kofoid, 1992; Tamayo et al., 2007). These regulatory systems mediate their responses by interacting with elaborate networks of primary and second messengers. Primary messengers are those which interact with the receptors present at the cell surface and relay its information across the cell membrane by conformational change in these receptors. These changes bring about an intracellular biosynthesis of second messengers which targets molecules within the cells. Often, this process allows the amplification of the original signal carried by the primary messengers into an intracellular signal capable of eliciting massive biochemical changes in the cell. The intracellular second messengers which operate exclusively in prokaryotes are c-di-GMP and guanosine 5′-diphosphate 3′-diphosphate (ppGpp) (Tamayo et al., 2007) while cAMP operates both in prokaryotes as well as eukaryotes.

cAMP is a universal second messenger as it is used by many regulatory systems of prokaryotes as well as eukaryotes. Since the discovery of cAMP (Rall and Sutherland, 1958) intensive research efforts have been focused on understanding the mechanisms of regulation of its intracellular concentration. cAMP is produced by adenylyl cyclases, which utilize ATP as a substrate and require a metal co-factor, usually Mg^{2+} or Mn^{2+} for catalysis (Tesmer et al., 1999). The enzyme phosphodiesterase provides the negative feed back by converting the cAMP to AMP (Butcher and Sutherland, 1962).

1.2 Adenylyl Cyclases

Adenylyl cyclases (ACs) catalyze the formation of cAMP from ATP. They are currently grouped into six classes (Barzu and Danchin, 1994). Class I ACs are involved in catabolite repression in enteric bacteria, e.g. *E. coli.* (Barzu and Danchin, 1994; Cases and de Lorenzo,

1998). Class II ACs are toxins secreted by *Bacillus anthracis* (Leppla, 1982)*, Bordetella purtussis* (Weiss et al., 1984) and *Pseudomonas aeruginosa* (Yahr et al., 1998)*.* Class III ACs are universal enzymes as they are present both in prokaryotes as well as eukaryotes. The classes IV, V, VI are minor classes as only few members are known and have been studied (Cotta MA, 1998; Sismeiro et al., 1998; Tellez-Sosa et al., 2002). Interestingly, class III ACs are often found to be multi-domain proteins (Fig. 1.1, Linder and Schultz, 2003) which opens an opportunity for several three dimensional interactions amongst different domains. Class III ACs are always functional as dimers, it can be a pseudoheterodimer as in the case of mammalian ACs (Tesmer et al., 1997; Tesmer et al., 1999; Whisnant R E, 1996; Zhang et al., 1997) or homodimers as found in bacterial ACs (Linder, 2006; Linder and Schultz, 2003) . They are further subdivided into class III a-d based on different signature motifs present at the dimer interface and the length of an arm region, that is the distance between a conserved glycine and the substrate defining aspartate and threonine/serine residues (Linder and Schultz, 2003).

1.2.1 Mammalian Adenylyl Cyclases

All mammalian ACs belong to class III ACs. There are ten distinct isoforms of mammalian ACs, isoforms I-IX are membranous proteins while the Xth isoform is a soluble protein. In the membrane bound ACs two catalytic domains (C1 and C2), also termed as cyclase homology domain (CHD), are tethered by two large membrane anchors M1 and M2 in a single polypeptide chain (Krupinski et al., 1989; Patel et al., 2001; Sunahara et al., 1996; Tang and Hurley, 1998). In mammalian ACs the two CHDs in a single protein form a pseudoheterodimer with a single ATP binding pocket (Tesmer et al., 1997). Six amino acids have been identified which are important for catalysis. Two aspartate residues coordinate two metal co-factors (Mg^{2+} or Mn^{2+}). They are contributed by the C1-CHD. The four other residues are donated by the C2-CHD. These are a substrate specifying lysine and aspartate pair and a transition state stabilizing arginine and asparagine couple (Sunahara et al., 1998; Tesmer et al., 1999). Each membrane anchor (M1 and M2) in the mammalian ACs has six putative transmembrane (TM) helices which make up more than 40% of the total protein. Presence of such a huge TM anchor might suggest an additional role beyond mere anchoring.

1.2.2 Bacterial Adenylyl Cyclases

Bacterial class III ACs have a single CHD hence all six catalytic residues are present on a single protein chain (Table 1.1). They are typically multi-domain proteins (Fig. 1.1 Linder and Schultz, 2003) and are functional only upon homodimerization, forming two catalytic centres. In contrast to mammalian ACs the mode of regulation of bacterial ACs is not well understood. Most of the N-terminal domains of the bacterial ACs are believed to regulate the cyclases but the mechanism of signal regulation in most of the ACs is enigmatic.

	Cation binding	Substrate binding	Catalysis	
Rv1625c CyaG	D D D D	K D K D	N R N R	**Class IIIa**
Rv1318 Rv1319 Rv1320 Rv3645 CyaB1	D D D D D D D D D D	K T K T K T K T K T	N R N R N R N R N R	**Class IIIb**
Rv1647 Rv1264 Rv2212 Rv0386 Rv0891c Rv1900 Rv2488c	D D D D D D D D D D D D D D	K D K D K D Q N R L N D Q N	N R N R N R N R N R H R D R	**Class IIIc**

Table 1.1: Overview of the six amino acids that form the catalytic centre of the class III ACs. The table shows ACs from M. tuberculosis genome and CyaG and CyaB1 ACs from cyanobacteria. Deviations of the conserved catalytic residues are highlighted in black. ACs are grouped by their subclassifications.
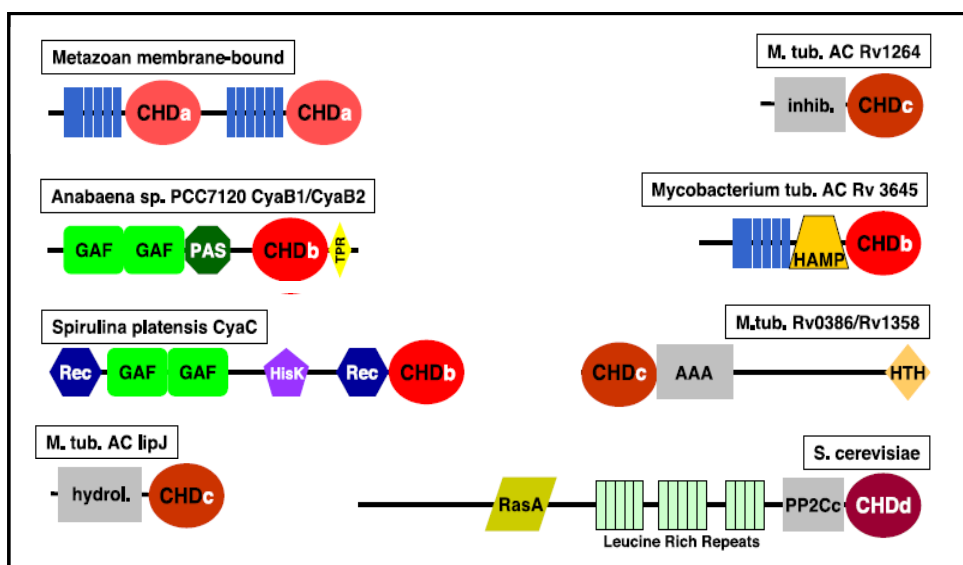
Figure 1.1: Schematic representation of various domain organisations of class III ACs. The black line represents the protein sequence approximately to scale. Domains are coded by shape and colour. CHD are sorted by the proposed subclassification IIIa to IIId. Blue vertical bars represent predicted transmembrane helices; GAF, HAMP, and PAS domains are explained in the text; AAA: ATPase associated with various activities (AAA-ATPase); HisK: histidine kinase domain; HTH: helix turn helix DNA binding domain; hydrol.: α/β hydrolase fold; inhib.: autoinhibitory domain; PP2C: protein phosphatase type 2C domain; RasA: RAS-associating domain; Rec: receiver domain; TPR: tetratricopeptide repeat (adapted from Linder and Schultz, 2003).

1.2.2.1 *Mycobacterium tuberculosis cyclases*

M. tuberculosis encodes 15 class III ACs as revealed from genome sequencing of *M. tuberculosis* H37Rv (Cole et al., 1998; Fleischmann et al., 2002). Out of 15 ACs only 12 are active (other three, Rv2435, Rv1358 and Rv1359 are inactive as they lack an indispensable aspartate residue in its catalytic centre). Among several soluble and membrane bound ACs there are one class IIIa, four class IIIb and seven class IIIc ACs (Table 1.1). Out of these 12 ACs found in *M. tuberculosis* 10 of them have several N-terminal regulatory domains associated with their CHDs. These domains include membrane anchors, a novel auto-inhibitory domain, AAA ATPase domains, helix turn helix DNA binding domains, an α / β hydrolase domain and HAMP domains.

Mycobacterial Rv3645 cyclase

Rv3645, a class IIIb AC, consists of six TM helices which are connected to the CHD via a HAMP domain. A similar domain organisation is found in three other mycobacterial ACs

(Rv1318, Rv1319 and Rv1320). The CHDs of all four ACs are active *in vitro* (Linder et al., 2004)*.* The catalytic activity of Rv3645 is enhanced when it is linked with its N-terminal HAMP region. HAMP-domains, named according to the protein in which these sequence motifs have been identified, **H**istidine *Kinase,* **A**denylyl *cyclase,* **M**ethyl *accepting chemotaxis proteins (MCPs) and* **P**hosphatases (Aravind and Ponting, 1999) are small signal transmitter modules which transmit extracellular signals to cytosolic effectors (Linder, 2006). According to structure predictions, HAMP domains comprise about 50 amino acids and fold into two amphipathic α-helices which are joined by a connector of variable length (Hulko et al., 2006). The sequence of HAMP domains shows a repeating seven residue pattern, whose positions are labelled a-g and in which hydrophobic residues occupy positions a and d (Fig. 1.2). This pattern corresponds to the heptad periodicity postulated by Crick as the hallmark of a coiled-coil structure (Crick, 1953; Mason and Arndt, 2004). Disruption of these predicted hydrophobic epitopes of the HAMP-domain in Rv3645 enhanced substrate affinity demonstrating the ability of HAMP to regulate AC activity (Linder et al., 2004). In ACs, MCPs *(methyl accepting chemotaxis proteins)* and histidine kinases where HAMPs were first identified, the first amphipathic helix of HAMP is believed to start immediately at the exit of the last TM domain, hence it is speculated that conformational changes within the TM spans are directly transmitted to the HAMP domains.

Figure 1.2: Alignment of HAMP domains. Heptad periodicity of amino acid a-g is indicated by highlighting residues a and d (grey), which form the hydrophobic core in the coiled-coil. AS1 and AS2 denote the border of the α-helices of the HAMP domain as deduced by the available NMR structure (Hulko et al., 2006).

Mycobacterial Rv1264 cyclase

Rv1264, a class IIIc AC, is linked to an N-terminal autoinhibitory domain. The N-terminus acts as a pH sensor as it senses an acidic pH which activates the enzyme several fold. In the absence of this N-terminal domain the activity of the CHD is drastically reduced hence this

domain is called an autoinhibitory domain. The crystal structure of Rv1264 in active and inhibited states explains the molecular basis of regulation of this pH sensitive AC (Linder et al., 2002; Tews et al., 2005).

Mycobacterial Rv1625c cyclase

Rv1625c, a class IIIa AC, has six TM spans and a single CHD, i.e. it resembles one half of the pseudoheterodimeric mammalian ACs (Guo et al., 2001). On duplicating Rv1625c by a head to tail concatenation an AC with a domain organisation exactly similar to the mammalian pseudoheterodimers is produced (Guo et al., 2005). Like other bacterial ACs, homodimerization is essential for catalytic activity of Rv1625c. The biochemical properties of the catalytic homodimer of Rv1625c were very similar to those of mammalian ACs. Rv1625c holoenzyme could be functionally expressed in *E. coli* as well as in stably transfected mammalian HEK293 cells confirming that its membrane targeting signal sequence is compatible with the bacterial and mammalian machinery for targeting and membrane insertion (Guo et al., 2001). Active chimeras of Rv1625c having heterologous membrane spans of Rv1625c and mammalian ACs established that the mycobacterial membrane anchors are not only compatible with those of mammalian ACs but also operate equally well in mammalian as well as bacterial cell membranes (Guo et al., 2005). All these biochemical properties of Rv1625c indicate that Rv1625c may constitute a direct progenitor to the mammalian pseudoheterodimeric ACs (Guo et al., 2005; Guo et al., 2001).

1.2.2.2 *Cyanobacterial Adenylyl cyclases*

Whole genomes of 38 cyanobacterial strains have been sequenced so far demonstrating an abundance of ACs and other signaling proteins. cAMP is an important signaling molecule in cyanobacteria (Ohmori and Okamoto, 2004).

Arthrospira platensis cyclase CyaG

Arthrospira platensis encodes 22 ACs as revealed from recent genome sequencing of *Arthrospira platensis* NIES39 (Fujisawa et al., 2010). This number is quite high compared to other cyanobacteria which have no more than 10 ACs. It seems that cAMP signaling systems are highly developed in *A. platensis* (Fujisawa et al., 2010). CyaG AC from Arthrospira, a class IIIa AC, has two TM spans, a HAMP domain, and a CHD. The primary structure of the

CHD of CyaG is more closely related to transmembrane ACs and guanylyl cyclases (GCs) than those of other cyanobacterial ACs. CyaG strictly operate as an AC and could be converted to a GC by replacing three key residues needed for ATP selection to those required for GTP selection (Kasahara et al., 2001).

Anabaena cyclase CyaB1

Anabaena sp. PCC7120 encodes 10 ACs with several signaling domains in a single protein as revealed from its complete genome. The genome encodes approximately 87 GAF domains in 62 putative proteins and 143 PAS domains in 61 proteins (Narikawa et al., 2004). CyaB1 AC, a class IIIb AC, consists of a tandem GAF domain (GAF-a and GAF-b), a PAS domain and CHD (Katayama and Ohmori, 1997). In CyaB1 binding of cAMP to GAF-b increases the Vmax of the AC, thus functioning as an auto-activating cAMP switch (Kanacher et al., 2002). GAF domains and PAS domains are small molecule binding regulatory domains found in many different proteins in nearly all the phyla (Aravind L, 1997; Ponting and Aravind, 1997; Zoraghi et al., 2005). The acronym GAF is derived from mammalian c**G**MP-activated phosphodiesterases, **A**denylyl cyclases and *E.coli* **F**ormate hydrogen lyase transcription activator (FhlA). The PAS domain module was first identified in the Drosophila clock protein **P**ER, the basic helix loop helix containing transcription factor **A**RNT (aryl hydrocarbon receptor nuclear translocator) in mammals and **S**IM (single minded protein) in insects (Nambu et al., 1991; Ponting and Aravind, 1997). PAS domains are involved in detecting changes in redox potential, light, oxygen, and small ligands, in addition to their role in mediating protein-protein interactions (Taylor and Zhulin, 1999; Zhulin et al., 1997).

1.3 *Bacterial Chemotaxis receptors*

Movement of cells in response to chemical attractants and repellents, i.e. chemotaxis is important for bacterial survival. *E. coli* has five chemosensory receptors known as MCPs. They are serine receptor (Tsr), aspartate receptor (Tar), galactose/ribose receptor (Trg), dipeptide receptor (Tap) and aerotaxis receptor (Aer) which elicit an attractant response towards serine, aspartate/maltose, ribose/galactose, dipeptides and oxygen, respectively (Blair, 1995; Falke et al., 1995; Stock and Mowbray, 1995). Salmonella has a slightly different organisation of chemoreceptors with Tap replaced by Tcp, a citrate/phenol sensor (Yamamoto and Imae, 1993). Tsr and Tar are the most extensively studied receptors as they

are most abundant in *E. coli*. They are usually present in several thousand copies per cell while Trg, Tap and Aer are much less abundant with only few hundred copies per cell (Li and Hazelbauer, 2004). Frequently the domain organisations of these TM receptors are similar to those of several bacterial ACs. They have a tripartite domain architecture as often present in membrane bound ACs. It consists of TM spans with an extended periplasmic domain, a signal conversion domain or the HAMP domain and an effector domain which is the chemotaxis domain (Fig. 1.3) (Aravind and Ponting, 1999; Boyd et al., 1983). Homodimerization is an essential feature for the receptors to be functional and the ligand binds to the periplasmic interface between the subunits (Kim et al., 1999; Kim et al., 2002; Milburn et al., 1991). Binding of the ligand initiates a cascade of downstream signaling pathways involving several histidine kinases which finally leads to changes in the swimming behaviour of *E. coli* (Adler, 1965; Ames and Parkinson, 1994; Hazelbauer et al., 2008).
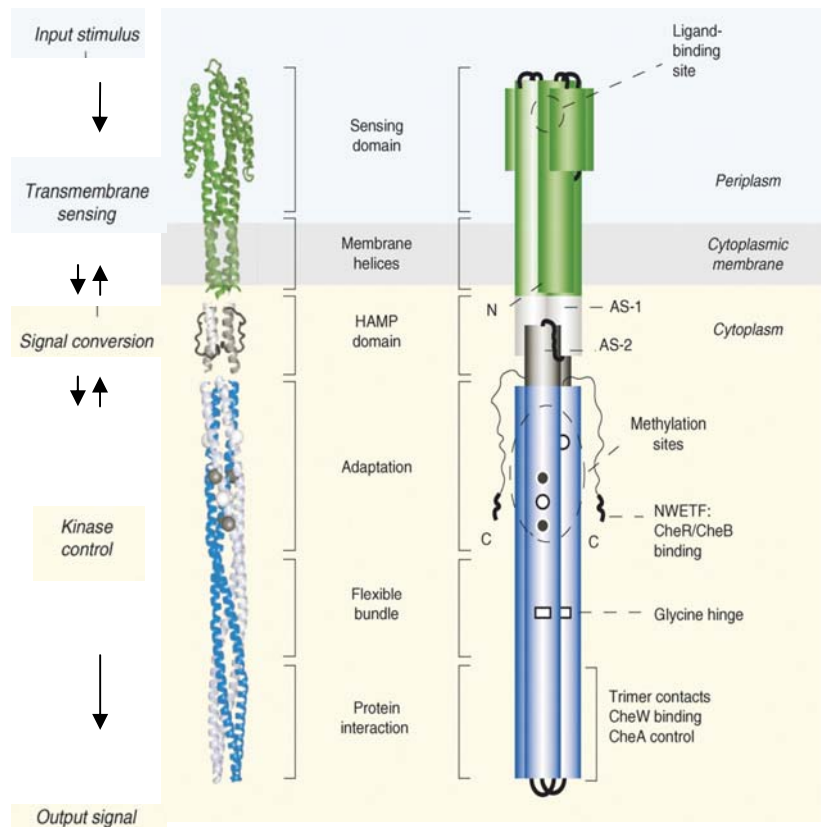
Figure 1.3: The chemoreceptor dimer. A ribbon diagram and a scheme showing the 3D organisation of the chemoreceptor dimer from E. coli. Modules are indicated on the left, roles or identities of the module in the middle and notable features on the right. The model is based on the electron microscopic structure of the receptor (Weis et al., 2003), X-ray structure of the periplasmic domain (Milburn et al., 1991), the X-ray structure of the cytoplasmic domain (Kim et al., 1999) and the model of signal conversion module based on NMR structure of the homologous HAMP domain (Hulko et al., 2006). The figure is adapted from Hazelbauer et al., 2008.

1.4 Aim

The functional significance of the huge membrane domains in many ACs still remains enigmatic and a matter of speculation since the first mammalian AC was cloned (Krupinski et al., 1989). Since the bacterial ACs have to homodimerize to be active hence an active AC has twelve TM helices as seen in mammalian counterparts. Several bacterial membrane bound ACs have six TMs, and a HAMP domain which connects the TM domains to the cyclase domain. This tripartite domain organisation is similar to the bacterial MCPs. Like bacterial ACs homodimerization is an essential feature for MCPs to be functional. Such a high similarity in domain organisation of bacterial ACs and MCPs suggested that the AC membrane anchors may have some sensory function. This idea was reinforced by the fact that the HAMP domains are usually present between the input and output modules of signaling proteins and are known as signal convertors, i.e. HAMP domains might be involved in transmission of extracellular signals to the CHD. To investigate whether these ACs have an ability to sense an extracellular signal, its TMs were replaced by the TMs from bacterial chemotaxis receptors. The aim of the work has been outlined below:

- Construction of active and functional chimeras of *E. coli* chemotaxis receptors and bacterial ACs.
- To explore the mechanism of signal regulation in the receptor/AC chimeras by swapping HAMP domains, transmembrane domains and ligand binding domains of these chimeras.
- To investigate the details of signal transmission through HAMP domains by the help of soluble receptor/AC chimeras.
- To investigate the functional significance of the transmembrane domains and the periplasmic domains of bacterial membrane bound ACs.
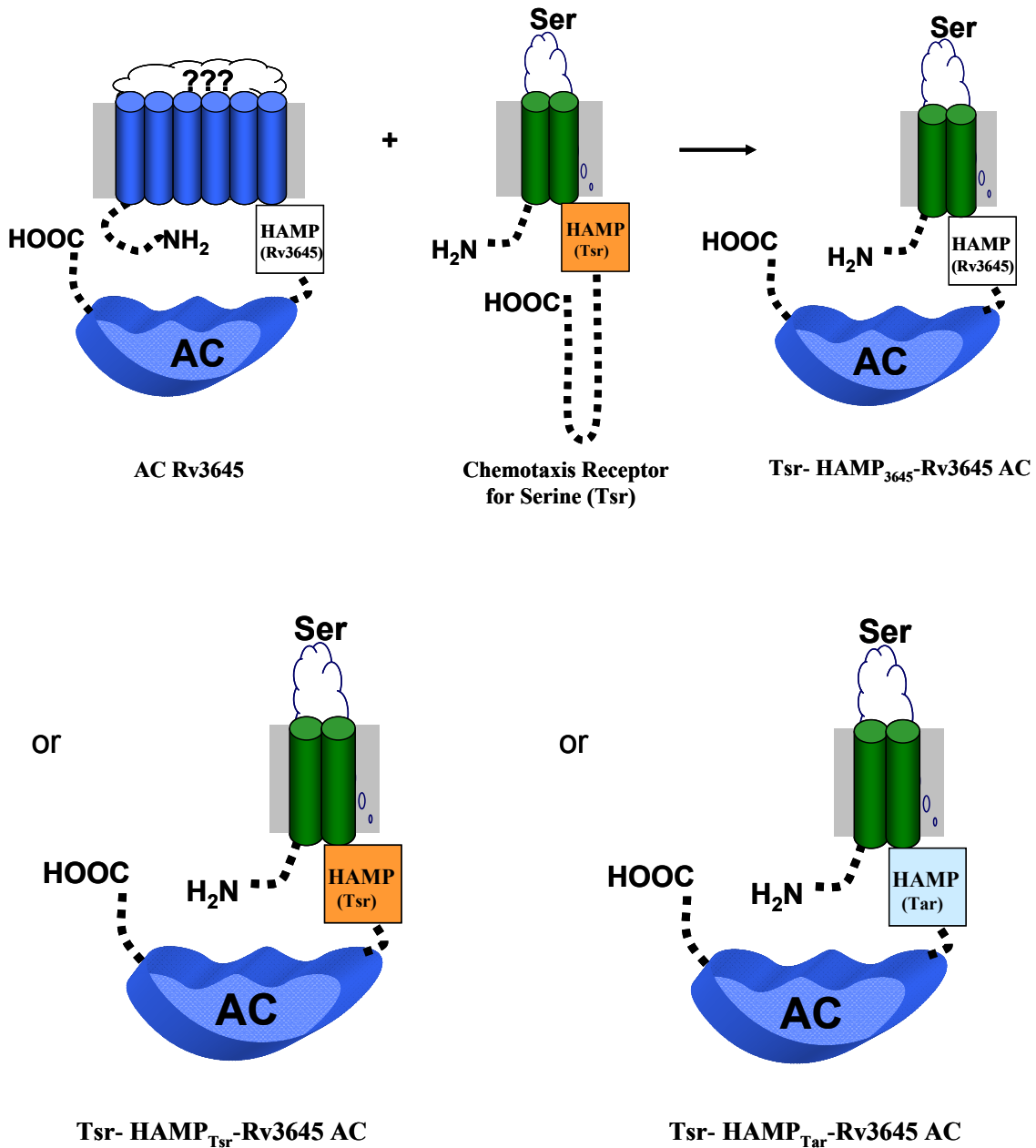
Figure 1.4: General pattern of chimeric ACs used in this study. As examples, the three chimeras examined in section 3.2.1 are depicted. In the figure, the membrane anchor of AC Rv3645 with six predicted α-helices (schematically abbreviated as six blue cylinders at the left) was replaced by the E. coli chemotaxis receptor Tsr, which has two predicted membrane-spanning α-helices (depicted as two green cylinders in the middle). The origin of the HAMP domains is colour-coded. The linkers interconnecting the membrane spanning α-helices have been omitted for clarity. Note that the proteins require dimerization for function.

2. MATERIALS AND METHODS

Only those sources are listed which are deemed important for repetition of the experiments described herein. All the chemicals and instruments were from usual suppliers.

2.1 Chemicals

Amersham Pharmacia Biotech, Freiburg (2,8- ^{3}H)- cAMP, ECL Plus Western Blotting detection system, hyperfilm ECL, Formamide.

Applegene, Heidelberg: Taq DNA-polymerase with 10x reaction buffer

Biomers.net GmbH, Ulm: Oligonucleotides (PCR and sequencing primers)

Dianova, Hamburg: secondary Goat antimouse IgG-F_c horseradish peroxidase conjugated antibodies

Hartmann Analytik, Braunschweig: (α-^{32}P)- ATP

Macherey-Nagel, Düren: Nucleotrap kit, Porablot PDVF- blotting membrane (2µm pore size)

New England Biolabs, Schwalbach/Taunus: BSA for molecular Biology, Restriction endonucleases, T4-Polynucleotide kinase and 10x kinase buffer

Novagen R&D systems, Wiesbaden: PET16b-Expression vector, *E. coli* BL-21 (DE3) [pREP4] cells

Promega, Madison (USA): Pfu DNA-polymerase enzyme with 10 x buffer, Wizard plus SV Plasmid Purification Kit (Minipreps)

PEQ LAB, Erlangen: KAPA HiFi proofreading DNA Polymerase

Qiagene, Hilden: Ni-NTA Agarose, pQE30, PQE60 and PQE80 expression vectors, purified mouse monoclonal RGS-His$_4$ antibody and Tetra-His antibody, Taq-DNA- polymerase

Roche (Boehringer), Mannheim: alkaline phosphatase, ATP, dNTPs, λ-DNA, Restriction endonucleases, Klenow-polymerase, Rapid DNA ligation Kit.

Schleicher& Schuell, Dassel: Whatmann paper 3MM, Protran BA 83 cellulose nitrate 0.2μm (200 x 200 mm) nucleic acid and protein transfer media

Stratagene, Heidelberg: Pbluescript II SK (-), E.coli XL 1- Blue cells

Sud-Laborbedarf GmbH: HiYieldR PCR Cean-Up / Gel Extraction Kit

Vivascience, Hannover: Vivaspin 500 μL and 2 mL (for protein concentration)

2.2   Equipments

Kontron-Hermle, Gosheim: Centrikon *H401 & ZK401*, Rotors *A6.14 (SS34) and A8.24* (GSA)

SLM, instruments, Urbana (USA): French Pressure Cell Press *FA-078-E1*, French Press cylinder, nylon balls and rubber rings

2.3   Buffers and Solutions:

2.3.1 Molecular Biology buffers and solutions

All solutions and buffers for molecular biology methods were either sterile-filtered (0.22 μm) or autoclaved for 20 min at 120°C (1bar). Milli Q water was used and pH was adjusted at RT, unless indicated otherwise.

Solutions for DNA treatment:

TAE	TE buffer
40 mM Tris/ Acetate pH 8.0	10 mM Tris/ HCl pH 7.5
1 mM EDTA	1 mM Na_2EDTA

10x TBE buffer

1000 mM Tris
890 mM Boric acid
25 mM Na$_2$EDTA

dNTP's

25 mM of each dNTP

4x Loading sample buffer (BX)

0.05 % Bromophenol blue
0.05 % Xylenecyanol
50 % Glycerol

Solutions for Bacterial culture media

LB-Agar

35 g/L LB Agar

LB-antibiotic-Agar plates

100 µg Ampicillin/ml LB Agar and/or
50 µg Kanamycin/mL LB Agar

LB-broth

20 g/L LB broth powder

2.3.2 Protein chemistry buffers and solutions

Protein purification with Ni-NTA agarose

Pellet washing buffer

50 mM Tris/ HCl pH 8.0
1 mM EDTA

Cell lysis buffer

50 mM Tris/HCl pH 8
2 mM α-thioglycerol
50 mM NaCl

Washing buffer A

50 mM Tris/HCl pH 8
250 mM NaCl
5 mM MgCl$_2$
15 mM imidazole, pH 8
10% Glycerol
0.16% α-Thioglycerol

Washing buffer B

50 mM Tris/HCl pH 8
0.16% α-Thioglycerol
5 mM MgCl$_2$
15 mM imidazole, pH 8
10% Glycerol

Elution buffer

50 mM Tris/HCl pH 8
5 mM MgCl$_2$
250 mM Imidazole
10% glycerol

Membrane Resuspension buffer

40 mM Tris/HCl buffer pH 8
1.6 mM α-Thioglycerol

Materials and Methods

Membrane solubilization buffer

50 mM Tris/HCl pH 7.5
1.6 mM α-Thioglycerol
10% glycerol
0.8% Dodecyl-β-D- maltoside (DDM)

Resolving Gel buffer

1.5 M Tris/HClpH8.8
100 mM NaCl
0.4% SDS

Stacking Gel Buffer

500 mM Tris/HCl pH 6.8
0.4% SDS

10x electrophoresis buffer

250 mM Tris
1.92 M Glycine
1% SDS

Coomassie staining solution

0.2% Brilliant Blue G-250
40% Methanol
10% Acetic acid

4x sample buffer

130 mM Tris/HCl pH 6.8
10% SDS
20% Glycerol
0.06% Bromophenol blue
10% β-mercaptoethanol

Destaining solution

10% Acetic acid
30% Methanol

Western Blot

TBS buffer (Tris Buffer Saline)

20 mM Tris/HCl pH 7.5
150 mM NaCl

Ponceau S staining solution

0.1% (w/v) Ponceau S
5% Acetic acid

M-TBS

5% Milk powder in TBS buffer

TBS-T

0.1% Tween 20 in TBS buffer

Towbin- Blot buffer

25 mM Tris/HCl
192 mM Glycine
20% Methanol

Cyclase Enzyme test

ATP Stock Solution

10 mM pH 7.5 (adjusted with NaOH)

10x AC Start solution
0.75 to10 mM ATP with
2.5-4x10^6 Bq/ml
[α^{32}P] ATP

AC Stop buffer
3 mM cAMP/Tris pH 7.5
3 mM ATP, 1.5% SDS

2x AC-Cocktail
100 mM Tris/HCl pH 7.5
6 mM MnCl$_2$
43.5% Glycerol
4 mM cAMP with 2-4x10^3 Bq/ml
6 mM creatine phosphate (CP)
0.46 mg/2 ml creatine kinase (CK)
(CP and CK only for membrane protein)

2.4   Bacterial strains

Different strains of bacterial competent cells have been used.

Strain	Supplier	Genotype	characteristics
E. coli XL1 Blue	Stratagene (Heidelberg)	*recA1 endA1 gyrA96 thi -1 hsdR17 supE44 relA1 lac [F´ proAB lacIqZΔM15 Tn10 (Tetr)]*	Cloning cells (tetracycline resistants)
E. coli DH5α		*F–, Φ 80dlacZΔM15, Δ (lacZYA-argF)U169, deoR, recA1, endA1, hsdR17(rK–, mK+), phoA, supE44, λ –, thi-1, gyrA96, relA1*	Cloning cells
E.coli BL21 (DE3) [pREP4]	Novagene (Wiesbade)	*F-, ompT, hsdSβ(rβ-mβ-), dcm, gal, (DE3) tonA*	Expression cells

2.5   Plasmids

The plasmids that have been used in the cloning of different constructs, their Supplier and characteristics are mentioned below

Plasmid	supplier	Characteristics
pBluescript II SK(-)	Stratagene (Heidelber)	Cloning vector, *lacZa*, ColE1-origin, f1(-)-origin, Amp^R
pQE30	Qiagene (Hilden)	Expression vector, N-terminal His6-tag
pQE60	Qiagene (Hilden)	Expression vector, C-terminal His6-tag
pQE80L	Qiagene (Hilden)	Expression vector, N-terminal His6-tag
pET-pQE30 MCS	Novagene (Wiesbade)	Expression vector, N-terminal His6-tag

2.6   Oligonucleotides

Restriction sites are in **bold**

s = sense primer   as = antisense primer

Sr. No.	Name	Sequence (5' 3')	Comment
1	T7 s	TAA TAC GAC TCA CTA TAG GG	p-Bluescript II SK (-)
2	T3 as	AAT TAA CCC TCA CTA AAG GG	p-Bluescript II SK(-)
3	U-PQE s	GAA TTC ATT AAA GAG GAG AAA	Universal for PQE30
4	R-PQE as	CAT TAC TGG ATC TAT CAA CAG G	Reverse for PQE30
5	Switch oligo XmnI s	GCT CAT CAT TGG AAA ACG TTC TTC GGG	
6	pQE Xho1 s	CGT CTT CAC CTC GAG AAA TCA TAA	

2.6.1 Cloning Primers

Tsr/Rv3645 chimeras

7	Tsr full s (BamHI)	AAA **GGA TCC** ATG TTA AAA CGT ATC AAA ATT G	From U.Kurz
8	Tsr Hamp as (Bgl II)	AAA **AGA TCT** CGC AAA CCG ACG GTA CGC ATC AGC TC	From L.Garcia
9	Tsr-as-fus 3645H(MfeI)	CGG **CAA TTG** ACA TTT TAA TAC CGA ACC AGA CGG	From U.Kurz
10	3645Hamp-s Fus-Tsr(MfeI)	TGT **CAA TTG** CCG ACC CGT TAC GCC AGT TG	From U.Kurz
11	R3645Hamp BglII as	GCG ACC GAA **CAG ATC T**CG CAA CCG	
12	Tsr Tar SphI as	CCA **GCA TGC** GTT TAT ACC GAA CCA GAC GGC GA A	From U.Kurz
13	Tar-SphI-s	AAA C**GC ATG C**TG CTT ACT CCG CTG GCA	From U.Kurz
14	Tsr(R69E) (BamHI) s	GCG TAA CAC CCT CAA CGA GGC GG**G GAT CC**G CTA CAT GAT G	Fusion primer
15	Tsr(R69E) (BamHI) as	CAT CAT GTA GC**G GAT CC**C CGC CTC GTT GAG GGT GTT ACG C	Fusion primer
16	Tsr(T156K) (Bpu10I) s	CGA GTT CTT TGA TCA **GCC TAA GC**A GGG ATA TCA GGA CGG	Fusion primer
17	Tsr(T156K) (Bpu10I) as	CCG TCC TGA TAT CCC TGC TTA GGC TGA TCA AAG AAC TCG	Fusion primer
18	Rv3645 SnaB1 as	AAA **TAC GTA** GCC GCG TACT TC GCT GGA	
19	Tsr Nco1 s	AAA **CCA TGG** ATG TTA AAA CGT ATC AAA ATT GTG ACC AGC	
20	Tsr-fus-Rv3645 as	ATT AAA GAA CAG GGC CAG CAC GAT CGC	Fusion primer
21	Rv3645-fus-Tsr s	ATC GTG CTG GCC CTG TTC TTT AAT GCC TTA AAG	Fusion primer
22	Rv3645-f-Tsr-as	CAT CAT CAG GAT GAT CCA CAT CGC CTG GC	Fusion primer

23	Tsr-fus-Rv3645-s	GCG ATG TGG ATC ATC CTG ATG ATG GCA CTG	Fusion primer
24	Rv3645-sty1-as	**GCC TTG G**AA CTT GTT GAC	
25	Rv3645BglIIs	AAA **AGA TCT** ATG GAT GCC GAG GCG TTC	
26	3645-Tsr AflII s	ATC GTG CTG GCC CTG TTC TTT AAT GCC **CTT AAG** AAT GAC AAA GAA	Fusion primer
27	Tsr-f-3645 AflIIas	ATT **CTT AAG** GGC ATT AAA GAA CAG GGC CAG CAC GAT CGC	Fusion primer
	Tar/Rv3645 chimeras		
28	Tar full (BamHI)s	AAA **GGA TCC** ATG ATT AAC CGT ATC CGC GTA	From U.Kurz
29	Tar HAMP as (BglII)	AAA **AGA TCT** CGC AAA GTG ACG GTG TCA GTC AAA GA	From U.Kurz
30	TsrHAMP s fusTar(NheI)	AAA **GCT AGC** CTG GTA GCG CCA ATG AAT CGC	From U.Kurz
31	Tar as fusTsr HMP(NheI)	AAA **GCT AGC** GCG AAT GCC GTA CCA CGC CAC	From U.Kurz
32	Tar Rv3645H as	GGC GAT CGA CAT GCG AAT GCC GTA CCA	Fusion primer
33	Rv3645 Hamp s	TAC GGC ATT CGC ATG TCG ATC GCC GAC	Fusion primer
	Tsr/CyaG chimeras		
34	Tsr Hamp XhoI as	AAA **CTC GAG** ACC GAC GGT ACG CAT CAG	
35	Xho sp Link AC s	AAA GGT ACC **CTC GAG** AAG ACT AAC CGC GAA TTA G	
36	Hind AC sp as	AAA **AAG CTT** TTA GGA AAT ATC TAC TAC TGG	
37	Tsr Afe1 as	AAA **AGC GCT** TAA TAC CGA ACC AGA C	
38	Afe1 sp Hamp s	AAA **AAG CGC T**GG ATT AGT GAA CCG ATT	

Tar/CyaG chimeras

39	Tar F1 Sp ACH as	TTC ACT AAT CCA GCG GCG AAT GCC GTA CCA CGC	Fusion primer
40	Sp ACH F1 Tar s	TGG TAC GGC ATT CGC CGC TGG ATT AGT GAA CCG	Fusion primer
41	Tar HAMP Xho as	AAA **CTC GAG** AGT GAC GGT GTC AGT CAA	
42	Tar(R69E)fwr (SacII)	CGC GCA TTA ACC TGA GTG AAT **CCG CGG** TAC GGA TGA TGA TGG	Fusion primer
43	Tar(R69E) (SacII) as	CCA TCA TCA TCC GTA **CCG CGG** ATT CAC TCA GGT TAA TGC GCG	Fusion primer
44	Tar(T154I) StyI s	CGC TCA GCC AAT CAA AGG AAT GCA AAA TGC AAT GGG C	Fusion primer
45	Tar (T154I) (StyI) as	GCC CAT TGC ATT TTG CAT T**CC TTG G**AT TGG CTG AGC G	Fusion primer
46	ACsp SnaB1 as	AAA **TAC GTA** GGA AAT ATC TAC TAC TGG	
47	Tar NdeI s	AAA **CAT ATG** ATG ATT AACCGTATCCGC G	
48	MBP BamH1s	AAA **GGA TCC** ATG AAA ATA AAA ACA GGT GC	
49	MBP HindIII as	AAA **AAG CTT** TTA CTT GGT GAT ACG AGT	
50	MBP seq	GGT CTG ACC TTC CTG GTT	Sequencing primer

Trg/CyaG chimeras

51	Trg HAMP XhoI as	AAA **CTC GAG** CCC TAC TGT CAT CCC CAG	
52	spACH_TRG s	TTT ATG GTG CTA CGT CGC TGG ATT AGT GAA CCG	Fusion primer
53	TRG_sp ACH as	TTC ACT AAT CCA GCG ACG TAG CAC CAT AAA TGT	Fusion primer
54	GBP BamH1 s	AAA **GGA TCC** ATG AAT AAG AAG GTG TTA	
55	GBP HindIII as	AAA **AAG CTT** TTA TTT CTT GCT GAA TTC AGC	

19

56	DBP seq	CAG CAG TAT CAA AAA GAT TCC	Seq. primer
Tap/CyaG chimeras			
57	Tap HAMP Xho1rvs	AAA **CTC GAG** ACT TAC CGT CCC ACG CAA	
58	sp ACH_TAP Fwr	CTG TGG TGG ACG CGC CGC TGG ATT AGT GAA CCG	Fusion primer
59	TAP_sp ACH as	TTC ACT AAT CCA GCG GCG CGT CCA CCA CAG CGC	Fusion primer
60	DBP Sph1 s	AAA **GCA TGC** ATG CGT ATT TCC TTG AAA	
61	DBP Sal 1Rv	AAA **GTC GAC** TTA TTC GAT AGA GAC GTT	
62	GBP seq	TCA GGC GAA AGA TAA GAT GGA	Sequencing primer
CyaG holoenzyme			
63	Sp ACH s	ATT CTC ACT GCT CGC TGG ATT AGT GAA CCG	Fusion primer
64	Sp memb as	ACT AAT CCA GCG AGC AGT GAG AAT ACC CAA	Fusion primer
65	Sp memb s	AAA GGA TCC ATG CGC TTA TTT ATT ATA CCC	
Tsr/Rv1264 chimeras			
66	Rv1264 Sac I s	AAA **GAG CTC** GAC AGG TCA CCG	
67	TSRH Sac I as	AAA **GAG CTC** CAC CGC CCA ACT CGG TGC CG	
68	Rv1264_A_ TSR s	ACC GAG TTG GGC GGT GCG GGA GCG CGA CAG GTC ACC	Fusion primer
69	TSR_A_ Rv 1264 as	GAC CTG TCG CGC TCC CGC ACC GCC CAA CTC GGT GCC	Fusion primer
70	TSR_AA_ Rv 1264 as	GAC CTG TCG CGC TCC CGC CGC ACC GCC CAA CTC GGT GCC	
71	Rv1264_AA_ Tsr s	ACC GAG TTG GGC GGT GCG GCG GGA GCG CGA CAG GTC ACC	Fusion primer

20

Tsr/CyaB1 chimeras

| 72 | CYAB1L Bgl II s | AAA **AGA TCT** GAT GTA TCG CTA CCT TAC ACC C | |
| 73 | CyaB1 kat as KpnI | AAA **GGT ACC** CTA CTT TGT GAA AAT TGT | Form I. Mansi |

Tsr and Tar/Rv1625c chimeras

74	Rv1625c AatII s	AAA **GAC GTC** TCG GTG CTG TTC GCC GAC ATC G	
75	Tsr F2 Rv1625 as	GCG CGC AGT ATC ACG CTT AAT ACC GAA CCA	Fusion primer
76	Rv1625F2Tsr Fwd	TTC GGT ATT AAG CGT GAT ACT GCG CGC GCG	Fusion primer
77	Tar F1Rv 1625 as	GCG CGC AGT ATC ACG GCG AAT GCC GTA CCA CGC	Fusion primer
78	Rv1625F1 Tar s	TGG TAC GGC ATT CGC CGT GAT ACT GCG CGC GCG	Fusion primer
79	Rv1625 (R-A) as	CCG **CTC GAG** CAG TAT CAG CCT TAA TAC C	XhoI
80	Rv1625 (R-AA) as	CCG **CTC GAG** CAG TAT CAG CAG CCT TAA TAC C	XhoI
81	Rv1625 R(-ve) as	CCG **CTC GAG** CAG TAT CCT TAA TAC C	XhoI
82	Tsr_1625 s	TGG TTC GGT ATT GAA CAC ACC GCG TTA	Fusion p.
83	Tsr_1625 as	CGC GGT GTG TTC AAT ACC GAA CCA GAC	Fusion p.
84	Rv1625SnaB1 as	AAA **TAC GTA** GAC CCC TGC CGT GCG GGG	
85	Rv1625-BamH1-s	AAA **GGA TCC** ATG GCG GCA AGA AAA TGC	
86	Tsr-fus1625-as	ATT AAA GAA CAG CAG GAA CTC CAG GGC	Fusion primer
87	Rv1625-fus-Tsr-s	CTG GAG TTC CTG CTG TTC TTT AAT GCC TTA AAG	Fusion primer
88	Rv1625-f-Tsr-as	GAC GGT CGT GAG CAG AAT CCA CAT CGC CCT	Fusion primer

89	Tsr-fus-Rv1625-s	ATG TGG ATT CTG CTC ACG ACC GTC TCC G	Fusion primer
90	Rv1625 Mfe1 as	GGA TCT TT**C AAT TG**C GCT	
91	Ach-f-1625 Stu1 as	CCG AGG CCT CTT CAA TAG C	Fusion primer
92	Tsrh-1625L Xho as	CCG **CTC GAG** CAG TAT CAC GCA GAC CGA CGG TAC GCA TCA G	
93	Ach-1625L Xho1 as	CCG **CTC GAG** CAG TAT CAC GCA GAA TAA ATG AAT CCC GCA AAT TCT G	
94	Tsr-Rv1625 Xho1 as	CCG **CTC GAG** CAG TAT CAC GCA GTT TAA TAC CGA ACC AGA C	

Tsr/Rv3645 mutants (transmembrane deletion chimeras)

95	Tsr SpeI s	GTG **ACT AGT** TTC TTT AAT GCT TTA AAG AAT GAC	
96	TsrohneTm StuI as2	AAA **AGG CCT** TAA TAC CGA ACC AGA CGG CAA TCC ACA TCG CCT GGC TATAGCT	
97	TsrohneTm StuI as3	AAA **AGG CCT** TAA TAC CGA ACC AGA CGG CGA AAA TCC ACA TCG CCT GGC TAT AGC T	
98	Tsr-V-StuI as	AAA **AGG CCT** TAA TAC CGA ACC AGA CCG CCT GGC TAT AGC T	
99	Tsr-AV-StuI as	AAA **AGG CCT** TAA TAC CGA ACC AGA CGG CCG CCT GGC TAT AGC T	
100	Tsr-FAV-StuI as	AAA **AGG CCT** TAA TAC CGA ACC AGA CGG CGA ACG CCT GGC TAT AGC T	
101	TsrohneTm StuI as1	AAA **AGG CCT** TAA TAC CGA ACC AGA CAA TCC ACA TCG CCT GGC TAT AGC T	
102	Tsr_Stu1_I as	GCG **AGG CCT** TAA TAC CGA ACC AGA CGG CGA AGA TAA TCC ACA TCG CCT G	
103	Tsr_Stu1_V as	GCG **AGG CCT** TAA TAC CGA ACC AGA CGG CGA AGA TGA CAA TCC ACA TCG CCT G	
104	Tsr_Stu1_A as	GCG **AGG CCT** TAA TAC CGA ACC AGA CGG CGA AGA TGA CCG CAA TCC ACA TCG CCT G	
105	Tsr_Stu1_V as	GCG **AGG CCT** TAA TAC CGA ACC AGA CGG CGA AGA TGA CCG CCA GTA CAA TCC ACA TCG CCT G	
106	Tsr_Stu1_VV as	GCG **AGG CCT** TAA TAC CGA ACC AGA CGG CGA AGA TGA CCG CCA GTA CGA CAA TCC ACA TCG CCT G	
107	TsrN'_VG_Stu1as	GCG **AGG CCT** TAA TAC CGA ACC AGA CGG CGA AGC CCA CCA GAA TCC ACA TCG CCT G	

108	TsrN'_VGV_Stu1as	GCG **AGG CCT** TAA TAC CGA ACC AGA CGG CGA ACA CGC CCA CCA GAA TCC ACA TCG CCT G	
109	TsrN'_M_Stu1as	GCG **AGG CCT** TAA TAC CGA ACC AGA CGG CGA ACA TCA CGC CCA CCA GAA TCC ACA TCG CCT G	
110	TsrN'_I_Stu1as	GCG **AGG CCT** TAA TAC CGA ACC AGA CGG CGA AGA TCA TCA CGC CCA CCA GAA TCC ACA TCG CCT G	
111	TsrN'_V_Stu1as	GCG **AGG CCT** TAA TAC CGA ACC AGA CGG CGA ACA GGA TCA TCA CGC CCA CCA GAA TCC ACA TCG CCTG	
112	TsrN'_VV_Stu1 as	GCG **AGG CCT** TAA TAC CGA ACC AGA CGG CGA ATA CCA GGA TCA TCA CGC CCA CCA GAA TCC ACA TCG CCT G	
113	Tsr-Af1503Tm1s	AAA **GGA TCC** ATG TTA AAA CGT ATC AAA GTG GTT GTT GCA AGC CTT GTG CCC CTG AGC GTT CTT GGT TAT CTT ACC ATA GCT GGG ATG ACA TCC TCT GCT GAA GAG GCG AAG AAT GAC AAA GAA AAT	BamHI
114	Tsr-Af1503 Tm2 as	GCG **AGG CCT** TTG TGA ACA CCG CCA GAA CGA TGA CGA AAA CTA TAG CAA TTG CCG CTG CAA TAC CCA AAG CGT AGT AAA CCT GGC TGT AGG AGG CAT T	StuI
115	Af1503TM1 fusTsr s	AAA **GGA TCC** ATG AAG TTG ACG CCC CAA ATT GTT TTG ATA GTG GTT GTT GCA AGC CTT GTG CCC CTG AGC GTT CTT GGT TAT CTT ACC ATA GCT GGG ATG ACA TCC TCT AAG AAT GAC AAA	BamHI
116	Tsr-TarTm2 as	GCG **AGG CCT** TAA TGC CGT ACC ACG CCA CCA GCA GAA TCA ATA CCA CCA CCA GCG CGA TAA CCG CCA GTT GCC ACT GGG CAA AGC TGT AGG AGG CAT TGTT	StuI
117	Tsr-Tar Tm1s	AAA **GGA TCC** ATG TTA AAA CGT ATC AAA GTA GTC ACG CTG TTG GTA ATG GTG CTG GGG GTA TTC GCA CTG TTA CAG CTT ATT TCC GGC AGT CTG TTT TTT TCT TCC TTA AAG AAT GAC AAA GAA	BamHI
118	Tsr mut s	ATT GTG **ACT AGT** TTA ATG CAG GGT TTG GCC CTT TAT GGC CCT TTA CAA CTG	SpeI
119	Tsr mut as	CAG CGA **GGC CT**T AAT ACT CAA CCA GAC GGC GGA GTT GAC CGC CTG TAC GAC GAT CAT CGC GCC CAC CTG ACT CCA CAT CGA CTG GCT ATA G	StuI
120	Tsr LL-RR s	ATT GTG **ACT AGT** TTA CGG CGG GTT TTG GC	SpeI
121	Tsr LL-RR as	GCG **AGG CCT** TAA TAC CGA	StuI
122	Tsr_StuI_L as	GCG**AGGCCT**TAATA CGAACCAG CGG CGA AGATGACCGCCAGAATCCACATCGCC G	StuI

2.7 Molecular biology methods

2.7.1 Polymerase chain reaction

The polymerase chain reaction used specific primers to amplify DNA fragments from plasmid or genomic DNA and for the introduction of endonuclease restriction sites. The annealing temperature was calculated with the formula:

$$T_a = 2 \ x \ (A + T) \ + \ 4 \ x \ (G + C)$$

If the annealing temperatures of the primers were different, the lower one was used. The reaction was run in 50 µl with a thermocycler with heatable lids to avoid volume and concentration changes through evaporation and condensation. The samples contained maximally 1 ng plasmid, 0.2 mM dNTP, 0.5 µM of each primer, 1 U Taq-DNA-Polymerase and 1 x of corresponding reaction buffer. The temperature was:

Denaturation		95 °C	5 min
25-30 cycles	denaturation	95 °C	1 min
	Primer annealing	T_a	1 min
	Extension	72 °C	1 min/ kb
Fill up		72 °C	5 min
Hold		4 °C	∞

For fusion PCR, Kappa DNA Polymerase was used. The reaction volume of 50 µl contained 10 ng of plasmid DNA, 0.3 mM dNTP mix, 0.3 µM of each primer, 0.02 U/µl Kappa Polymerase and 1 x reaction buffer. The temperature was:

Denaturation		95 °C	2 min
25-30 cycles	denaturation	98 °C	20 sec
	Primer annealing	T_a	15sec
	Extension	68 °C	30sec/kb
Fill up		68 °C	5 min
Hold		4 °C	∞

2.7.2 Agarose electrophoresis

Agarose electrophoresis was used to determine the yield, purity and size of DNA and to check digestion by a restriction enzyme. Agarose was dissolved in TAE-buffer and melted in a microwave oven. The solution was poured into a mould in which a well-forming comb was fitted, the agarose concentration was chosen according to the expected size (bp) of the DNA fragments:

≥ 2000 bp	0.8 - 1 %
500 - 2000 bp	1 - 1.8
≤ 500 bp	2 %

TAE buffer was used for electrophoresis, agarose gels were placed in a horizontal electrophoresis apparatus, the DNA samples were mixed with loading sample buffer, and electrophoresis was carried out at 80-100 V for 30-45 min at room temperature. The size markers EcoRI/Hind III-digested λ- DNA (λ Marker: 21226, 5184, 4973, 4277, 3530, 2027, 1904, 1584, 1330, 983, 831, 564, 125 bp) and MspI/SspI digested pBluescript II SK (-) Vector (π Marker: 489, 404, 312, 270, 242/241, 215, 190, 157, 147, 110, 67, 57, 34, 26 bp) were co-electrophoresed with DNA samples. For detection of the DNA, gels were emerged in an ethidium bromide bath (0.01 mg/ml) for 2 min., the gel was run for another 10 min and DNA fragments were visualized on a UV-light box (302 nm) and photographed.

2.7.3 Photometric determination of DNA concentration

Nucleic acid content was measured at 260 nm. An OD of 1 corresponds to 50 µg/ml for double stranded DNA. The ratio OD260/OD280 was calculated to estimate purity. An OD ratio of > 1.8 was desirable.

2.7.4 Enzymatic digestion

Digestion of DNA by restriction enzymes was carried out by incubating the double-stranded DNA molecules with the appropriate amount of restriction enzyme at the optimal temperature required and in the corresponding buffer as recommended by the supplier. In the case of simultaneous digestion with 2 or more enzymes, the most compatible buffer was used.

2.7.5 Generation of blunt ends

After PCR, the Klenow fragment of the DNA polymerase I was used. For blunting, maximally 500 ng DNA, 1 µl of 10X Klenow buffer and 0.8 µl of Klenow polymerase (1U/µl) in a 10 µl reaction volume were mixed (10 min, 37°C). Then, 1µl of dNTP (20 mM) was added and the incubation continued for 30 min. The sample was heated to 70°C for 10 min for inactivation.

2.7.6 5'-phosphorylation of PCR products

To ligate blunt ended-PCR products into the EcoRV site of pBluescript, the DNA should be phosphorylated. The Klenow treated DNA solution, 1 mM ATP, 10U T4 polynucleotide kinase and 1X T4-PNK buffer in 15 µl were incubated at 37°C for 1 hr.

2.7.7 Dephosphorylation of plasmid vectors

To avoid re-ligation of the vector without insert, the 5'-phosphate was removed using Alkaline phosphatase, 500 ng DNA, 1U/pmol of enzyme and 1X dephosphorylation buffer (10 µl; 37 °C, 1 hour).

2.7.8 Ligation of the DNA fragment

DNA fragments were ligated using the Rapid Ligation Kit according to the instructions of the manufacturer. The molar ratio of vector to insert should be 1:3 in case of one insert and 1:1:1 in case of two inserts.

2.7.9 Transformation of recombinant DNA

The entire DNA ligation reaction (21µl from a rapid ligation kit protocol) was added to competent cells (100µl), mixed gently and incubated on ice for 10 min, cells were then heat-shocked at 42°C for exactly 1 min. and incubated on ice for 2 min. 500 µl of the LB-broth (without antibiotic) were added and cells were incubated for 45 min. (210 rpm). 100-200 µl of the mixture were spread on LB agar plate with antibiotic. Plates were incubated in an inverted position (to prevent water condensation) 12-16 hrs at 37°C. If pBluescript was used for cloning, then a blue-white screen is made by spreading 40 µl of 0.1 M IPTG and 40 µl of X-GAL (2%) over the LB agar plate. Bacteria carrying recombinant DNA form

white colonies while those without insert are blue.

2.7.10 Isolation and Purification of DNA

Plasmid DNA was isolated from bacterial cultures using commercial miniprep kits. The standard protocol of the Wizard Plus Minipreps DNA Purification system (PROMEGA) was carried out using 5 ml of overnight bacterial culture (12-16 hr by 37°C) and vacuum manifold. The DNA was eluted with sterile water. The plasmid could be used directly for DNA sequencing and restriction digestion. DNA fragments separated through electrophoresis were excised from the agarose gel using a scalpel and extracted with the help of High Yield PCR cleanup and gel extraction kit following the protocol of the manufacturer.

2.7.11 DNA sequencing

DNA was sequenced with the ABI Big Dye terminator v3.1 cycle sequencing kit. For single stranded DNA 25-50 ng and for double stranded DNA 150-300 ng of DNA was usually taken. 5 µl of DNA was taken and mixed with 4 µl of ABI mix provided in the kit and then 0.6 picomoles of sequencing primers were added. The volume of the reaction was adjusted to 10 µl. The eppendorf tube with this reaction mixture was given a short spin and run in the thermocycler.

Phase	Temperature	Time	Cycles
Denaturation	96ºC	1 min	
Rapid thermal ramp	96ºC	10 sec	25
Rapid thermal ramp	50 ºC	5 sec	
Rapid thermal ramp	60ºC	4min	
Hold	4 ºC	∞	

*Rapid thermal ramp is 1°C/ second

After the PCR reaction the mixture was given a short spin and the mix was transferred to 1.5 ml eppendorf tubes. 40 µl of 75% isopropanol was added (10 min; RT) and then centrifuged (13,000 rpm, 30 min). Isopropanol was taken out carefully without touching the pellet. The pellet was washed with 140 µl of 80% ethanol for 5 min, vacuum dried and resuspended in 15 µl of Hi Di buffer. The pellet was kept in Hi Di buffer for 15 min and then vortexed.

2.7.12 Permanent cultures

600 µl of overnight bacterial culture was taken and resuspended in 400 µl of autoclaved glycerol and stored at -80°C.

2.7.13 Cloning

All clonings were done either into pBluescripII (SK-) vector and then transferred to expression vector or cloned directly into expression vectors. The expression vectors used were pQE30, pQE80L, pQE80 Δ XhoI, Δ NcoI (modified by A. Schultz) or pETDUET hence all the clones had an N-terminal MRGSHis$_6$ Tag or a C-terminal S-Tag when cloned into the MCS II of pETDUET. Below are all clones used in this thesis. Restriction sites used for cloning are marked. The primers used for cloning are marked with arrows with the No.s listed in 2.6.1. The primers which overlap each other in the figure below are fusion primers.

2.7.13.1 Cloning of CyaG and CyaG chimeras

CyaG CHD+Linker
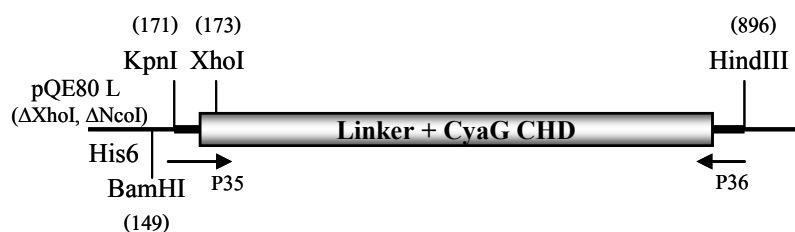
Figure 2.1: End clone of CyaG CHD in pQE80L (ΔXhoI, ΔNcoI); cloned as KpnI/HindIII into the expression vector. A silent restriction site for XhoI was inserted into the clone by PCR.
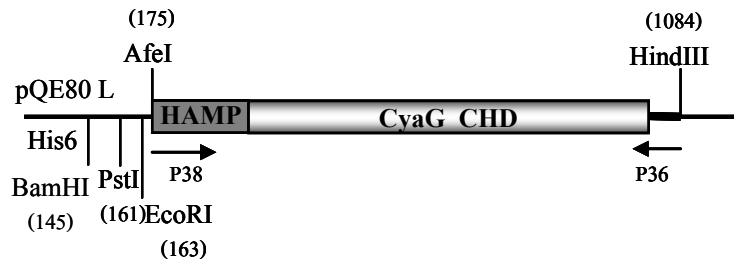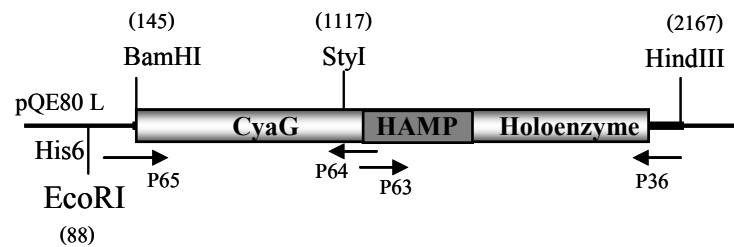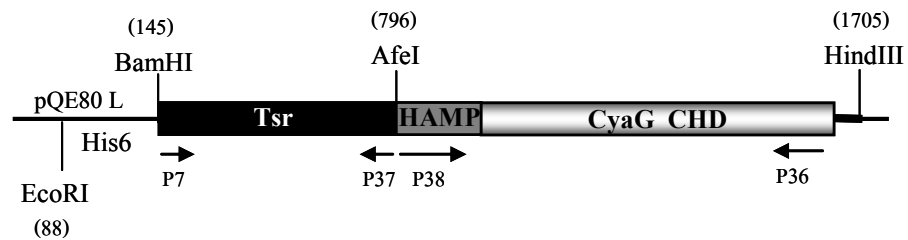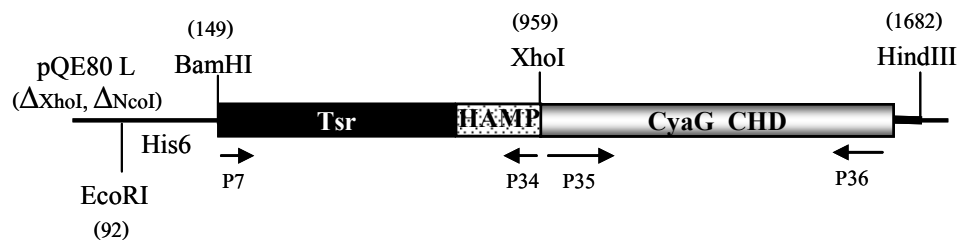
Figure 2.2: ***A***. *CyaG (HAMP+CHD) in pQE80L, cloned at the EcoRV site of pBluescrip and then transferred to the expression vector as BamHI/HindIII. A silent restriction site for AfeI was inserted by PCR;* ***B***. *CyaG Holoenzyme in pQE80L, cloned by fusion PCR and then transferred into the vector as BamHI/HindIII;* ***C***. *Tsr-HAMP_{CyaG}-CyaG in pQE80L;* ***D***. *Tsr-HAMP_{Tsr}-CyaG in pQE80L (ΔXhoI, ΔNcoI), both the CyaG chimeras were cloned by triple ligation into the vector as BamHI/AfeI/HindIII and BamHI/XhoI/HindIII, respectively. Different fragments are colour coded with the names in the middle.*

Tsr-HAMP_{Tsr} (I288S)-CyaG and Tsr-HAMP_{Tsr} (I288S)(I291A)-CyaG

The construct Tsr-HAMP$_{Tsr}$-Rv3645 with similar mutations i.e. I288S single mutant and double mutant I288S, I291A were available. They were used as template to amplify the Tsr fragment with P7 and P34 primers. The PCR product was digested with BamHI/XhoI and cloned into the CyaG (CHD+Linker) clone digested with BamHI/XhoI.

A. **Tar-HAMP$_{CyaG}$ -CyaG**
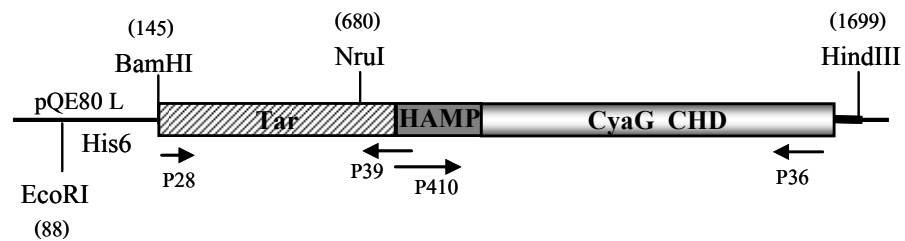
B. **Tar-HAMP$_{Tar}$ -CyaG**
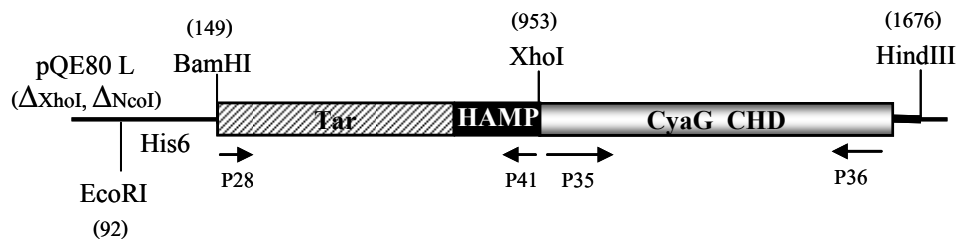
*Figure 2.3: **A**. Tar-HAMP$_{CyaG}$-CyaG in pQE80L, cloned by fusion PCR and then transferred into the vector as BamHI/HindIII; **B**. Tar-HAMP$_{Tar}$-CyaG in pQE80L (Δ XhoI, Δ NcoI), cloned as BamHI/XhoI into CyaG (CHD+Linker). CyaG (CHD+Linker) was digested with BamHI/XhoI and used as a vector.*
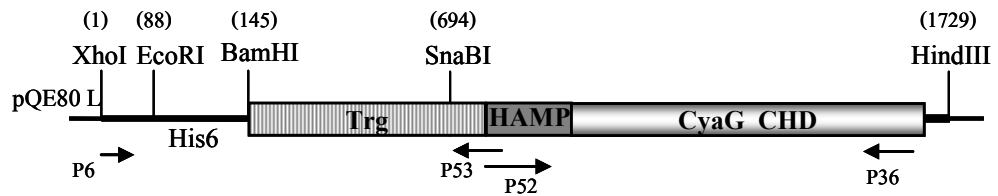
A. **Trg-HAMP$_{CyaG}$-CyaG**

B. **Trg-HAMP$_{Trg}$-CyaG**
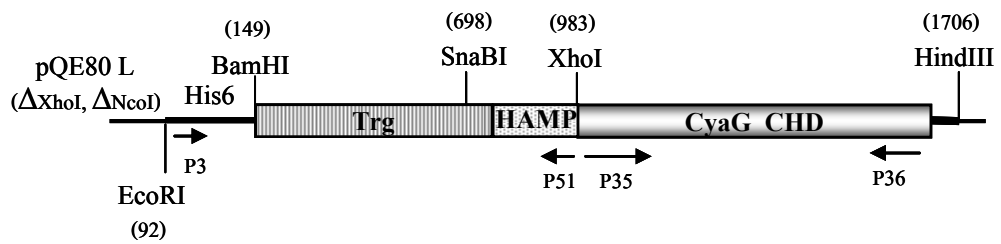
C. **Tap-HAMP$_{CyaG}$-CyaG**
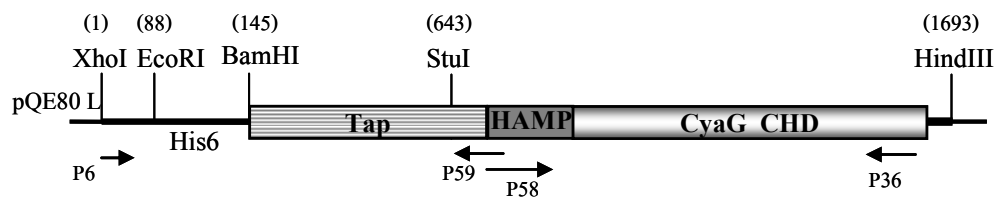
D. **Tap-HAMP$_{Tap}$-CyaG**
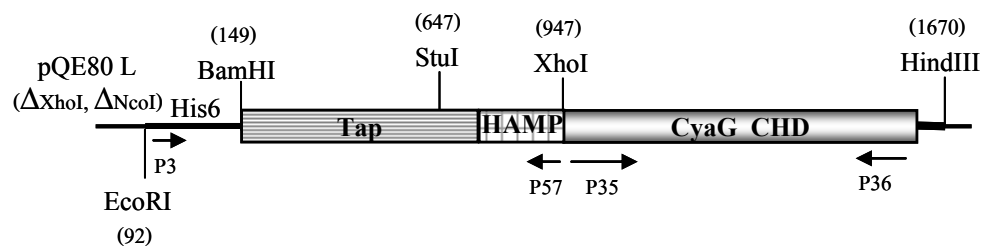
*Figure 2.4: **A**. Trg-HAMP$_{CyaG}$-CyaG in pQE80L, cloned by fusion PCR and then transferred into the vector as BamHI/HindIII; **B**. Trg-HAMP$_{Trg}$-CyaG in pQE80L (Δ XhoI, Δ NcoI), cloned with BamHI/XhoI into CyaG (CHD+Linker); **C**. Tap-HAMP$_{CyaG}$-CyaG in pQE80L, cloned by fusion PCR and then transferred into the vector with BamHI/HindIII; **D**. Tap-HAMP$_{Tap}$-CyaG in pQE80L (Δ XhoI, Δ NcoI) vector, cloned as BamHI/XhoI into CyaG (CHD+Linker).*

2.7.13.2 Periplasmic Binding proteins (MBP, GBP, DBP)

Maltose binding protein (MBP), galactose binding protein (GBP) and dipeptide binding protein (DBP) were amplified from *E. coli* K12. Primers used for MBP were P48 and P49. It was cloned as BamHI and HindIII into pQE80L. The primers used for GBP were P54 and P55. It was cloned as BamHI and HindIII into pQE80L. DBP was cloned into pQE80L as SphI and SalI and the primers used for amplification were P60 and P61.

2.7.13.3 Cloning of Rv3645 chimeras

Six chimeras of Rv3645 AC were used. Five of them were cloned by U.Kurz (Fig. not shown). All five were cloned into pQE30 vector as BamHI and HindIII. Tsr-HAMP$_{Tsr}$-Rv3645 and Tar-HAMP$_{Tar}$-Rv3645 chimeras were cloned by inserting a silent BglII restriction site. Tsr-HAMP$_{Tar}$-Rv3645 and Tar-HAMP$_{Tsr}$-Rv3645 were cloned by inserting silent SphI and NheI restriction sites, respectively. Tsr-HAMP$_{3645}$-Rv3645 was cloned by inserting a silent MfeI restriction site.

Tar-HAMP$_{3645}$-Rv3645
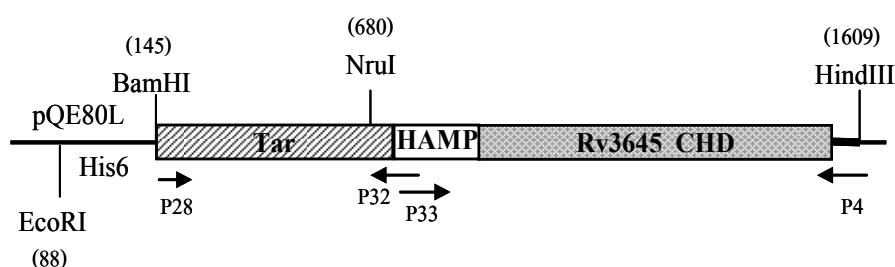
Figure 2.5: *Tar-HAMP$_{3645}$-Rv3645 chimera in pQE80L, cloned by fusion PCR with respective primers. It was ligated into the vector using BamHI and HindIII restriction sites.*

2.7.13.4 Cloning of Rv1264 chimeras

A. Tsr-HAMP$_{Tsr}$-Rv1264
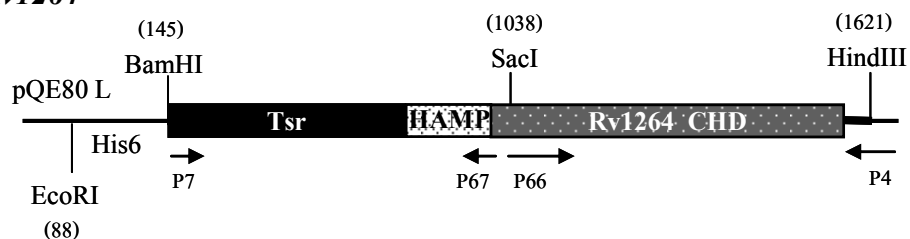
B. Tsr-HAMP$_{Tsr}$-(+A)-Rv1264 and Tsr-HAMP$_{Tsr}$-(+AA)-Rv1264
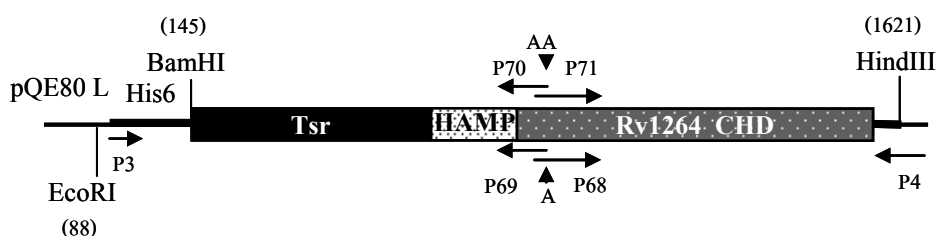
Figure 2.6: **A**. *Tsr-HAMP$_{Tsr}$-Rv1264 chimera was cloned into pQE80L via BamHI and HindIII. A silent SacI site was inserted to fuse the Tsr and Rv1264 fragments;* **B**. *Both Tsr-HAMP$_{Tsr}$-(+A)-Rv1264 and Tsr-HAMP$_{Tsr}$-(+AA)-Rv1264 chimeras were cloned into pQE80L via BamHI and HindIII. Fusion PCR with respective primers indicated in the figure was used for assembling different domains.*

2.7.13.5 Cloning of CyaB1 chimeras

Tsr-HAMP$_{Tsr}$-CyaB1

The CyaB1 part of the chimera was obtained by digesting the PDE10.CyaB1 pBLUE construct (obtained from A. Schultz) with XhoI and KpnI and ligating it into Tsr-HAMP$_{Tsr}$ pBLUE restricted with XhoI/KpnI. The assembled chimera was transferred to pQE30 via BamHI and HindIII.

A. *Tsr-HAMP$_{Tsr}$ (ΔPAS)-CyaB1*
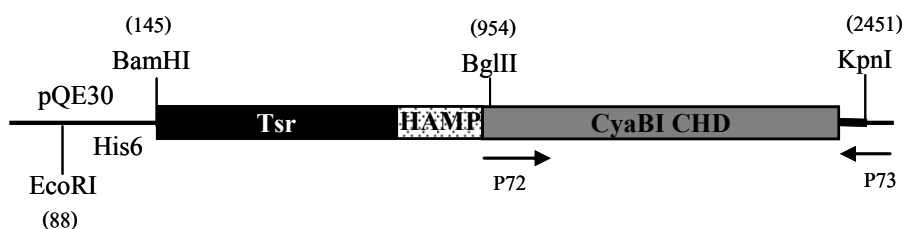
2.7.13.6 Cloning of Rv1625c chimeras

B. *Tsr-HAMP$_{Tsr}$-Rv1625c*
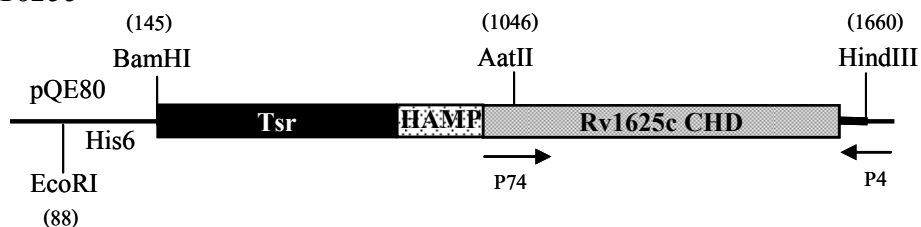
C. *Tsr-H(Tsr)-Rv1625c*
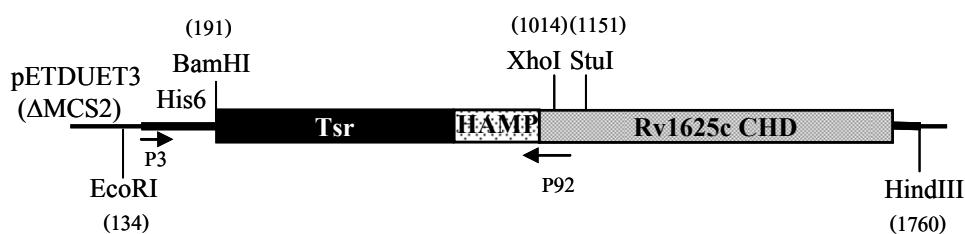
Figure 2.7: **A**. *Tsr-HAMP$_{Tsr}$ (ΔPAS)-CyaB1in pQE30, cloned by amplifying CyaB1 CHD with primers indicated in the figure and ligated into Tsr-HAMP$_{Tsr}$-Rv3645 pBLUE restricted with BglII and KpnI. The assembled clone was transferred from pBLUE to pQE30 via BamHI and KpnI;* **B**. *Tsr-HAMP$_{Tsr}$-Rv1625c in pQE80L, Rv1625CHD was amplified by respective primers as indicated and then ligated into Tsr-HAMP$_{Tsr}$-Rv3645 pBLUE restricted with AatII and HindIII, the clone was transferred to pQE80L via BamHI and HindIII;* **C**. *Tsr-H(Tsr)-Rv1625c in pETDUET3, the Tsr fragment was amplified and ligated to Rv1625c holoenzyme as BamHI and XhoI and then transferred into pETDUET3 via BamHI and HindIII.*

A. *Tsr-H(Ap)L(Ap)-Rv1625c*
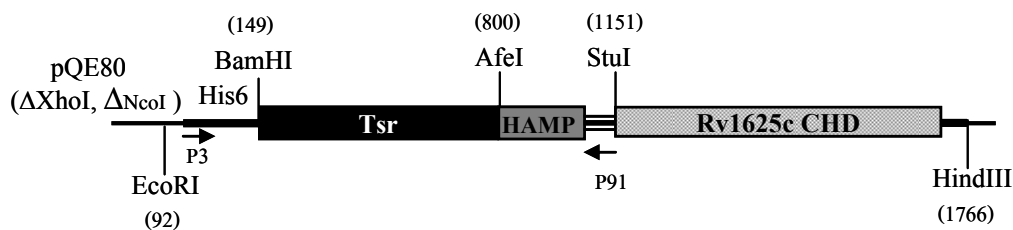
B. *Tsr-H(Ap)L(1625)-Rv1625c*
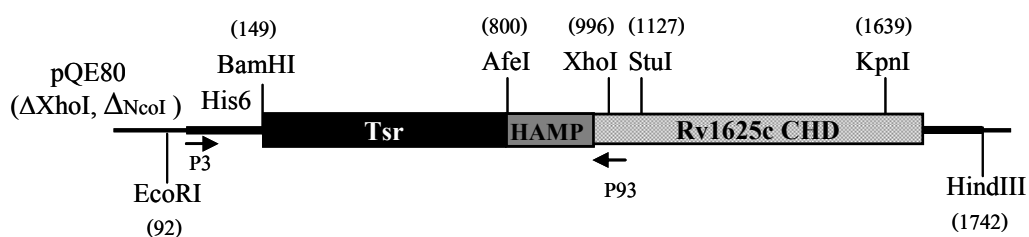
C. *Nt(1625)Tsr-H(Tsr)-Rv1625c*
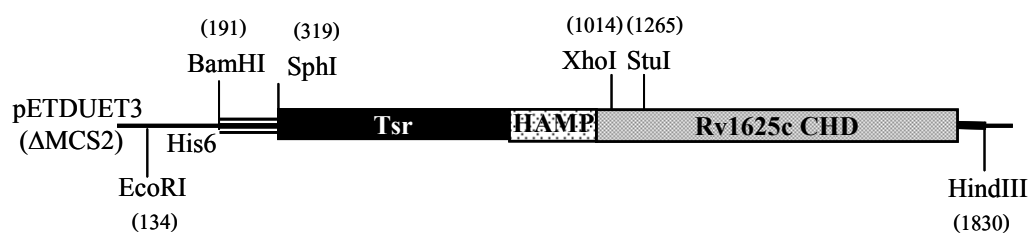
D. *Tar-Rv1625c*
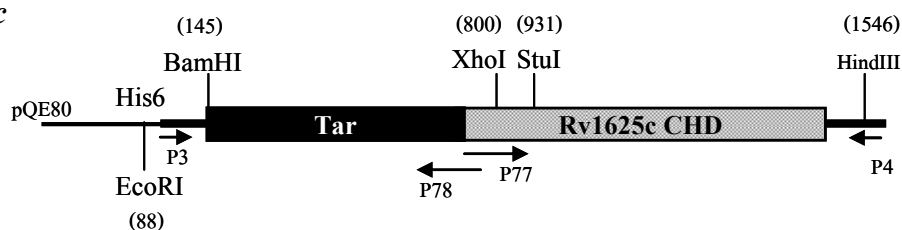
Figure 2.8: **A**. *Tsr-H(Ap)L(Ap)-Rv1625c in pQE80L (Δ XhoI, Δ NcoI), Tsr-H(Ap)L(Ap) fragment was amplified with primers as indicated and cloned as BamHI/StuI into Tsr-H(Tsr)-Rv1625c restricted with BamHI/StuI; **B**. Tsr-H(Ap)L(1625)-Rv1625c in pQE80L (Δ XhoI, Δ NcoI), Tsr-H(Ap)L(1625)-Rv1625c fragment was amplified with primers as indicated and cloned as BamHI/XhoI into Tsr-H(Tsr)-Rv1625 restricted with BamHI/XhoI; **C**. Nt(1625)Tsr-H(Tsr)-Rv1625c in pETDUET3, N-terminal sequence of Rv1625c holoenzyme was cloned in front of Tsr-H(Tsr)-Rv1625c via BamHI and SphI. SphI was introduced as a silent site; **D**. Tar-Rv1625c in pQE80L, Tar-Rv1625c was cloned by fusion PCR with respective primers as indicated and ligated via BamHI/HindIII to BamHI/HindIII restricted pQE80L.*
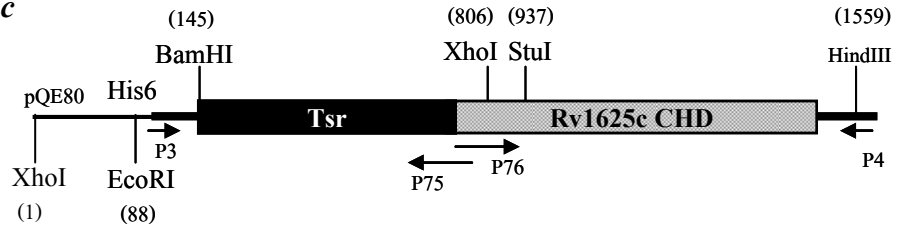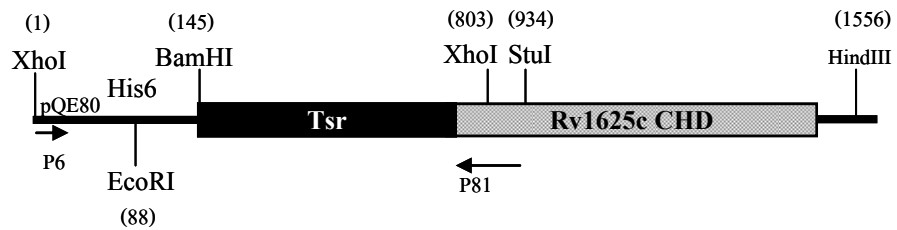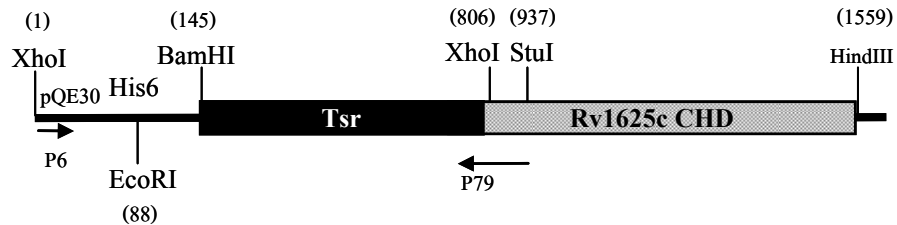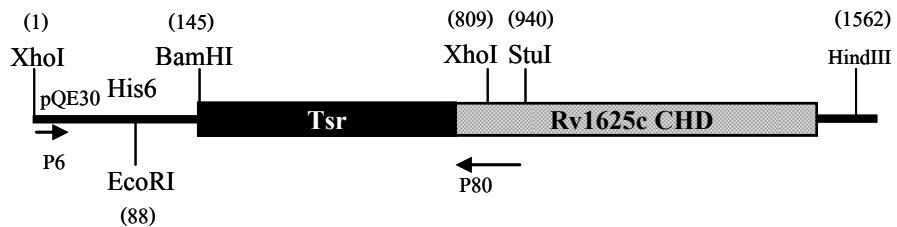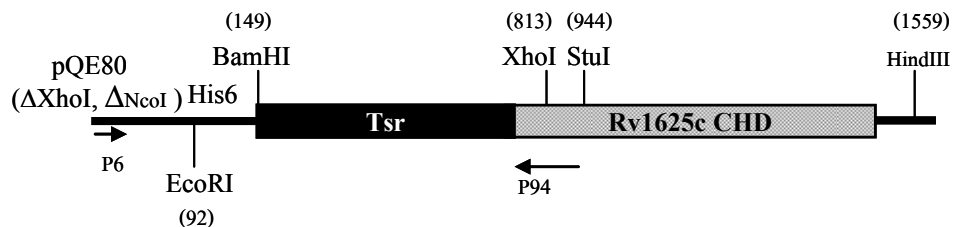
*Figure 2.9: **A**. Tsr (+R) Rv1625c in pQE80L, Tsr (+R) Rv1625c was cloned by fusion PCR with respective primers as indicated and ligated via BamHI/HindIII to BamHI/HindIII restricted pQE80L; **B**.Tsr-Rv1625c in pQE80L; **C**. Tsr (+A) Rv1625c in pQE80L; **D**. Tsr (+AA) Rv1625c in pQE30. The chimeras from B to D was amplified by their respective primers as indicated and cloned via Xho1/XhoI into Tsr (+R) Rv1625c pQE80L construct restricted with XhoI/XhoI; **E**. Tsr (+LR) Rv1625c in pQE80L (Δ XhoI, Δ NcoI), cloned as BamHI/XhoI into Rv1625c holoenzyme restricted with BamHI/XhoI.*

A. Nt (1625)Tsr-Rv1625c
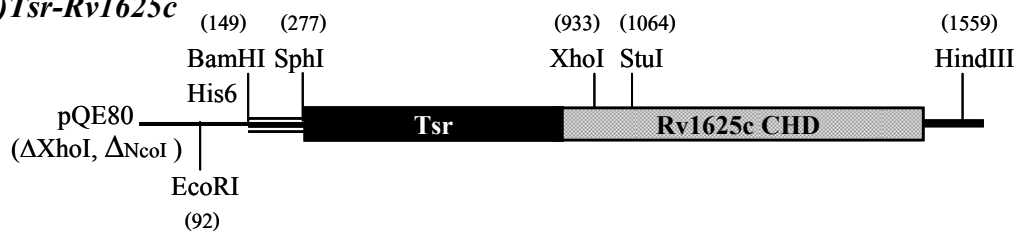
2.7.13.7 Chimeras with swapped transmembranes and ligand binding domains

B. Tsr-Tar (TM1,TM2) Rv3645
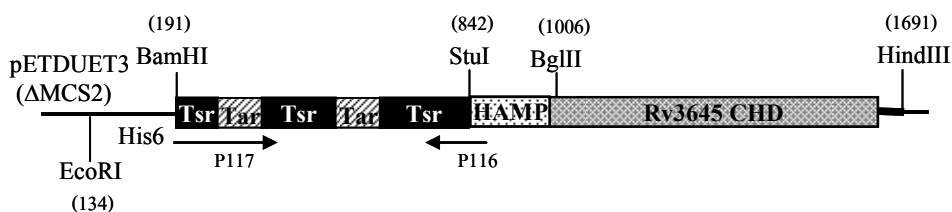
C. Tsr-Af1503 (TM1,TM2) Rv3645 (I)
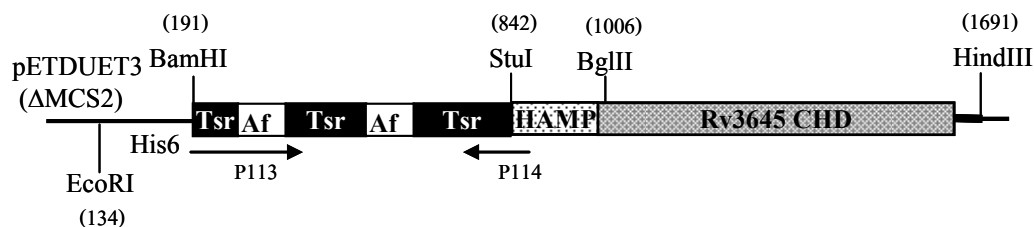
D. Tsr-Af1503 (TM1,TM2) Rv3645(II)
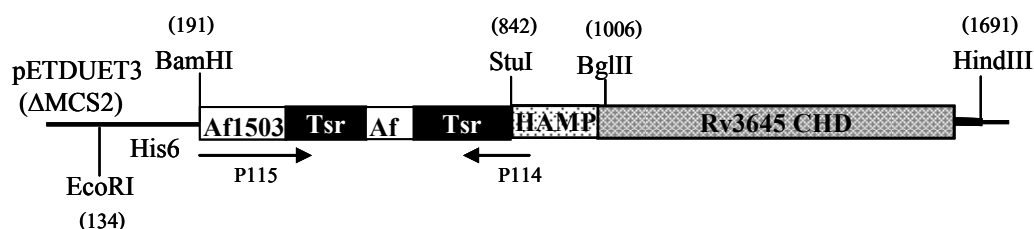
*Figure 2.10: **A**. Nt (1625) Tsr-Rv1625c in pQE80L (Δ XhoI, Δ NcoI), N-terminal sequence of Rv1625c holoenzyme was cloned in front of Tsr(+R)Rv1625c via BamHI and SphI. SphI was introduced as a silent site; **B**. Tsr-Tar (TM1,TM2) Rv3645 in pETDUET 3 (ΔMCSII); **C**. Tsr-Af1503(TM1,TM2) Rv3645 in pETDUET 3 (ΔMCSII)(I); **D**. Tsr-Af1503 (TM1,TM2) Rv3645 in pETDUET 3 (ΔMCSII)(II). The chimeras from B to D were amplified by their respective primers as indicated and cloned as BamHI/StuI into Tsr-HAMP_{Tsr}-Rv3645 pETDUET 3 (obtained from A. Schultz) restricted with BamHI/StuI.*

...

A. TM (1-5)-Tsr-TM6-HAMP$_{(Rv3645)}$-Rv3645
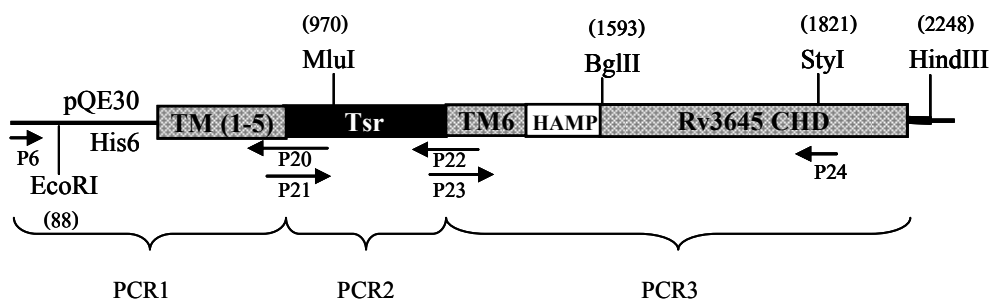
B. TM (1-5)-Tsr-TM6-Rv1625c
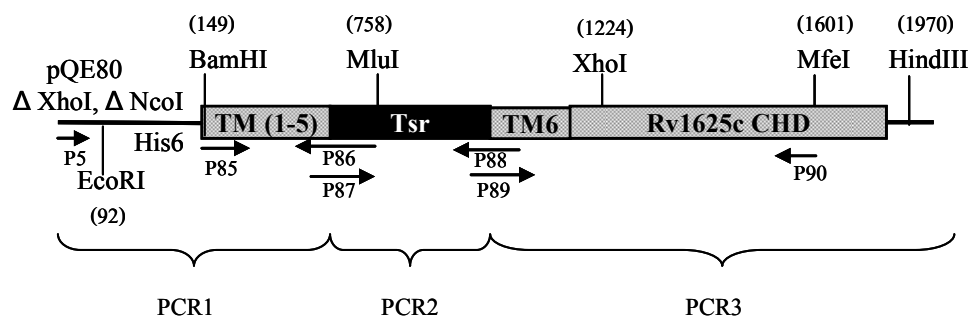
C. Tsr-TM5/TM6-Rv3645
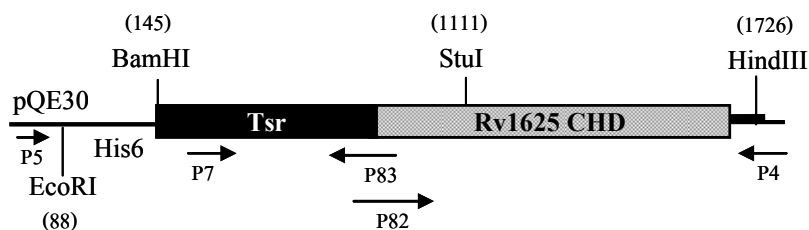
Figure 2.11: **A.** *TM (1-5)-Tsr-TM6-HAMP$_{(Rv3645)}$-Rv3645 in pQE30, PCR2 amplified by P21 and P22 and PCR3 amplified by P23 and P24. PCR2 and PCR3 assembled via fusion PCR. PCR1 amplified by P6 and P20. PCR1 and (PCR2+PCR3) assembled via fusion PCR. The final fusion product was digested with EcoRI/BglII and cloned into Tsr-HAMP$_{3645}$-Rv3645 pQE30 construct restricted with EcoRI/BglII;* **B.** *TM (1-5)-Tsr-TM6-Rv1625c in pQE80 (Δ XhoI, Δ NcoI), PCR2 amplified by P87 and P88 and PCR3 amplified by P89 and P90. PCR2 and PCR3 assembled via fusion PCR. PCR1 amplified by P5 and P86. PCR1 and (PCR2+PCR3) assembled via fusion PCR. The final fusion product was digested with BamHI/XhoI and cloned into Rv1625c holoenzyme restricted with BamHI/XhoI;* **C.** *Tsr-TM5/TM6-Rv3645 in pQE30, cloned by fusion PCR and ligated via EcoRI/HindIII to pQE30 restricted with EcoRI/HindIII.*

2.7.13.8 Transmembrane deletion chimeras

Tsr-(ΔTM1&TM2)-HAMP$_{Tsr}$-Rv3645 pETDUET3 (ΔMCSII) chimera was cloned by A. Schultz. TsrΔTm1C'Rv3645Mt1-Mt12 and TsrΔTm1N'Rv3645Mt1-Mt6 were cloned into the above chimera via SpeI/StuI. The forward primer (P95) was common for all the chimeras while the reverse primer varied. Primers, P98, P99, P100, P101, P96, P97, P102, P103, P104, P122, P105 and P106 were used to clone TsrΔTm1C'Rv3645Mt1-Mt12 chimeras respectively. Primers, P107, P108, p109, P110, P111 and P112 were used to clone TsrΔTm1N'Rv3645Mt1-Mt6 chimeras respectively. Tsr-(mut)-Rv3645 and Tsr-(LL-RR)-Rv3645 were cloned via SpeI /StuI into Tsr-(ΔTM1&TM2)-HAMP$_{Tsr}$-Rv3645 construct restricted with SpeI/StuI. Tsr-(mut)-Rv3645 chimera was cloned via P118 and P119 primers. Tsr-(LL-RR)-Rv3645 chimera was cloned via p120 and P121 primers. Tsr-(ΔTM1)-HAMP$_{Tsr}$-Rv3645 was prepared by subcloning. Tsr-HAMP$_{Tsr}$-Rv3645 pETDUET3 was restricted with BlpI. The insert released was cloned into Tsr-(ΔTM1&TM2)-HAMP$_{Tsr}$-Rv3645 digested with BlpI.
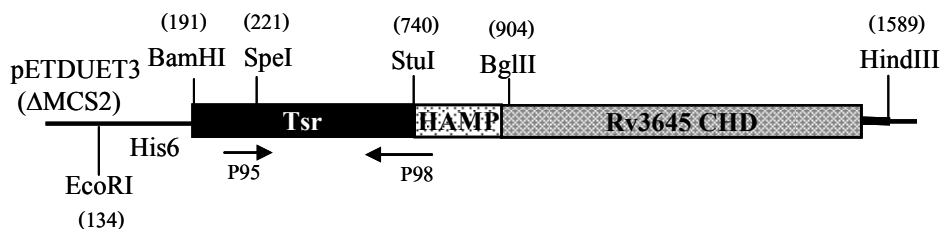
Figure 2.12: TsrΔTm1C'Rv3645Mt1 in pETDUET 3 (Δ MCSII), cloned via SpeI/StuI.

2.7.13.9 Tsr and Tar receptor mutant chimeras

Tsr-(R69E)-HAMP$_{Tsr}$-Rv3645 and Tsr-(T156K)-HAMP$_{Tsr}$-Rv3645 were cloned into pQE30. The individual mutants were then subcloned into MCSI and MSCII of pETDUET 3. Tsr-(R69E, T156K)-HAMP$_{Tsr}$-Rv3645 pETDUET was cloned by A. Schultz. Tar-(R69E)-HAMP$_{CyaG}$-CyaG and Tar-(I154I)-HAMP$_{CyaG}$-CyaG were cloned into pQE80L. The individual mutants were subcloned into MCSI and MSCII of pETDUET 3. Tar-(R69E, T154I)-HAMP$_{CyaG}$-CyaG pETDUET was cloned by A. Schultz.

A. Tsr-(R69E)-HAMP$_{Tsr}$-Rv3645
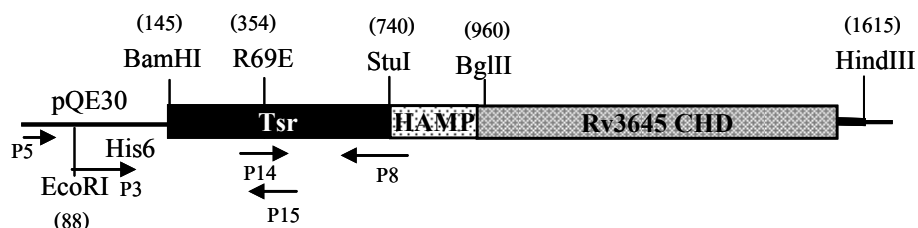
B. Tsr-(T156K)-HAMP$_{Tsr}$-Rv3645
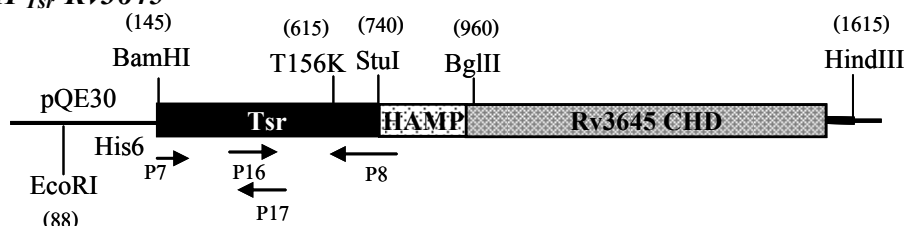
C. Tar-(R69E)-HAMP$_{CyaG}$-CyaG
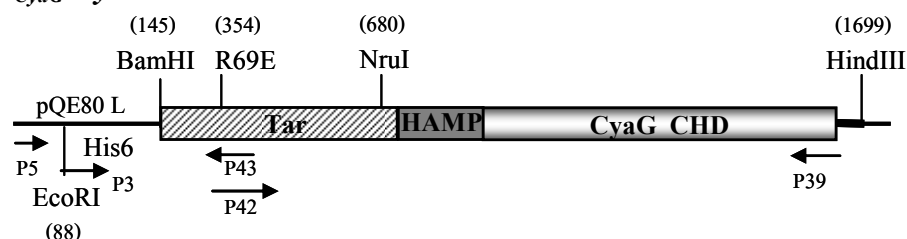
D. Tar-(T154I)-HAMP$_{CyaG}$-CyaG
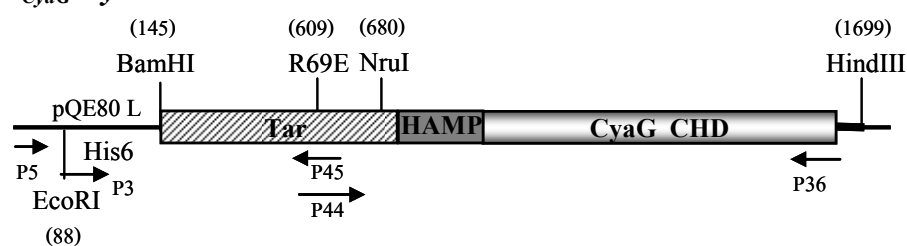
*Figure 2.13: **A.** Tsr-(R69E)-HAMP$_{Tsr}$-Rv3645 in pQE30, cloned by fusion PCR and then ligated to the Tsr-HAMP$_{Tsr}$-Rv3645 restricted with EcoRI/BglII; **B.** Tsr-(T156K)-HAMP$_{Tsr}$-Rv3645 in pQE30, cloned by fusion PCR and then ligated to the Tsr-HAMP$_{Tsr}$-Rv3645 restricted with EcoRI/BglII; **C**. Tar-(R69E)-HAMP$_{CyaG}$-CyaG in pQE80L, cloned by fusion PCR and then ligated to Tar-HAMP$_{CyaG}$-CyaG restricted with BamHI/NruI; **D**. Tar-(T154I)-HAMP$_{CyaG}$-CyaG in pQE80L, cloned by fusion PCR and then ligated to Tar-HAMP$_{CyaG}$-CyaG restricted with BamHI/HindIII.*

2.8 Protein Chemistry methods

2.8.1 Preculture

All proteins were expressed in *E. coli* BL-21 (DE3) [pREP4], for preculture 5 ml of LB-broth containing 100 µg/ml Ampicillin and 50 µg/ml Kanamycin were inoculated with a small amount of permanent culture (overnight, 37°C, 210 rpm).

2.8.2 Expression

The 5 ml preculture was the inoculum for 200 ml LB-broth containing 100 µg/ml Ampicillin and 50 µg/ml Kanamycin (30°C, 210 rpm) It was grown to an OD$_{600}$ between 0.4-0.6 (approx. 2-3 hrs). The expression of Rv3645, Rv1264 and Rv1625c chimeras were with 0.1 mM IPTG (22°C, 210 rpm) for 4-5 hours. CyaG AC chimeras were induced by 0.1 mM IPTG (18°C, 210 rpm) for overnight. CyaB1 AC chimeras were induced by 0.06 mM IPTG (16°C, 210 rpm) for overnight. 10 mM MgCl$_2$ was added wherever CyaB1 CHD was in the construct. Cells were harvested (15 min., 5000 x g, 4°C), the supernatant was discarded and the pellet washed with 20 ml of cell wash buffer, centrifuged (15 min, 5000 x g, 4°C) and stored at -80°C.

2.8.3 Purification of soluble proteins from *E. coli*

The frozen pellets were thawed on ice (10-15 min), suspended in 25 ml of cell lysis buffer, passed twice through a French Press (1000 psi) and the homogenate was centrifuged (30 min, 20000 x g, 4°C). The supernatant was mixed with 200 µl Ni-NTA-Agarose resin. The resin was washed with wash buffer B thrice before adding it to the supernatant. The supernatant was rocked for 2 hrs in ice. Samples were centrifuged (5 min, 2500 x g, 4°C). The supernatant was discarded, and Ni-NTA resin was brought into a 10 ml syringe with an attached Wizard mini-column, washed with 2 ml washing buffer A and B and protein was eluted with 600 µl elution buffer.

2.8.4 Preparation of membrane fractions from *E. coli*

The frozen pellets were thawed on ice (10-15 min), suspended in 20 ml of cell lysis buffer, passed twice through the French Press (1000 psi) and the homogenate was centrifuged (30

min, 5000 x g, 4°C) to remove the debris. The supernatant was ultracentrifuged (1 hr, 100,000 x g, 4°C). The supernatant was discarded and pellet was resuspended in 2-3 ml of membrane resuspension buffer and stored at -20°C or -80°C depending on stability of the protein.

2.8.5 Solubilization of the membrane protein

Membranes prepared in section 2.8.4 were resuspended in membrane solubilisation buffer and kept on ice for 2 hours. After two hours the suspension was ultracentrifuged (1 hr, 100,000 x g, 4°C). The supernatant was used further to purify the protein via Ni-NTA as described in 2.8.3. The pellet after ultracentrifugation as well as supernatant was run on SDS PAGE to see the extent of solubilization of the membrane. The membrane was used for another round of solubilization if it was incomplete.

2.8.6 Purification of periplasmic protein by osmotic shock

Periplasmic binding proteins GBP, MBP and DBP were purified by osmotic shock (Neu and Heppel, 1965). Cells were harvested and washed thrice at 5000 g with 0.01 M Tris/HCl pH 8 buffer (cold). The washed pellet (1 gm, wet weight) was suspended in 40 ml of 0.03 M Tris/HCl and 40% sucrose. The suspended pellet was kept on a rotatory shaker in 1 L flask (180 rpm, 10 min, 24°C) and then centrifuged (10 min, 13,000 g, 4°C). The supernatant was discarded and the well drained pellet was mixed with volume of cold water equal to that of the original volume of the suspension. The suspension was mixed in an ice bath on a rotary shaker for 10 min and centrifuged (13,000 g, 10 min, 4°C), and the supernatant fluid, hereafter termed as the cold water wash, was taken for SDS PAGE analysis. All the periplasmic proteins were released into the water. Since GBP, MBP and DBP were overexpressed hence their concentration was relatively more compared to other periplasmic binding proteins.

2.8.7 Bio-Rad Protein determination

A concentration of 1 mg/ml of BSA was used as a standard. 4-12 μg of protein were pipetted to 800 μl distilled water and vortexed. 200 μl of Dye reagent concentrate (5 x) was added, vortexed and the absorbances at OD_{595} were measured and protein concentrations were calculated according to the calibration curve.

2.8.8 Densitometry for protein determination

Proteins were also quantitated by densitometry. The programme called Image J which is a java based application (http://rsb.info.nih.gov/ij/) was used. A protein standard was taken which was usually a purified protein. The standard was estimated by Bio-Rad. The membrane proteins which had to be estimated were run on the SDS PAGE along with the control and then the Western blot was carried out. The photographic film containing the protein bands after developing the blot was scanned by the photographic scanner. The band intensities of the proteins were calculated by using Image J. Intensities were compared with the standard and the amount of the protein was calculated accordingly.

2.8.9 Bioinformatics

DNA sequences were analyzed by DNASTAR. Multiple alignments of protein sequences were done using the Megalign feature of DNASTAR followed by manual correction by the GENEDOC. Secondary structure prediction was done by using the programme [COILS] (Lupas et al., 1991). Transmembrane regions in individual proteins were predicted using TMPRED, HMMTOP 2.0 and DAS programme with default parameters (Cserzo et al., 1997; Hofmann and Stoffel, 1993; Tusnady and Simon, 2001). For documentation of the programmes see, (http://www.ch.embnet.org/software/TMPRED_form.html) and (http://www.enzim.hu/hmmtop/). The domain analysis was done by the SMART programme (Simple Modular Architecture Research Tool) and NCBI (National Center for Biotechnology Information) conserved domain search (Letunic et al., 2006; Marchler-Bauer and Bryant, 2004; Schultz et al., 1998). For documentation of the programmes see, (http://smart.embl.de/) and (http://www.ncbi.nlm.nih. gov/Structure/cdd/wrpsb.cgi).

2.8.10 SDS-PAGE

The expression and M. wt. of the protein was determined by SDS PAGE (Laemmli, 1970). Protein was mixed with 4 X sample buffer, heated for 95°C for 5 min and loaded on the gel. Membrane protein was mixed with 4 X sample buffer and kept at 37°C for 15 min. Gels were run at a constant current: 20 mA, 200V, 1 hr, stained with coomassie blue for 30 min with gentle agitation, decolourised for 20-25 min and washed with water until the bands are clearly detected. Usually, SDS-PAGE was carried out simultaneously with pellets and supernatant of *E.coli* containing an empty vector as a control. Protein marker components

(10 μl containing 1μg of each protein) were from Peq Lab (Peq gold). (http://www.peqlab.de/wcms/en/pdf/27-1010.pdf).

2.8.11 Western Blot

For immunochemical detection, proteins were transferred after SDS-PAGE to PVDF-membrane through semi-Dry-Electrotransfer (Towbin et al., 1979). The blot membrane was successively soaked in methanol, water and Towbin buffer each for 2 min. Three Whatman 3 MM papers were soaked in Towbin buffer and laid on the anode plate. The blot membrane was laid over them, the gel and finally three soaked Whatman papers again on the side of the cathode plate. Protein transfer was carried out for 2-3 hr at 20V and 2.5 mA/cm^2. The gel was stained in coomassie brilliant blue to check transfer efficiency. The membrane was stained in Ponceau S for about 5 min, then it was decolourized with deionized water until the protein bands were clear enough to mark the marker bands with a pencil. The membrane was blocked with M-TBS buffer for at least 1 hr at RT or overnight at 4°C and washed with TBS-T buffer (2 x 10 sec, 2 x 5 min). It was then incubated with the primary antibody (mouse monoclonal RGS-His$_6$ antibody 1:2000, or Tetra-His antibody 1:1000 diluted in M-TBS) for 1 hr. After washing (TBS-T, 2 x 15 min) it was incubated with the secondary antibody (goat anti-mouse IgG-F$_c$ or goat anti-rabbit IgG-F$_c$ horseradish peroxidase conjugated antibodies 1:5000 diluted in M-TBS) for 1 hr and then washed as above with TBS-T. The chemiluminescent reaction with the ECL Plus Western Blotting Detection Kit (Amersham) was carried out according to the manufacturer's instructions and it was detected on hyperfilm-ECL after its exposure to the detection reaction (from 10 sec to 5 min).

2.8.12 Adenylyl cyclase assay

The AC activity was tested by measuring the amount of ^{32}P cAMP formed from α^{32}P ATP used as a substrate (Salomon et al., 1974). Assay volume was 100 μl which contained 40 μl of protein sample including ligands (serine, aspartate etc) and 50 μl of AC test cocktail and 10 μl of ATP start solution. The final concentrations of substances were: 50 mM Tris/HCl buffer pH 7.5, 22% glycerol, 6 mM MnCl$_2$, 200, 750 or 75 μM ATP depending upon which AC was analyzed. The reaction was terminated by addition of 150 μl stop buffer. 10 μl of [2, 8-^{3}H]-cAMP were added as internal standard followed by 750 μl of water and the mixture was poured into a Dowex column (9 ×1 cm glass column with 1.2 g Dowex 50). After

washing with 3.5 ml water, the samples were eluted with 5 ml water in Al_2O_3 columns (10 × 0.5 cm plastic column with 1 g active neutral Al_2O_3 90). Samples were immediately eluted with 4.5 ml 0.1 M Tris/HCl pH 7.5, mixed with 4 ml of Ultima Gold XR Scintillator solution and counted. Dowex columns were regenerated with 1×5 ml 2M HCl, 1×10 ml water and 1×5 ml water, Al_2O_3 columns with 2×5 ml 0.1 M TRIS/HCl pH 7.5. Specific activity, (pmol/mg/min) was calculated with the following formula.

$$A = \frac{Substrate\,(\mu M) \times 100\,\mu l}{time\,(\min)} \times \frac{1000}{protein\,(\mu g)} \times \frac{cpm\left[{}^{3}H\right]_{total}}{cpm\left[{}^{3}H\right]_{sample} - 3\%\left[{}^{32}P\right]_{sample}} \times \frac{cpm\left[{}^{32}P\right]_{sample} - cpm\left[{}^{32}P\right]_{blank}}{cpm\left[{}^{32}P\right]_{total}}$$

3% of the phosphorous counts were subtracted from the tritium values because of the spill over of ${}^{32}P$ into the tritium channel. Activities lower than double background (in cpm) were considered zero.

3. Results

Transmembrane signal propagation has been studied using *E. coli* chemotaxis proteins and AC chimeras. Both the proteins have similar tripartite domain organizations. The domains are divided according to their function, they have a sensory domain which senses the signal (TM domains enclosing the periplasmic loop), signal conversion domain (HAMP domain) and output domain (CHD or the chemotaxis domain). In this study two completely unrelated proteins are brought together such that the sensory module is from the chemotaxis receptor and the output module is from the ACs connected to each other via the HAMP domain either from the AC or from the MCP, hence we have a unique chimera having both the chemosensory function as well as the AC function.

The results have been divided into five groups

❖ HAMP mediated signal transmission by different subclasses of bacterial ACs.

❖ Transmembrane signaling by non HAMP containing ACs.

❖ Swapping of TMs and ligand binding domains between the chimeras to understand the sequence specific or domain specific interactions of TM and AC chimeras.

❖ An effort to get soluble and functional chimeric proteins with transmembrane deletion constructs.

❖ Exploring ligands for the Arthrospira cyclase CyaG holoenzyme.

3.1 HAMP mediated Signal Transduction by a Class IIIa AC

Mycobacterial Rv3645 AC, a class IIIb AC was connected to Tsr and Tar receptor which resulted in active and functional chimeras (described later in section 3.2). To check whether the class IIIa isoform could also be connected in similar way to MCPs as observed in case of Rv3645 AC, CyaG from *A. platensis* was used for making chemotaxis receptor chimeras. CyaG is a class IIIa AC and a variant which is present in all mammalian ACs. It has two TMs, a HAMP domain and a CHD. A detailed biochemical characterization of CyaG AC was carried out prior to generating chemotaxis receptor/CyaG chimeras.

3.1.1 Biochemical Characterization of CyaG AC

3.1.1.a Construct design, expression and purification of CyaG CHD

The CyaG CHD (L490- S671) was cloned along with its linker (L432-L490) which is 63 aa long. The construct was cloned into pQE80 (Δ XhoI, Δ NcoI) and expressed in *E. coli* BL21 (DE3) [pREP4] (37°C, 30°C or 16°C; 4, 5 or 16 h). Protein was most active and stable at 30°C, at 37°C it was mostly degraded (Fig. 3.1 c). A Western blot was carried out with an anti-RGSHis$_6$ antibody which showed a single band at the expected size of 28 kDa. The isoelectronic point (pI) was predicted to be 7.4.
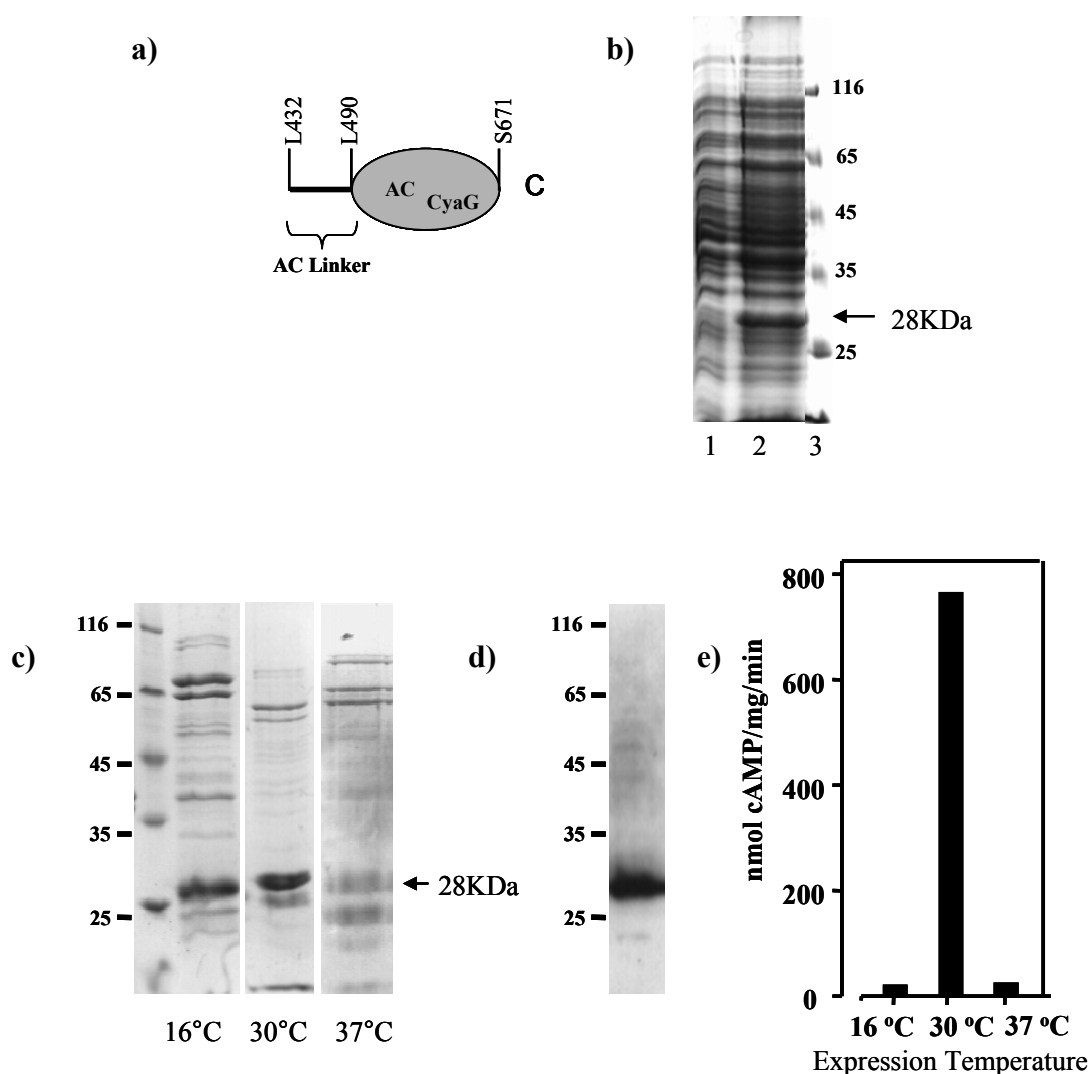
*Figure 3.1: **a)** Scheme of construct; **b)** expression of CyaG CHD, lane 1: un-induced sample; lane 2: expressed protein; lane 3: marker; **c)** purified protein expressed at 16°C; 30°C and 37°C; **d)** Western blot, 0.5μg of protein; **e)** AC activity (750 μM ATP, 10 min, 5 μg of protein, 37°C, Tris/HCl pH 7.5).*

3.1.1.b *Expression and purification of HAMP$_{CyaG}$ -CyaG*

The construct was cloned into pQE80L, transformed into *E. coli* BL21 (DE3) [pREP4] and expressed (37°C, 30°C, or 16°C; 4, 5 or 16 h) by adding 0.1 mM IPTG. The protein was purified as described in the section 2.8.3. The expression of the protein was similar at all the temperatures (Fig. 3.2 b) but the temperature used to express and purify the protein finally was 16°C. The activity of the protein was low as compared to the CHD alone (compare Fig. 3.1 e and 3.2 e). 12% SDS PAGE showed a faint band of the purified protein with several prominent non-specific bands. Most of the protein remained in inclusion bodies. A Western blot was carried out with an anti-RGS His$_6$ antibody which showed a single band suggesting that the other bands were unrelated proteins and not degradation products or higher order oligomers of HAMP$_{CyaG}$-CyaG (Fig. 3.2 c). Specific activity was independent of the protein concentration over the testable range (3.2 e). The predicted pI was 7.1.
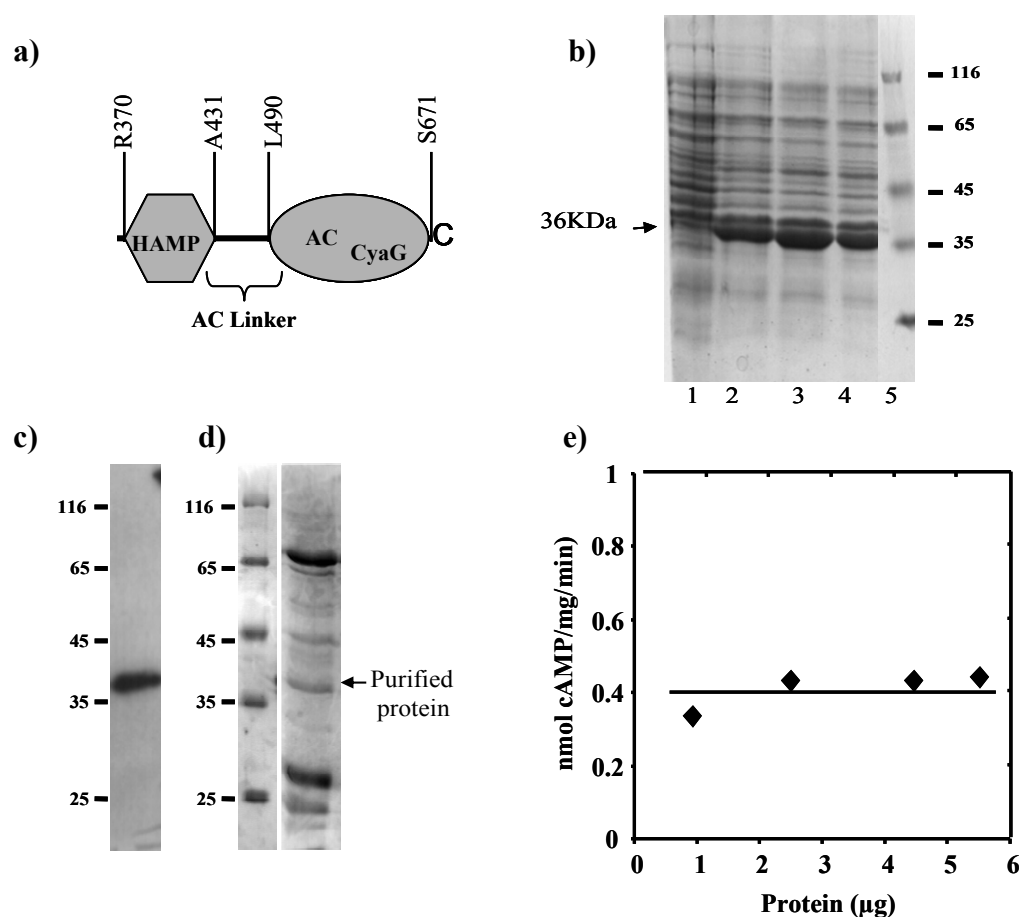
Figure 3.2: **a)** *Scheme of HAMP$_{CyaG}$-CyaG;* **b)** *expression of protein at different temperatures, lane 1: un-induced sample, lane 2: expression at 37°C, lane 3: expression at 30°C, lane 4: expression at 16°C, lane 5: marker;* **c)** *Western blot, 0.5µg of protein;* **d)** *Purification;* **e)** *Protein dependence (750 µMATP, 10 min, 37°C, Tris/HCl pH 7.5).*

3.1.1.c *Expression and Purification of CyaG holoenzyme*

Genomic DNA from *A. platensis* was isolated from a miniculture by standard procedures (http://pubs.nrc-cnrc.gc.ca/ispmb/ispmb18/R00-048.pdf) and used as a template. The gene sequence of CyaG retrieved from NCBI database was slightly different from that obtained by sequencing the PCR product. The product was cloned into pQE80L and expressed in *E. coli* BL21 (DE3) [pREP4] (18°C, 0.1 mM IPTG, overnight). Membrane fractions were isolated and solubilized with 0.8 mM n-dodecy-β-D maltoside (DDM) and then purified finally with Ni-NTA affinity chromatography. Protein could not be solubilized since most of the protein was still there in the membrane fraction (data not shown).The AC assay was performed as described in section 2.8.12 with membrane fraction, flowthru (protein lysate after Ni-NTA interaction) and purified protein sample. Since AC assay is very sensitive and can detect picomoles of protein, AC activity could be seen both in the flowthru as well as in the purified protein sample even though the protein band was not visible on the SDS gel. The AC activity was low in soluble fractions as compared to the membrane fractions. The protein had predicted pI of 5.5. Specific activity of AC holoenzyme was independent of the protein concentration over the testable range (Fig. 3.3 d).
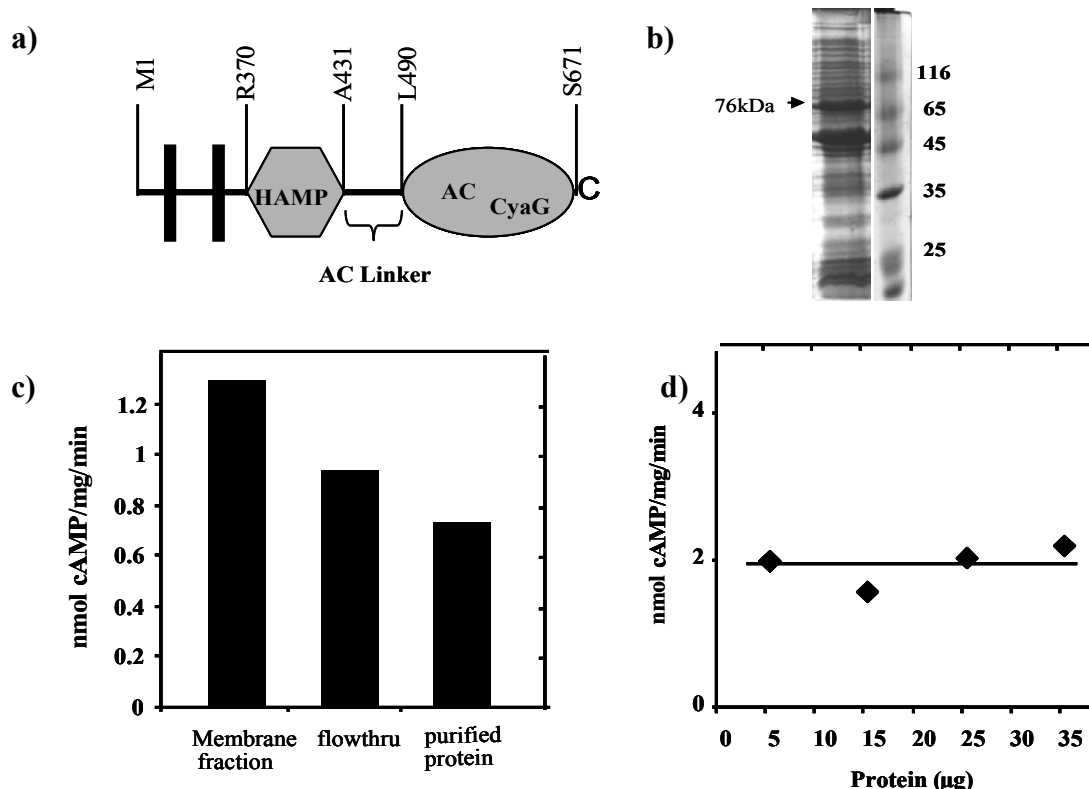
*Figure 3.3: **a)** Scheme of CyaG holoenzyme; **b)** 12% SDS PAGE of CyaG holoenzyme membrane fraction; **c)** AC activity: membrane fraction, flowthru (protein lysate after Ni-NTA interaction) and purified protein; **d)** Protein dependence (0.1 mM Tris/HCl pH 7.5, 10 min, 37°C, 750 μM ATP).*

3.1.2 Biochemical Characterization of Tsr/CyaG AC chimeras

3.1.2.a Tsr-HAMP$_{CyaG}$-CyaG AC

Construct design, expression and membrane preparation

The N-terminus of the chimera is from Tsr receptor (M1-K215) linked to the HAMP$_{CyaG}$ domain (R370- A431) and the CyaG CHD along with its linker (L432- S671) (Fig. 3.4 a). The chimera was cloned into pQE80 (Δ XhoI, Δ NcoI) and expressed in *E. coli* BL21 (DE3) [pREP4] (18°C, 0.1 mM IPTG, overnight). Membranes were isolated and stored at -20°C. A Western blot was carried out with an anti-RGSHis$_6$ antibody to confirm the presence of the chimeric protein because it was difficult to identify the expressed protein on the SDS PAGE due to its low expression and to exclude the presence of proteolytic fragments. The protein had a predicted pI of 6.8.
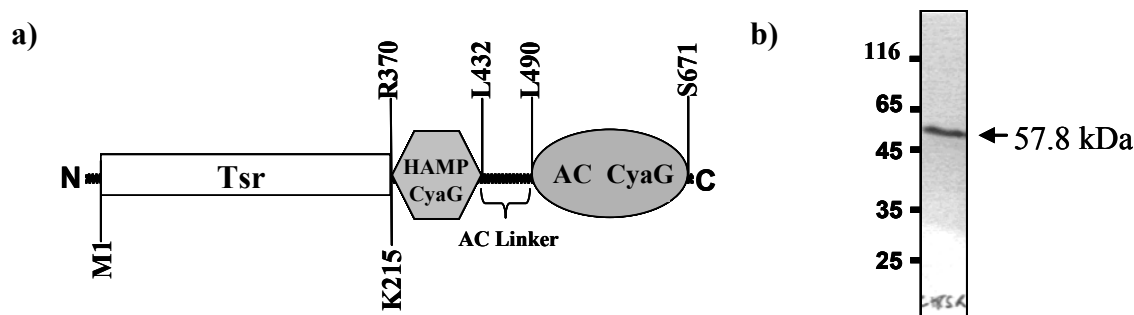
*Figure 3.4: **a)** Domain organization of the chimera; **b)** Western blot, 4μg of protein.*

Regulation by serine

Increase in serine concentrations lead to decrease in specific activity of the protein. Basal activity was 11.9 ± 0.1 nmol/mg/min. Inhibition was 58 ± 5% and the IC$_{50}$ for serine was 6 ± 3 μM, calculated from the serine concentration response curve (Fig. 3.5 a). 100 μM aspartate did not affect AC activity suggesting that inhibition was specific. The specificity was tested further by using other amino acids (asparagine, glutamine, arginine and threonine) and also a sugar (ribose). Inhibition was specific for serine (Fig. 3.5 b).
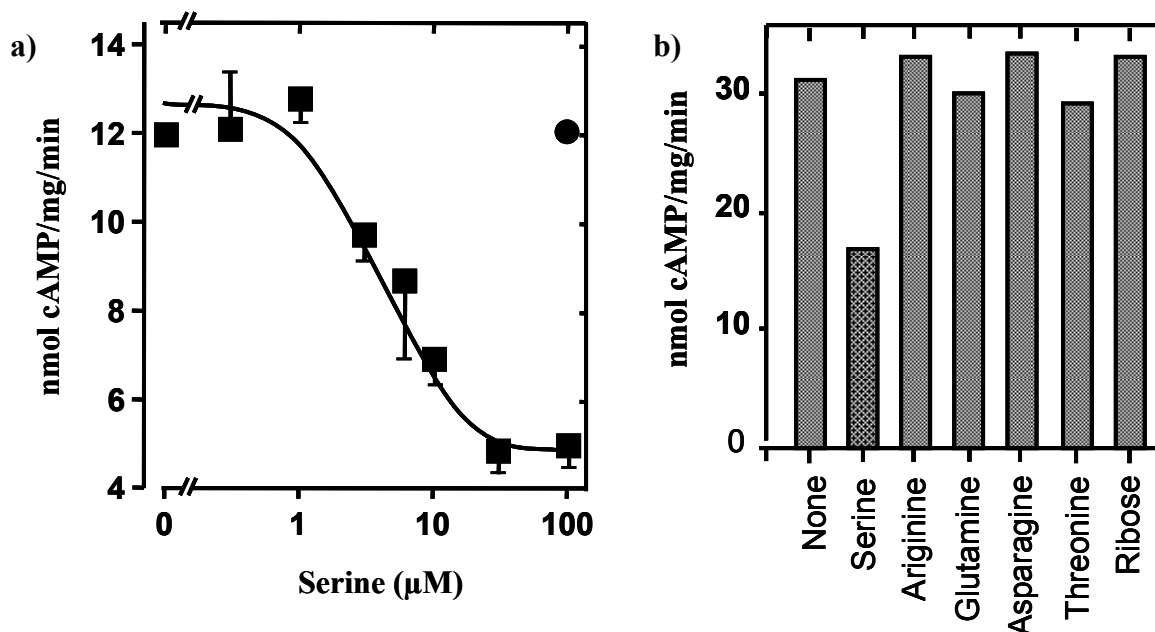
*Figure 3.5: **a)** Serine concentration response curve, n = 6, Filled circle: 100 µM aspartate as control; **b)** Specificity of the chimera for serine (1mM aa and sugar). Assay conditions: 750 µM ATP, 10 min, 10 µg of protein, 37°C, Tris/HCl pH 7.5.*

Protein dependence

On increasing the protein concentration, there was a notable decrease in specific activity of the chimera. The protein dependency graph suggests that the protein is already present as a active homodimer at lower concentrations, on increasing the protein concentration there is formation of less active oligomers and hence a decrease in AC activity.
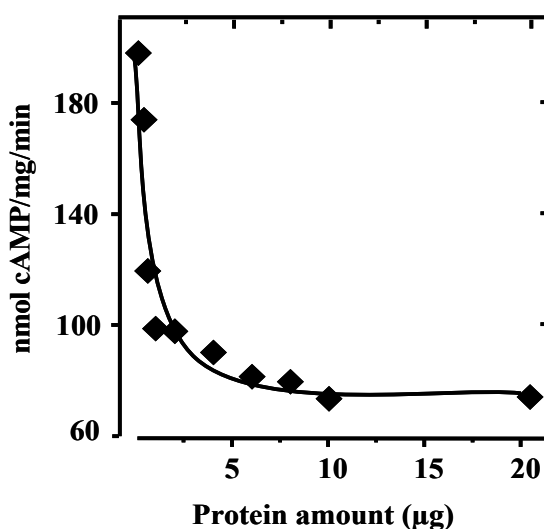
Figure 3.6: Protein dependence (0.1 mM Tris/HCl pH 7.5, 10 min, 37°C, 750 µM ATP, protein 0.1µg to 20 µg).

Temperature dependence

On increasing the temperature there was a continuous increase in specific activity up to 55°C after which there was a decrease in specific activity. The velocity of the reaction is directly proportional to the temperature of the reaction, the velocity increases until the enzyme starts to denature. The same was observed in this assay. The assay was carried out at a temperature range of 20°C to 65°C. The temperature optimum was 55°C. The activation energy of 93.5 kJ/mol was derived by an Arrhenius plot.
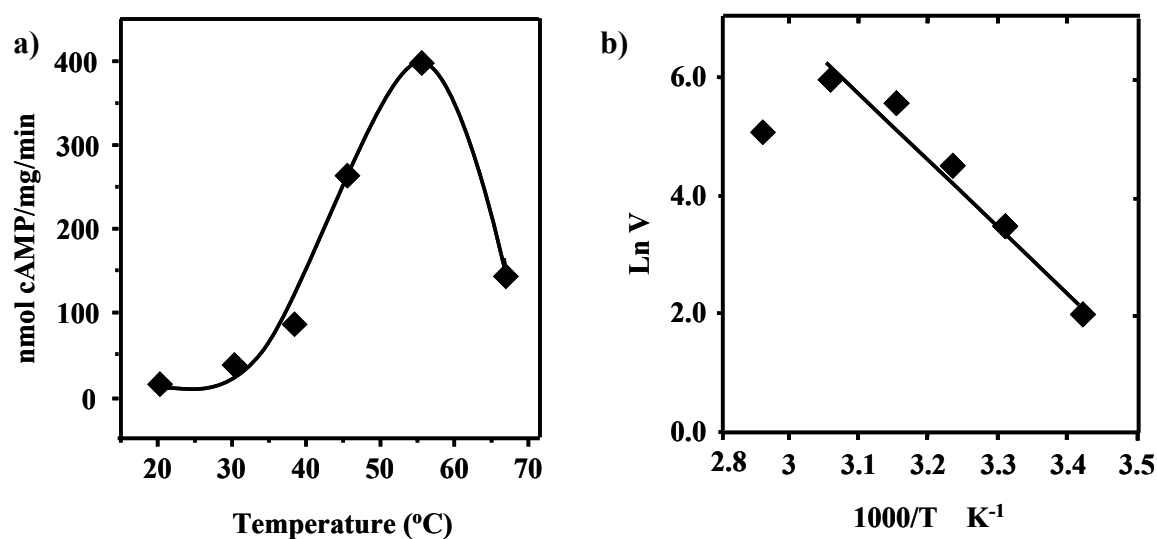
Figure 3.7: ***a)*** *Temperature dependence;* ***b)*** *Arrhenius plot, y = -11.255x+ 40.67, R^2 = 0.97, Ea = 93.5 kJ/mol. Assay conditions: 750 μM ATP, 10 min, 10 μg of protein, 37°C, Tris/HCl pH 7.5.*

pH dependence

The AC activity increases on increasing the pH of the reaction until it reaches its optimum after which there is a decrease on further increasing the pH. The pH optimum was 7.5. Tris/HCl buffer was used for the pH range from 7 to 8.5. MES/Tris buffer was used for pH 6.5. Since the pKa value of Tris/HCl buffer is 8 hence its buffer capacity is for pH 8 ± 1. It should not be used for a pH below pH 7.

Time dependence

AC activity increased linearly with time. 10 μg of protein was assayed at a time range from 2-30 min.
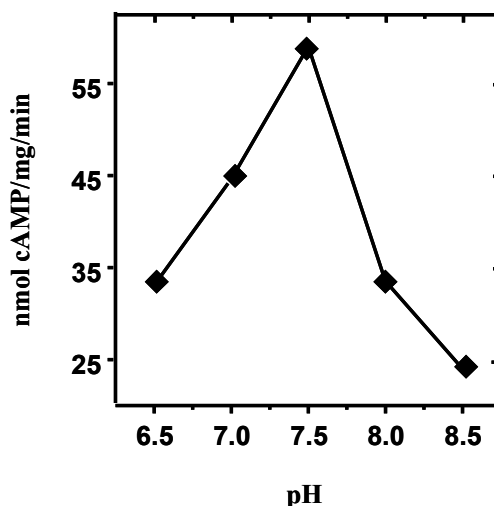
Figure 3.8: pH dependence (750 µM ATP, 10 min, 10 µg of protein, MES/Tris pH 6.5, Tris/HCl pH (7-8.5), 37°C).
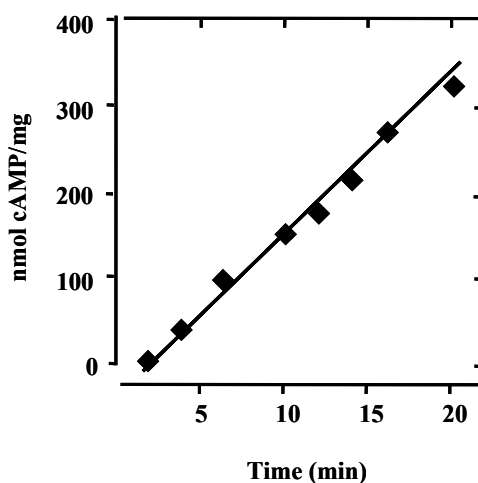
Figure 3.9: Time dependence (750 µM ATP, 10 µg of protein, Tris/HCl pH 7.5, 37°C).

Substrate kinetics

On increasing the ATP concentration there was a hyperbolic increase in specific activity giving an ideal Michaelis-Menten curve. The ATP range of 100-2000 µM was used to measure the substrate kinetics of the chimera. Lineweaver-Burk plot showed that in absence of serine the chimera had a Km of 818 ± 120 µM ATP and Vmax of 44 ± 10 nmol/mg/min while in presence of serine the chimera had a Km of 896 ± 135 µM ATP and Vmax of 34 ± 6 nmol/mg/min. Since at 750 µM ATP concentration, specific activity already starts to level off this ATP concentration was used further on. The Hill coefficient as calculated from the Hill

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plot was 1.13 (R^2 = 0.94) for the reaction without serine and 1.15 (R^2 = 0.97) for the reaction with serine. Since the chimera had a Hill coefficient slightly greater than one it showed no cooperativity.
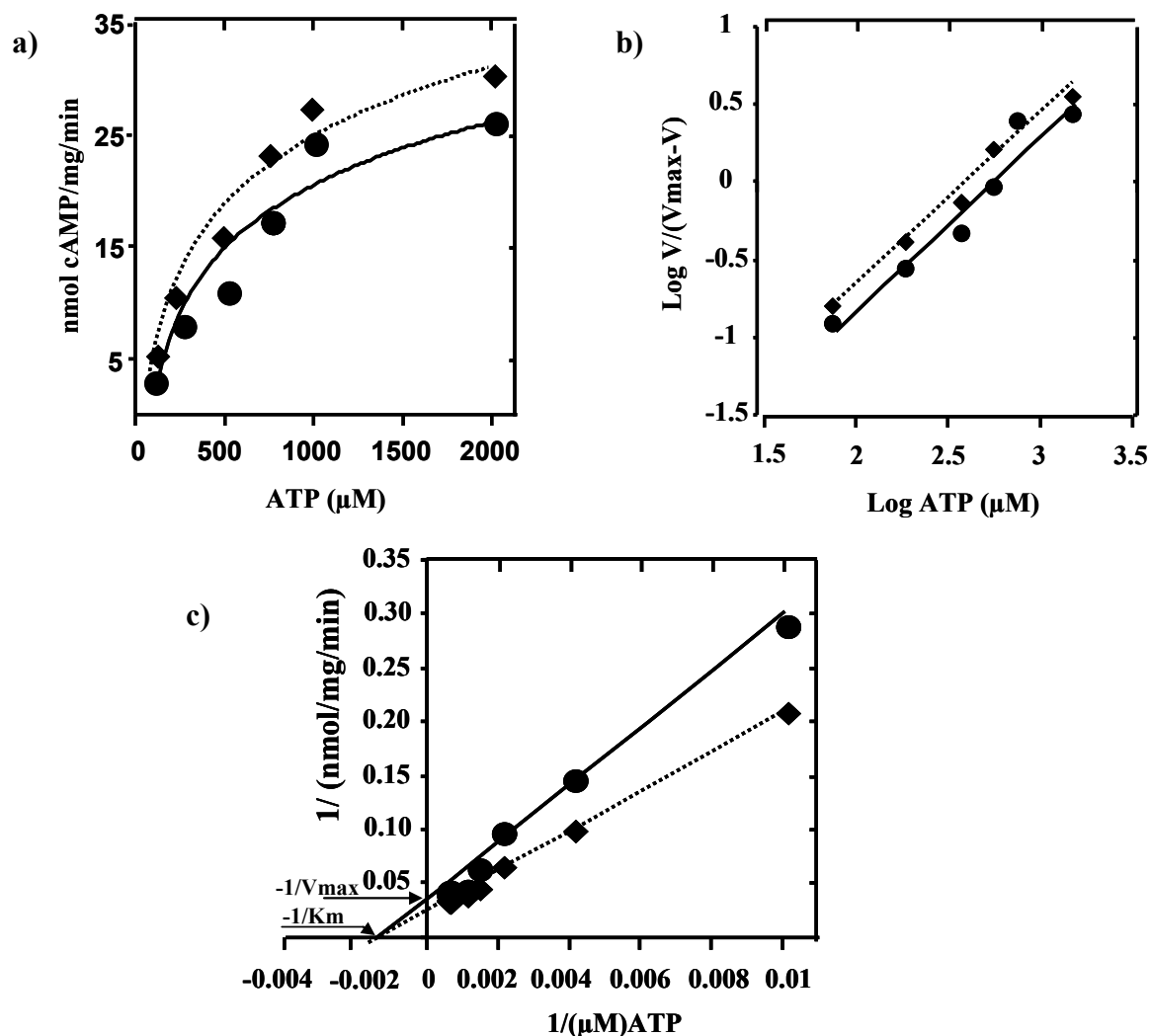
*Figure 3.10: Substrate kinetics, **a)** Michaelis-Menten plot; **b)** Hill plot; **c)** Lineweaver-Burk plot; circles: with 1mM serine; diamonds: without serine; Assay conditions: 0.1 mM Tris/HCl pH 7.5, 10 min, 37°C.*

3.1.2.b Tsr-HAMP$_{Tsr}$-CyaG AC

Construct design, expression and membrane preparation

The N-terminus of the chimera is from Tsr receptor (M1-K215) including the HAMP$_{Tsr}$ domain (A216-G268) linked to the CyaG CHD (L432-S671). The chimera was cloned into

pQE80 (Δ XhoI, Δ NcoI) and expressed in *E. coli* BL21 (DE3) [pREP4] (18°C, 0.1 mM IPTG, overnight). Membrane fractions were prepared and stored at -20°C. A Western blot was carried out with an anti-RGSHis$_6$ antibody which gave a single band at the expected size of 58 kDa. The protein had a predicted pI of 6.8.
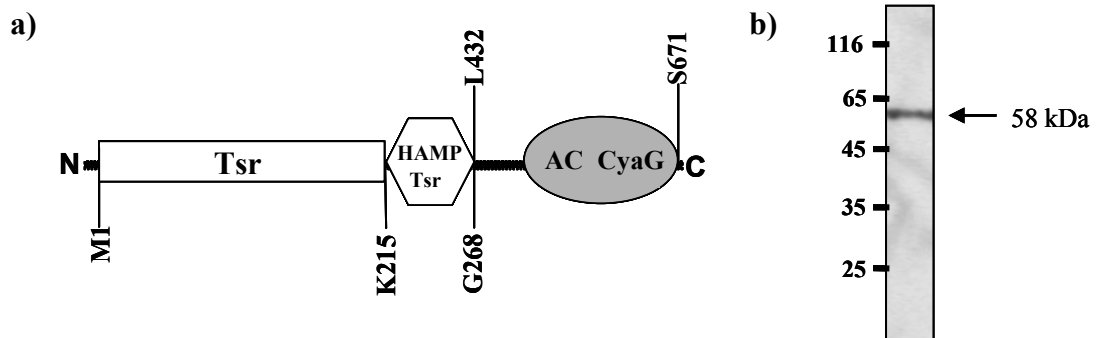
*Figure 3.11 **a)** The domain organization of the chimera; **b)** Western blot, 4µg of protein.*

Regulation by serine

Serine did not regulate. This failure of signaling of the chimera could not be due to misfolding of the protein as the basal activity of the protein was relatively high, 270 ± 76 nmol/mg/min. One reason not to observe signaling may be the fact that the activity of the protein was so high that it may be outside of the range for regulation.
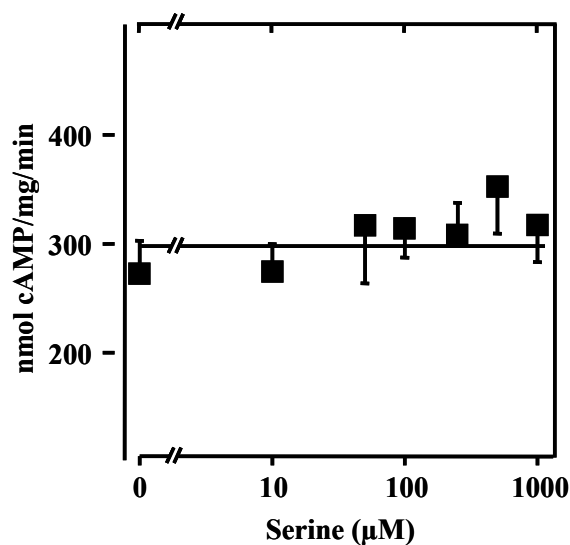
Figure 3.12: Serine concentration response curve; Assay conditions: 0.1 mM Tris/HCl pH 7.5, ATP 750 µM, 10 min 10 µg protein, 37°C, n = 8.

3.1.2.c Tsr-HAMP$_{Tsr}$(I288S)-CyaG and Tsr-HAMP$_{Tsr}$(I288S)(I291A)-CyaG

Tsr-HAMP$_{Tsr}$-CyaG chimera was not regulated by serine. This might be due to its high activity. To reduce the activity of this construct a single mutant (I288S) and a double mutant (I288S, I291A) were designed. When comparing the HAMP domain from CyaG with Tsr/Tar HAMPs there are two positions which differ (Fig. 3.13 a, marked by an arrow). Position 288 (S in CyaG and I in Tsr) and position 291 (S in CyaG and I in Tsr). Af1503 numbering is used as a reference. As it is known from Af1503 constructs (L. Garcia-Mondejar, unpublished) mutation of S288I shifts the HAMP into an "ON" state. In Tsr-HAMP$_{Tsr}$-CyaG chimera there is an "I" in position 288 hence an I288S mutation was generated to make the construct less active. The basal activity of the mutant protein decreased from 270 to 4.7 nmol/mg/min (Fig. 3.13 b) yet there was no change in specific activity of the mutant chimera at 1 mM and 10 mM serine. The amount of protein taken for the assay was same in both cases as confirmed by densitometry hence the reduction in specific activity could not be due to differences in the expression level of the proteins. The double mutant S288I/I291A did not affect the basal activity considerably compared with the S288I protein (Fig. 3.13 c). The basal activity of the double mutant was 7 ± 2 nmol/mg/min. The double mutant also did not display regulation by serine.
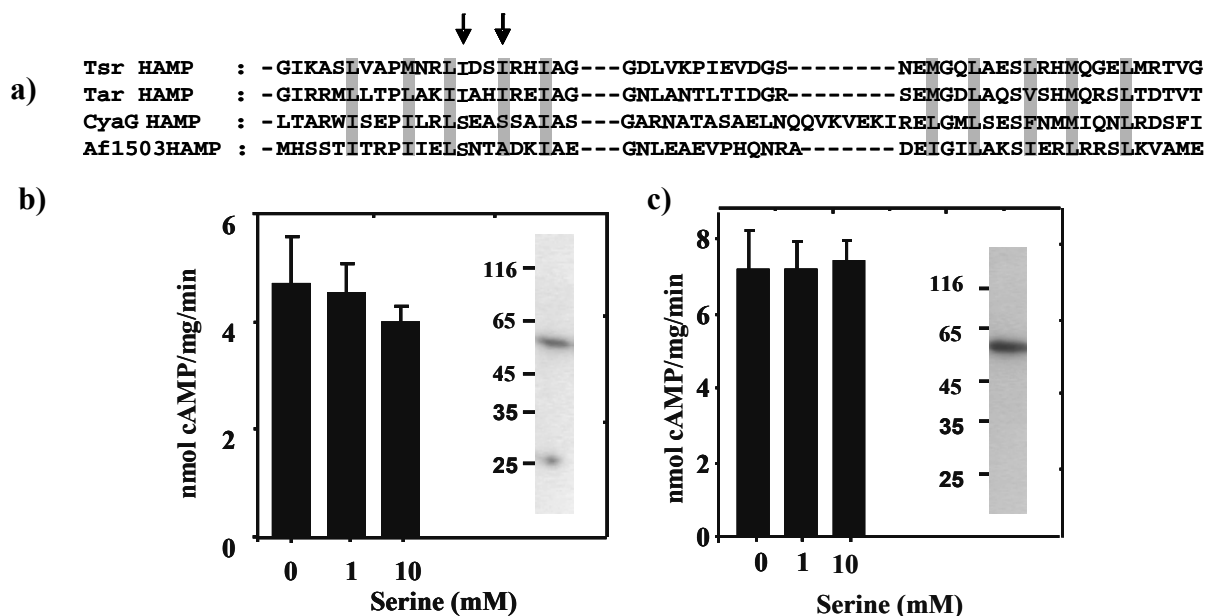
Figure 3.13: a) Alignment of Tsr, Tar, CyaG and Af1503 HAMP domains. The residues which were mutated are marked with an arrow, residues shaded grey are the (a and d) core hydrophobic residues present in HAMP domains; b) Serine concentration responses for Tsr-HAMP$_{Tsr}$(I288S)-CyaG *chimera and Western blot with an anti-RGSHis$_6$ antibody, M.wt. 58 kDa, 4 µg protein; c) Serine concentration responses for* Tsr-HAMP$_{Tsr}$(I288S)(I291A)-CyaG *chimera and Western blot with an anti-RGSHis$_6$ antibody, M.wt. 58 kDa, 4 µg protein. Assay conditions: 0.1 mM Tris/HCl pH 7.5, ATP 750 µM, 10 min, 10 µg protein, 37°C, n = 4.*

3.1.3 Biochemical Characterization of Tar/CyaG AC chimeras

3.1.3.a Tar-HAMP$_{CyaG}$-CyaG AC

Construct design, expression and membrane preparation

The N-terminus of the chimera is from Tar receptor (M1-R213) linked to the HAMP$_{CyaG}$ domain (R370-A431) and the CyaG CHD (L432-S671). The chimera was cloned into pQE80 (Δ XhoI, Δ NcoI) and expressed in *E. coli* BL21 (DE3) [pREP4] (18°C, 0.1 mM IPTG, overnight). Membranes were isolated and stored at -20°C. A Western blot was carried out with an anti-RGSHis$_6$ antibody which gave a single band at the expected size of 59 kDa. The protein had a predicted pI of 8.5.
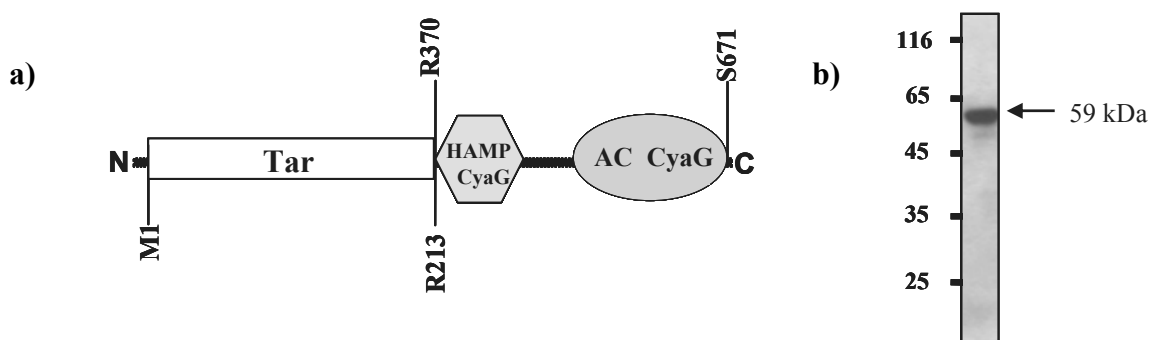
*Figure 3.14: **a)** Domain organization of the chimera; **b)** Western blot, 5 µg of protein.*

Regulation by aspartate

Increasing the aspartate concentration decreased AC activity. Inhibition was 45 ± 7% and the IC$_{50}$ for aspartate was 32 ± 8 µM (Fig. 3.15 a). 1 mM serine as a control did not affect AC activity suggesting that inhibition was specific. The specificity was tested further by using other amino acids (asparagine, serine and threonine) and sugars (ribose and galactose) which confirmed that the inhibition was specific for aspartate (Fig. 3.15 b)
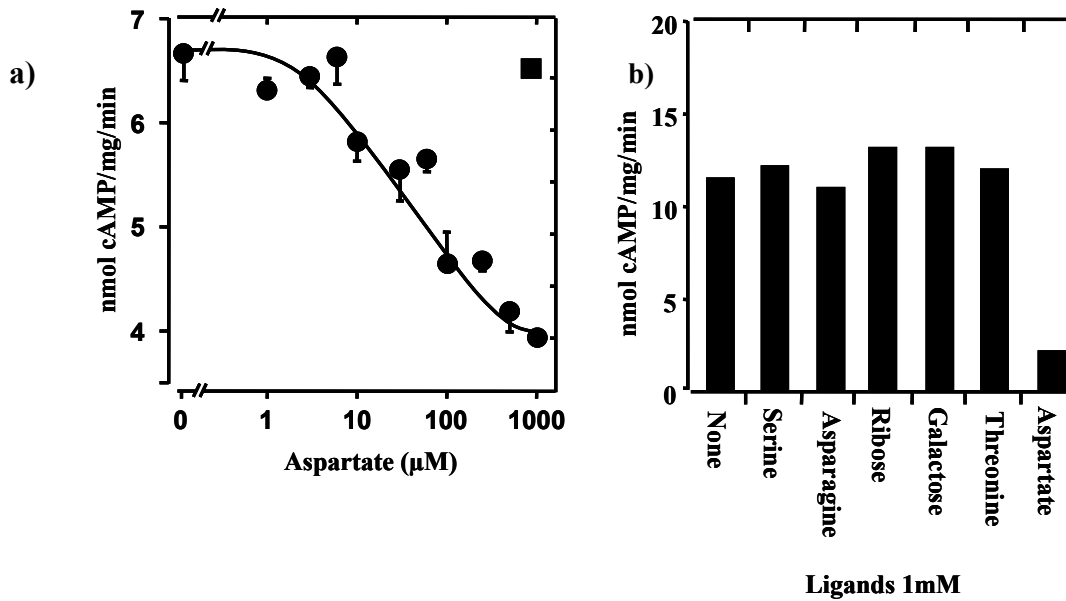
Figure 3.15: ***a****) Aspartate concentration response curve, n = 2. Square: 1 mM serine as a control;* ***b)*** *Specificity tested with other aa and galactose and ribose (1mM). Assay conditions: 0.1mM Tris/HCl pH 7.5, ATP 750 µM, 10 min, 37°C, 10 µg of protein.*

Temperature dependence

On increasing the temperature there was a continuous increase in specific activity of the protein. The temperature optimum was 55°C, which was similar to the construct with the Tsr receptor. The activation energy of 56.5 kJ/mol was derived by an Arrhenius plot (Fig. 3.16 b). The Arrhenius plot was drawn with the temperatures where the specific activity increased linearly.
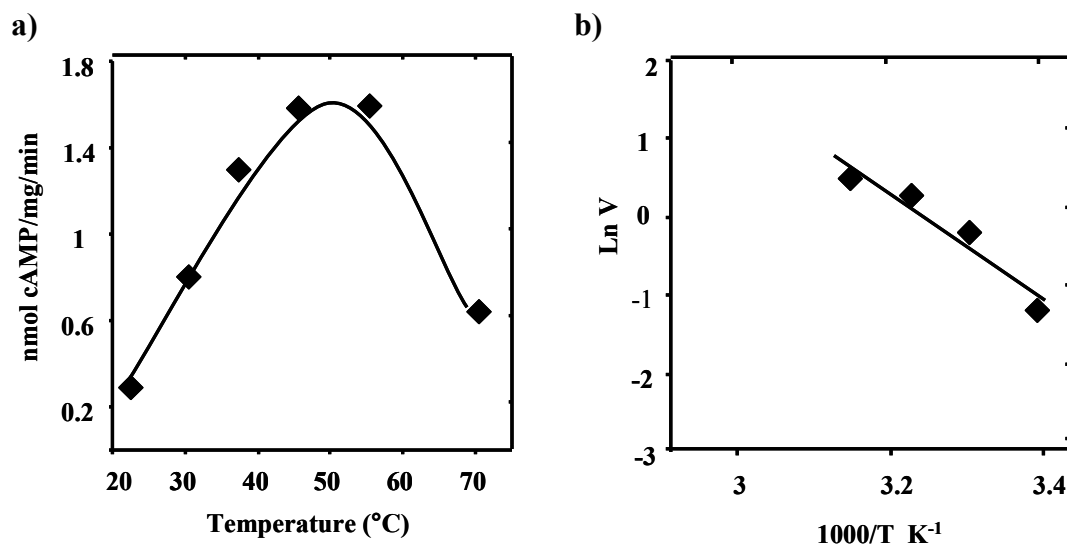
Figure 3.16: ***a)*** *Temperature dependence; Assay conditions: 750 µM ATP, 10 min, 10 µg of protein, 0.1 mM Tris/HCl pH 7.5;* ***b)*** *Arrhenius plot.*

pH dependence

On increasing the pH there was increase in AC activity. The optimum pH was 7.
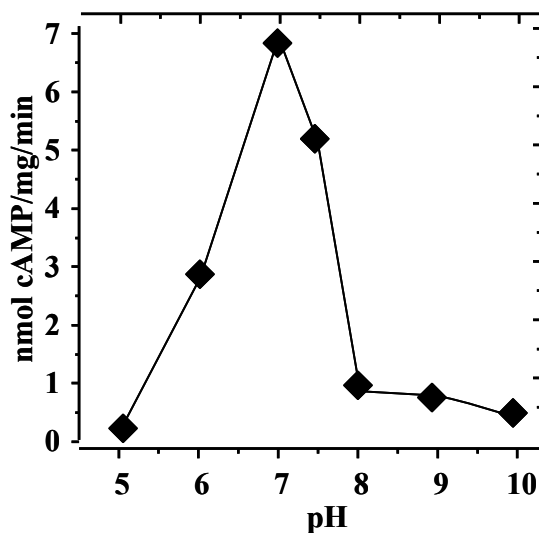
Figure 3.17: pH dependence. Assay conditions: 750 μM ATP, 10 min, 10 μg of protein, 37°C, Acetate buffer for pH5, MES/Tris pH for pH 6, Tris/HCl buffer for pH 7, 7.5 & 8 and glycine NaOH buffer for pH 9 & 10.

Substrate kinetics

Lineweaver-Burk plot shows that in absence of aspartate the enzyme had the Km of 12 mM ATP and a Vmax of 75 nmol/mg/min while in presence of aspartate the Km was 9 mM ATP and the Vmax was 45 nmol/mg/min. The Hill coefficient was 1.6 (R^2 = 0.99) for the reaction with aspartate and 1.46 (R^2 = 0.99) without aspartate indicating the possibility of positive cooperativity.
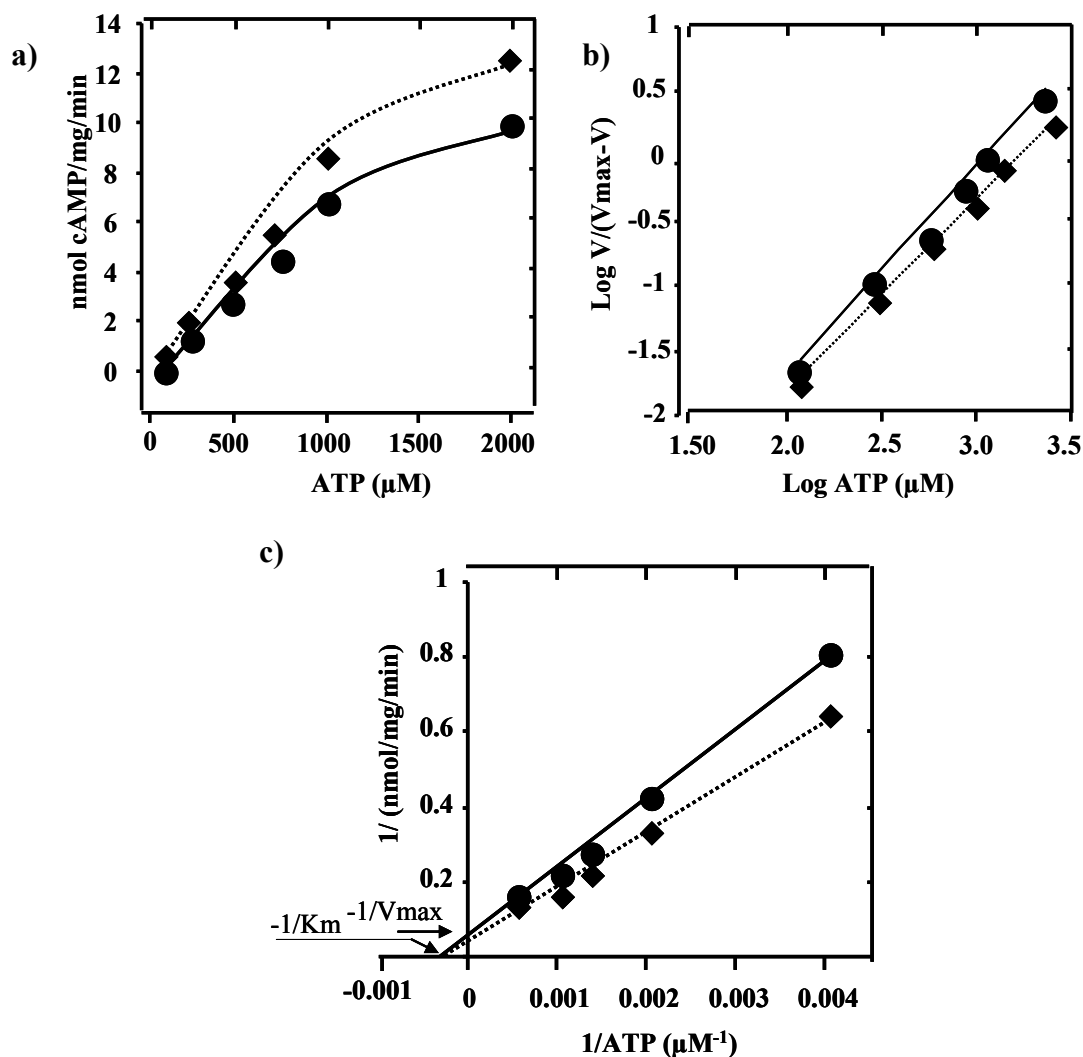
*Figure 3.18: Substrate kinetics, **a)** Michaelis-Menten plot; **b)** Hill plot; **c)** Lineweaver-Burk plot; circles: with 1mM aspartate; diamonds: without aspartate. Assay conditions: 0.1 mM Tris/HCl pH 7.5, 10 min, 10 μg of protein, 37°C.*

3.1.3.b Tar-HAMP$_{Tar}$-CyaG AC chimera

Construct design, expression and membrane preparation

The N-terminus of the chimera is from the Tar receptor (M1-R213) including the HAMP$_{Tar}$ domain (R214-T267) linked to the CyaG CHD (L432-S671). The chimera was cloned into pQE80 (Δ XhoI, Δ NcoI) and expressed in *E. coli* BL-21 (DE3) [pREP4] (18°C, 0.1 mM IPTG, overnight). Membrane fractions were prepared and stored at -20°C. A Western blotting

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was carried out with an anti-RGSHis$_6$ antibody to check the expression and lack of degradation fragments of the protein which gave a single band of 57.9 kDa (Fig. 3.19 b). The protein had a predicted pI of 8.5.
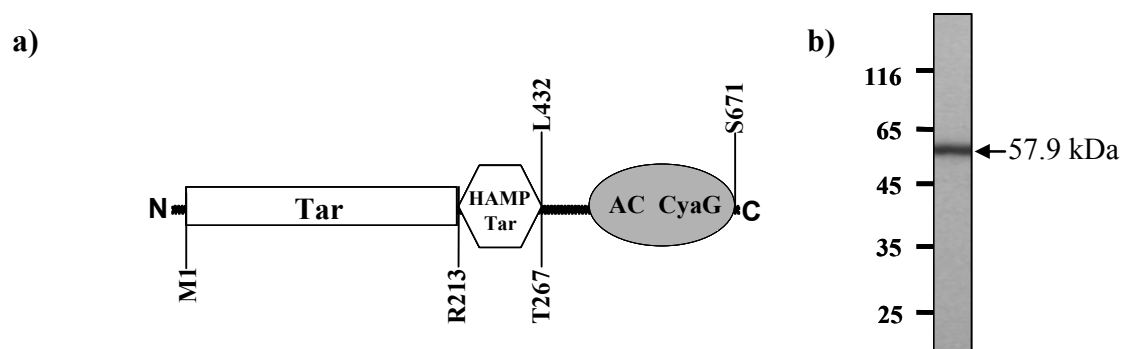
*Figure 3.19: **a)** Domain organization of the chimera; **b)** Western blot, 4µg of protein.*

Regulation by aspartate

Aspartate did not regulate. Basal activity of the protein was 36 ± 6 nmol/mg/min.
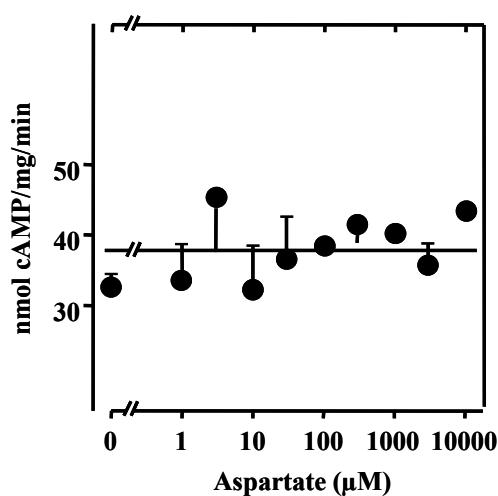
Figure 3.20: Aspartate concentration response curve; Assay conditions: 0.1 mM Tris/HCl pH 7.5, ATP 750 µM, 10 min, 10 µg of protein, 37°C, n = 2.

3.1.4 Biochemical Characterization of Trg and Tap Receptor/CyaG AC Chimeras

E. coli MCPs also include Trg and Tap receptors beside Tsr and Tar. Trg receptor binds galactose/ribose while Tap receptor binds dipeptides. Binding of respective ligands in case of

Trg and Tap is not direct as observed in the case of Tsr and Tar chemoreceptors. They need an additional periplasmic binding protein to bring about the binding of the ligands. GBP and RBP are the periplasmic binding proteins which bind galactose and ribose, respectively. Tap receptor needs DBP for binding dipeptides.

3.1.4.a Trg-HAMP$_{CyaG}$-CyaG AC and Trg-HAMP$_{Trg}$-CyaG AC

Construct design, expression and membrane preparation

The N-terminus of the chimera start with Trg (M1-R223) followed by HAMP$_{Trg}$ (R224-G276) (Fig. 3.21 a) or HAMP$_{CyaG}$ (R370-A431) (Fig. 3.21 b) linked to CyaG CHD (L432-S671). The chimeras were cloned into pQE80 (Δ XhoI, Δ NcoI) and expressed in *E. coli* BL21 (DE3) [pREP4] (18°C, 0.1 mM IPTG, overnight). Membrane fractions were prepared and stored at -20°C. A Western blot was carried out with an anti-RGSHis$_6$ antibody to check expression and degradation of the protein (Fig. 3.21). Trg-HAMP$_{CyaG}$-CyaG AC chimera had a predicted pI of 9.3 while Trg-HAMP$_{Trg}$-CyaG AC chimera had predicted pI of 9.
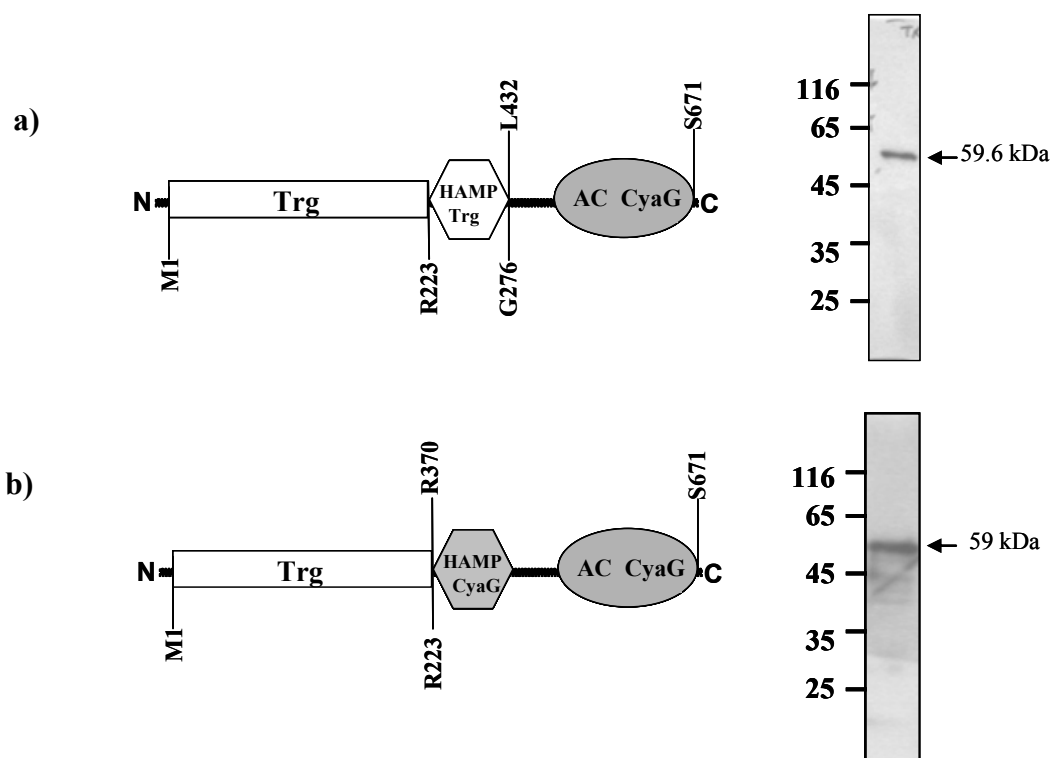
*Figure 3.21: **a)** Domain organization of Trg-HAMP$_{Trg}$-CyaG AC chimera and corresponding Western blot, 4µg of protein; **b)** Domain organization of Trg-HAMP$_{CyaG}$-CyaG AC chimera and corresponding Western blot, 4µg of protein.*

Regulation by galactose

Increasing the galactose concentration did not affect specific activity of the chimeric proteins. It is known that the Trg receptor needs a periplasmic binding protein called as GBP/RBP for chemotaxis (Hazelbauer and Adler, 1971; Kalckar, 1971; Miller et al., 1980). The ligand (galactose/ribose) first binds to these periplasmic GBP/RBP proteins which together bring about the conformational change in the periplasmic receptor. Since the AC assays were done with membrane fractions these binding proteins were absent. This may be the reason that regulation was not detected.
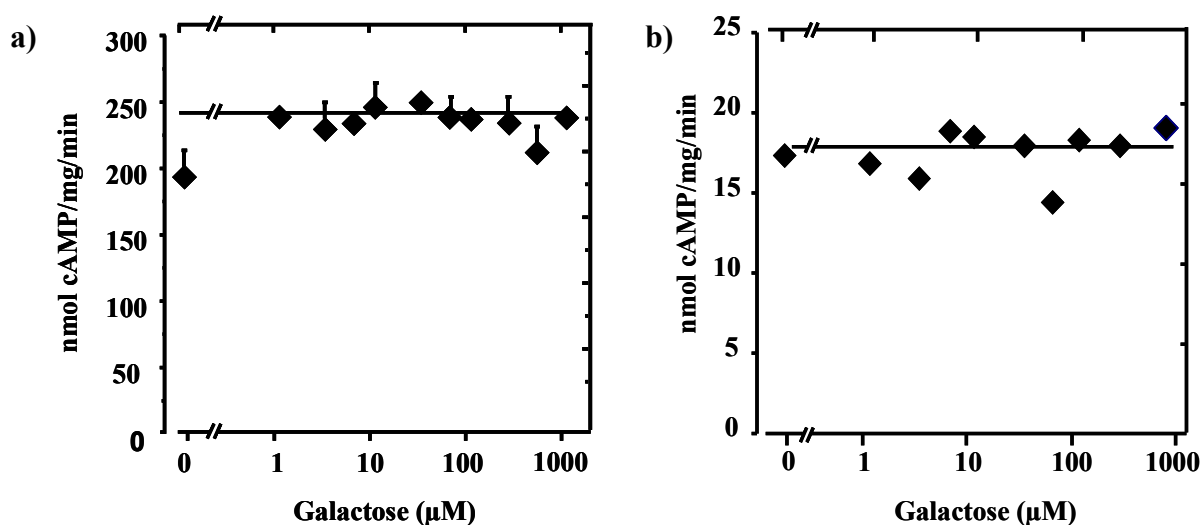
Figure 3.22: Galactose concentration response curves; **a)** Trg-HAMP$_{Trg}$-CyaG **b)** Trg-HAMP$_{CyaG}$-CyaG; Assay conditions: 0.1 mM Tris/HCl pH 7.5, ATP 750 µM, 10 min, 37°C, 10 µg of protein, n = 4.

Expression and Purification GBP

GBP was amplified along with its signal sequence from *E. coli* K12. The product was cloned into pQE80 and expressed in *E. coli* BL21 (DE3) [pREP4] (24°C, 0.1 mM IPTG, 4 h). The protein was purified by the osmotic shock method as described in section 2.8.6 (Neu and Heppel, 1965). The presence of the protein was confirmed by SDS PAGE and a Western blot with an anti-RGSHis$_6$ antibody gave a single band at the expected size (Fig. 3.23 a). Concentration response curves with the purified GBP protein were carried out to determine the concentration of GBP required for binding the receptor protein. On increasing the GBP concentrations there was no change in specific activity of the chimeric protein (Fig. 3.23 c). Two concentrations of galactose (0.5mM and 1mM) and approximately 3 µM of chimeric protein were used for the AC assay. Since the amount of GBP required for binding galactose

could not be determined from the graph, equal amount of chimeric protein and GBP were taken i.e. ~3 µM. Increasing galactose concentrations at fixed concentration of GBP did not effect the specific activity of the AC (Fig. 3.23 d and e). GBP had a predicted pI of 5.7.
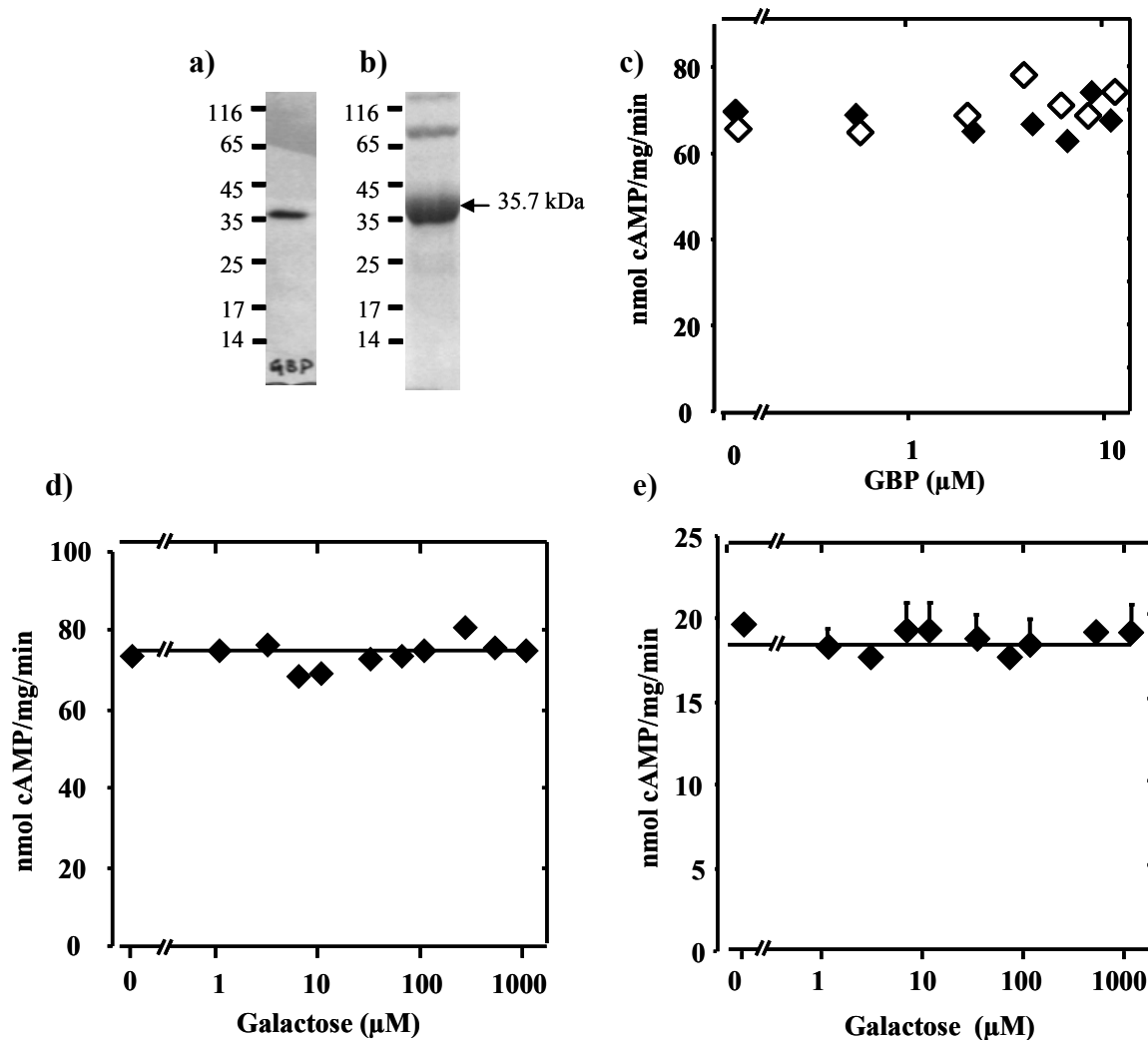
*Figure 3.23: **a)** Western blot of GBP, 1µg protein; **b)** 12% SDS PAGE of GBP, 5 µg protein; **c)** Concentration response curve with GBP: + 0.5 mM galactose (open diamonds) and + 1mM galactose (filled diamonds; Trg-HAMP$_{Trg}$-CyaG, 20 µg, n = 2); **d)** Galactose concentration response curve of Trg-HAMP$_{Trg}$-CyaG chimera, 3 µM GBP, ~3 µM of AC protein; **e)** Galactose concentration response curve of Trg-HAMP$_{CyaG}$-CyaG chimera, 3µM GBP and ~3 µM of AC protein. Assay conditions: 0.1 mM Tris/HCl pH 7.5; ATP 750 µM; 10 min; 37°C.*

Signal regulation with the Trg chimeras was not observed *in vitro*. This may be due to the fact that the concentration of GBP and ligand required for binding to each other as well as the concentration of the ligand-bound GBP complex to bind to the receptor might be below the

threshold for efficient signal regulation. Since *E. coli* has these periplasmic binding proteins, signal regulation of the chimeras was checked *in vivo* as well. *In vivo* analysis was carried out using a MacConkey assay as well as by β-galactosidase assays. The principle behind the MacConkey assay is that when *E. coli* (AC knockout strains) are transformed with an active AC and plated on MacConkey agar plates containing maltose or lactose, *E. coli* uses the maltose/lactose as the carbon source and ferments them into acids such as lactate, acetate, succinate and formate which are secreted and lower the pH of the medium. This is visualized on MacConkey agar plates with phenol red as a pH indicator by the appearance of red colonies. cAMP produced by these active ACs binds to the cAMP receptor protein (CRP) also called catabolite gene activator protein. cAMP/CRP complex in turn binds to the lactose operon and brings about the fermentation of lactose. When the AC deficient strain of *E. coli* (cya-99) alone is transformed on MacConkey plates, it produces white colonies because of its inability to ferment maltose/lactose.

Trg-HAMP$_{CyaG}$-CyaG chimera was transformed into the AC knockout strain of *E. coli* BTH101 and plated on MacConkey agar plates with maltose. A filter strip soaked with 1M galactose was placed in the middle of the plate. Galactose diffuses into the agar hence forms a gradient with high concentration in the vicinity of the filter strip and lower at the periphery. On incubating the plate at 37°C for 24 hours, red colonies were observed near the filter strip and white colonies at the periphery. This indicated that galactose activated the AC. Since inhibition of cAMP production was observed with both serine and aspartate chimeras the result was further analyzed by β-galactosidase assays. *E. coli cya* cultures transformed with Trg-HAMP$_{CyaG}$-CyaG AC were grown for 60, 75 and 90 min in presence of various galactose concentrations. After cell lysis β-galactosidase was determined spectrophotometrically. β-galactosidase is encoded by lacZ gene of the lactose operon and is responsible for the breakdown of lactose. Decrease in cAMP results in a decrease in β-galactosidase formation. β-galactosidase recognizes a synthetic compound o-nitrophenyl-β-D-galactoside (ONPG) as a substrate and cleaves it into o-nitrophenol which has a yellow color. Production of o-nitrophenol per unit time is proportional to the concentration of β-galactosidase, thus, the production of yellow color was used to determine enzyme concentrations. The assay indicated that galactose inhibited AC activity in contrast to the results on the MacConkey plates. At 60 min there was no inhibition, at 75 min a slight inhibition could be seen and at 90 min the inhibition was more prominent. The inhibition was approximately 35%. May be the

activation seen on the MacConkey was from a nonspecific interaction of galactose with the maltose operon.

a)

1 M galactose

MacConkey agar + maltose plate

b)
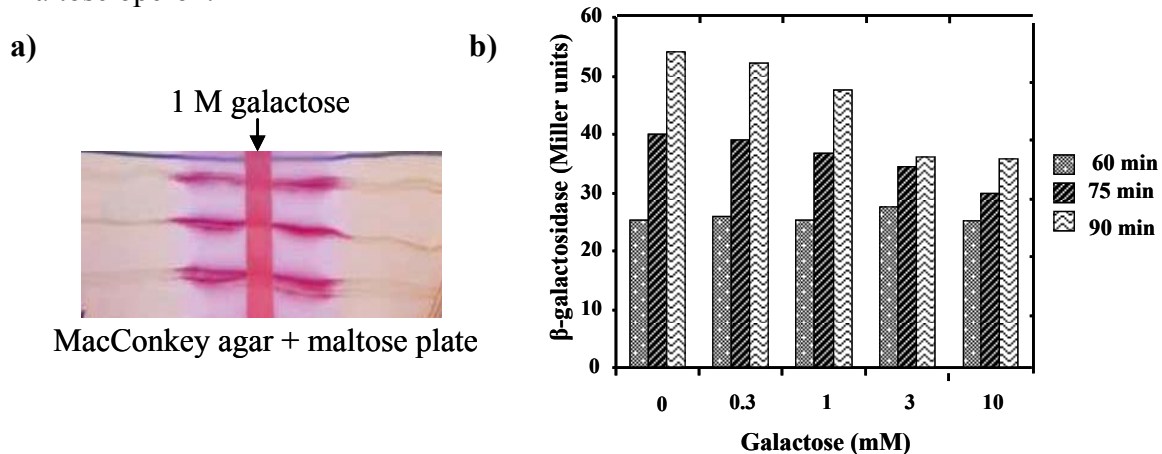
*Figure 3.24: **a)** MacConkey assay, formation of red colonies near the filter strip and white colonies at the periphery indicating the activation of AC at high galactose concentrations; **b)** β-galactosidase assay, n = 2.*

3.1.4.b Tap-HAMP$_{CyaG}$-CyaG AC and Tap-HAMP$_{Tap}$-CyaG AC

Construct design, expression and membrane preparation

The N-terminus of the chimera starts with Tap (M1-T210) followed by HAMP$_{Tap}$ (R211-S264) or HAMP$_{CyaG}$ (R370-L432) (figure 3.25) which is linked to CyaG CHD (L432-S671). The chimeras were cloned into pQE80 and expressed into *E. coli* BL21 (DE3) [pREP4] (18°C, 0.1 mM IPTG, overnight). Membrane was prepared and stored at -20°C. A Western blot was carried out with an anti-RGSHis$_6$ antibody which gave a single band confirming the presence of intact, undegraded protein. The predicted pI's of Tap-HAMP$_{CyaG}$-CyaG AC and Tap-HAMP$_{Tap}$-CyaG AC were 7.6 and 8.4, respectively.
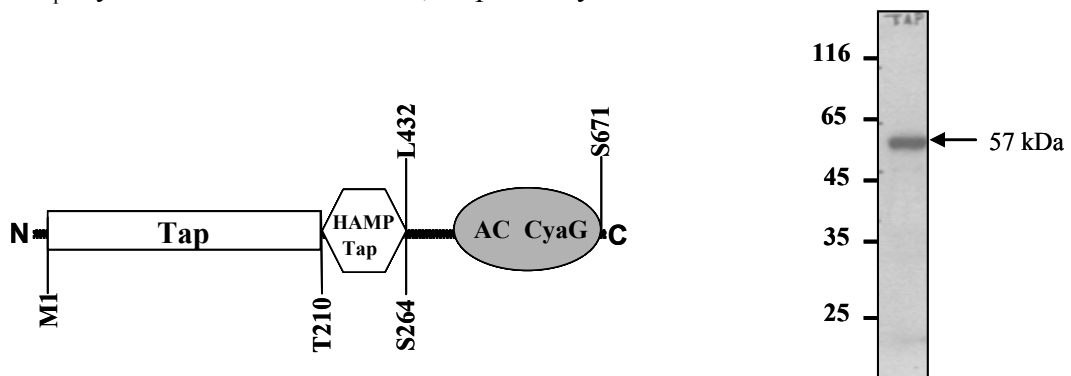
*Figure 3.25: **a)** Domain organization of Tap-HAMP$_{Tap}$-CyaG AC chimera with corresponding Western blot, 4μg protein.*

66
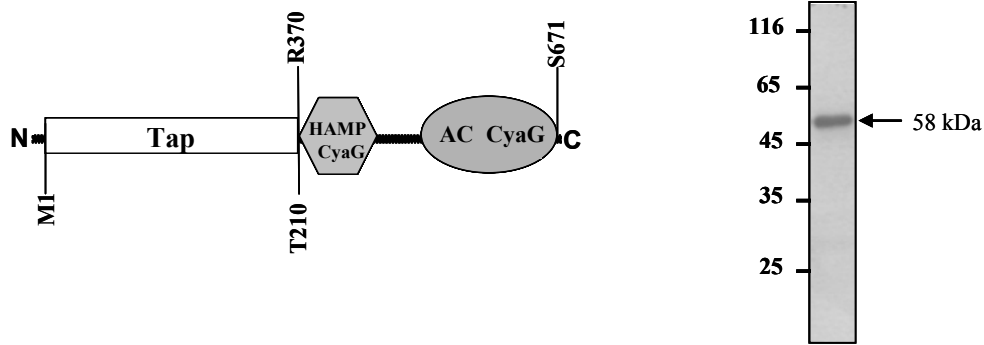
b) *Domain organization of Tap-HAMP$_{CyaG}$-CyaG chimera with corresponding Western blot, 4 µg protein.*

Regulation by glycylglycine

Increasing concentration of the dipeptide glycylglycine had no effect on the AC activity of the chimeras. Like Trg receptor the dipeptide receptor Tap also requires a periplasmic binding protein which binds dipeptides and brings about the conformational change in the receptor leading to chemotaxis (Abouhamad et al., 1991; Manson et al., 1986). DBP was amplified from *E. coli* K12. The product was cloned into pQE80 and expressed in *E. coli* BL21 (DE3) [pREP4]. The expression and purification procedures for the protein were those used for GBP. A Western blot was carried out with an anti-RGSHis$_6$ antibody which gave a single band confirming the presence of intact, undegraded protein (Fig. 3.26 a). Increase in the glycylglycine concentration in presence of 3 µM of purified DBP did not affect the AC activities. *In vivo* experiments with MacConkey and β-galactosidase were negative (data not shown). The predicted pI of DBP was 6.9.
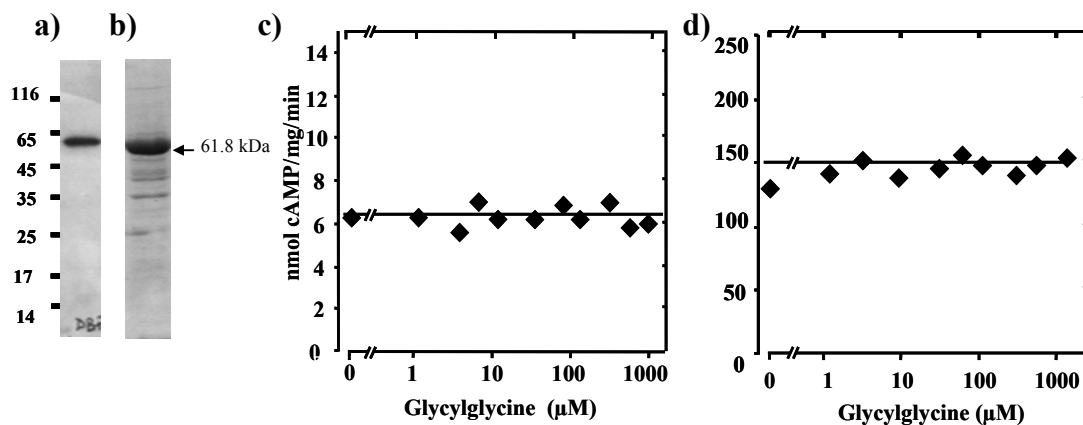
Figure 3.26: **a)** *Western blot of DBP, 1 µg protein;* **b)** *12% SDS PAGE of purified DBP, 5 µg;* **c)** *Glycylglycine concentration response of Tap-HAMP$_{CyaG}$-CyaG chimera;* **d)** *Glycylglycine concentration response of Tap-HAMP$_{Tap}$-CyaG chimera; Assay conditions: 0.1mM Tris/HCl pH 7.5, ATP 750 µM, chimeric protein ~3 µM and DBP 3 µM, 10 min, 37°C, n = 4.*

3.2 HAMP mediated Signal Transmission by Class IIIb ACs

Class IIIb CHD is distinguished by the replacement of the substrate defining aspartate with a hydroxyl amino acid, mostly threonine, occasionally serine. To investigate whether this difference has any effect on the signaling of the chimeric proteins, Rv3645 AC which is a class IIIb AC from mycobacteria was connected to Tsr and Tar in a similar way as done for CyaG chimeras.

3.2.1 Biochemical characterization of Tsr/Rv3645c AC chimeras

3.2.1.a Tsr-HAMP$_{Tsr}$-Rv3645 AC

Construct design, expression and membrane preparation

The N-terminus of the chimera starts with Tsr (M1-K215) followed by HAMP$_{Tsr}$ (A216-G268). Rv3645 has a linker of 34 aa which connects the HAMP$_{Tsr}$ domain to Rv3645 CHD (L331-G549). The chimera was cloned into pQE30 and expressed in *E. coli* BL21 (DE3) [pREP4] (22°C, 0.1 mM IPTG, 5 h). Membrane fractions were prepared as described in section 2.8.4 and stored at -20°C. A Western blot was carried out with an anti-RGS-His$_6$ antibody which showed a single band at the expected size of 54 kDa. The protein had a predicted pI of 5.6.
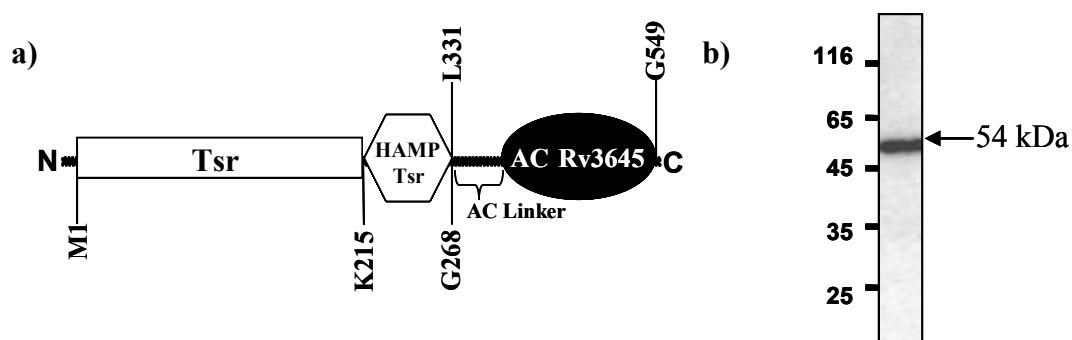
*Figure 3.27: **a)** Domain organization of the chimera; **b)** Western blot, 4µg protein.*

Regulation by serine

On increasing the serine concentration specific activity decreased. The inhibition was specific since 1 mM aspartate had no effect. The chimera was tested with the 20 proteinogenic amino acids. None of the other amino acids inhibited the AC activity which confirmed receptor specificity (Fig. 3.28 b). The IC_{50} for serine was 18.5 ± 7 µM and the maximum serine inhibition was $71 \pm 3\%$ (Fig. 3.28 a).
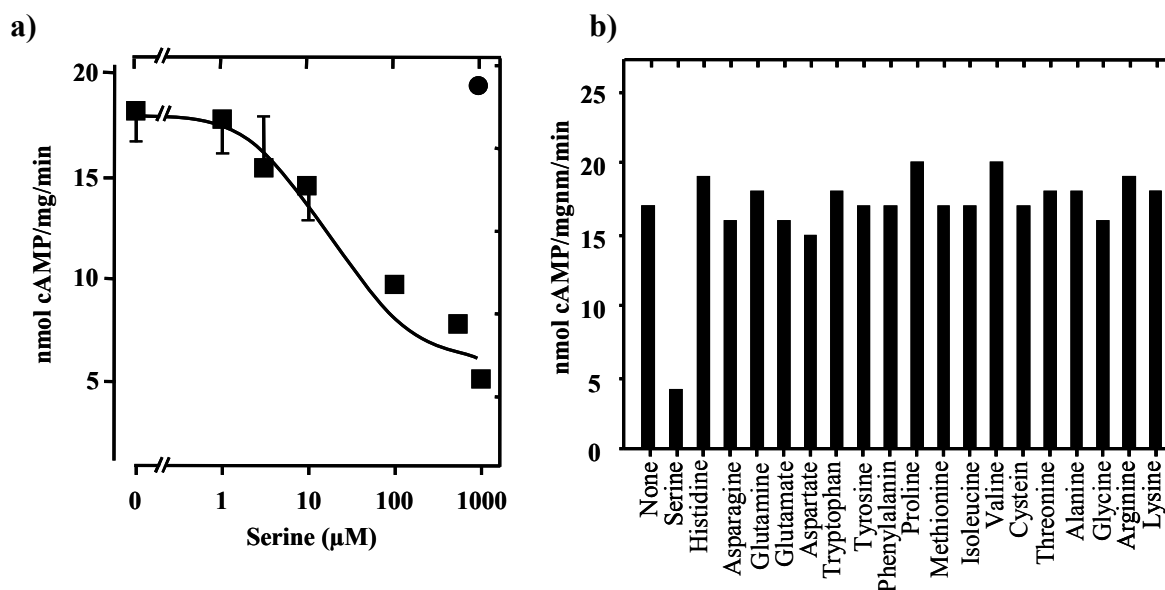
*Figure 3.28: **a)** Serine concentration response curve, circle: 1 mM aspartate, n = 5; **b)** Serine specificity, 1 mM of all 20 amino acids. Assay conditions: 0.1 mM Tris/HCl, 37°C, 10 min, 200 µM ATP, 20 µg protein.*

Substrate kinetics

Substrate kinetics were carried out with 50-400 µM ATP. Km and Vmax of the chimera were calculated from the Lineweaver Burk plot. In the absence of serine the Km was 0.5 mM ATP and Vmax was 36 nmol/mg/min while in presence of 1 mM serine the Km was 1.7 mM and Vmax was 22 nmol/mg/min. The AC activity starts to level off approximately at 200 µM ATP concentration which was the same as observed initially by J. Linder for Rv3645 CHD. This ATP concentration was used for all further experiments with Rv3645 AC chimeras. The Hill coefficient of the construct without serine was 1 ($R^2 = 0.99$) and with serine 0.94 ($R^2 = 0.97$) indicating no cooperativity.

a)

b)

c)
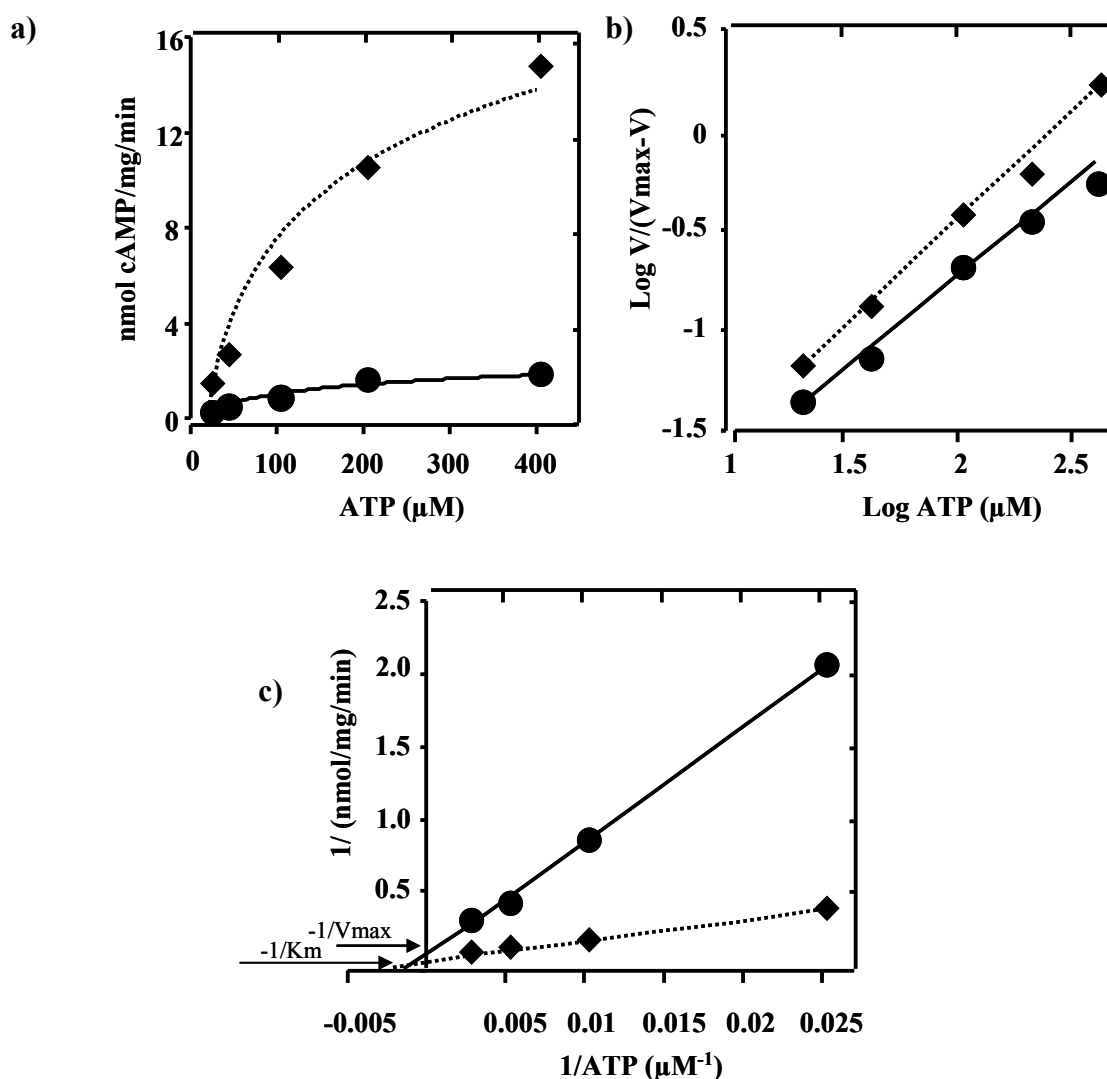
*Figure 3.29: Substrate kinetics, **a)** Michaelis-Menten plot; **b)** Hill plot; **c)** Lineweaver-Burk plot; circles: with 1mM serine; diamonds: without serine. Assay conditions: 0.1 mM Tris/HCl pH 7.5, 37°C, 10 min, 20 µg protein.*

3.2.1.b Tsr-HAMP$_{3645}$-Rv3645 AC

Construct design, expression and membrane preparation

The N-terminus of the chimera is from Tsr receptor (M1-K215) linked to HAMP$_{3645}$ (M278-R330) and Rv3645 CHD (L331-G549). The chimera was cloned into pQE30 and expressed in *E. coli* BL21 (DE3) [pREP4] (22°C, 0.1 mM IPTG, 5 h). Membranes were prepared as described in section 2.8.4 and stored at -20°C. A Western blot was carried out with an anti-

70

RGSHis$_6$ antibody which showed a single band at the expected size of 54.6 kDa. The predicted pI of the protein was 5.6.
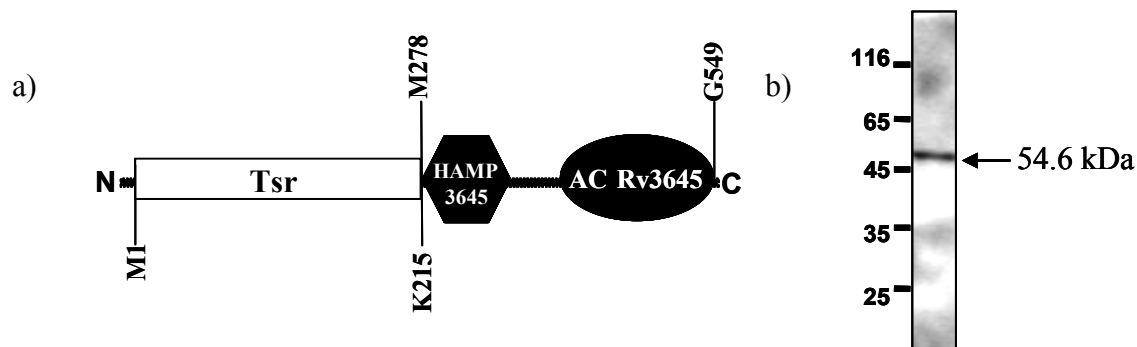
*Figure 3.30: **a)** Domain organization of the chimera; **b)** Western blot, 4 µg protein.*

Regulation by serine

On increasing the serine concentration specific activity decreased. The inhibition was specific for serine as 1 mM aspartate had no affect on activity. The IC$_{50}$ for serine was 15 ± 2 µM and the maximum serine inhibition was $45 \pm 5\%$ (Fig. 3.31 a).

Substrate kinetics

Substrate kinetics were carried out with 50-500 µM ATP. Km and the Vmax of the reaction were calculated from Lineweaver-Burk plot. In the absence of serine the Km of the chimera was 351 µM ATP and Vmax was 10.4 nmol/mg/min while in presence of serine the Km was 369 µM and Vmax was 6.6 nmol/mg/min. The Hill coefficient without serine was 1.16 ($R^2 = 0.97$) and with serine 1.17 ($R^2 = 0.98$) indicating no cooperativity.
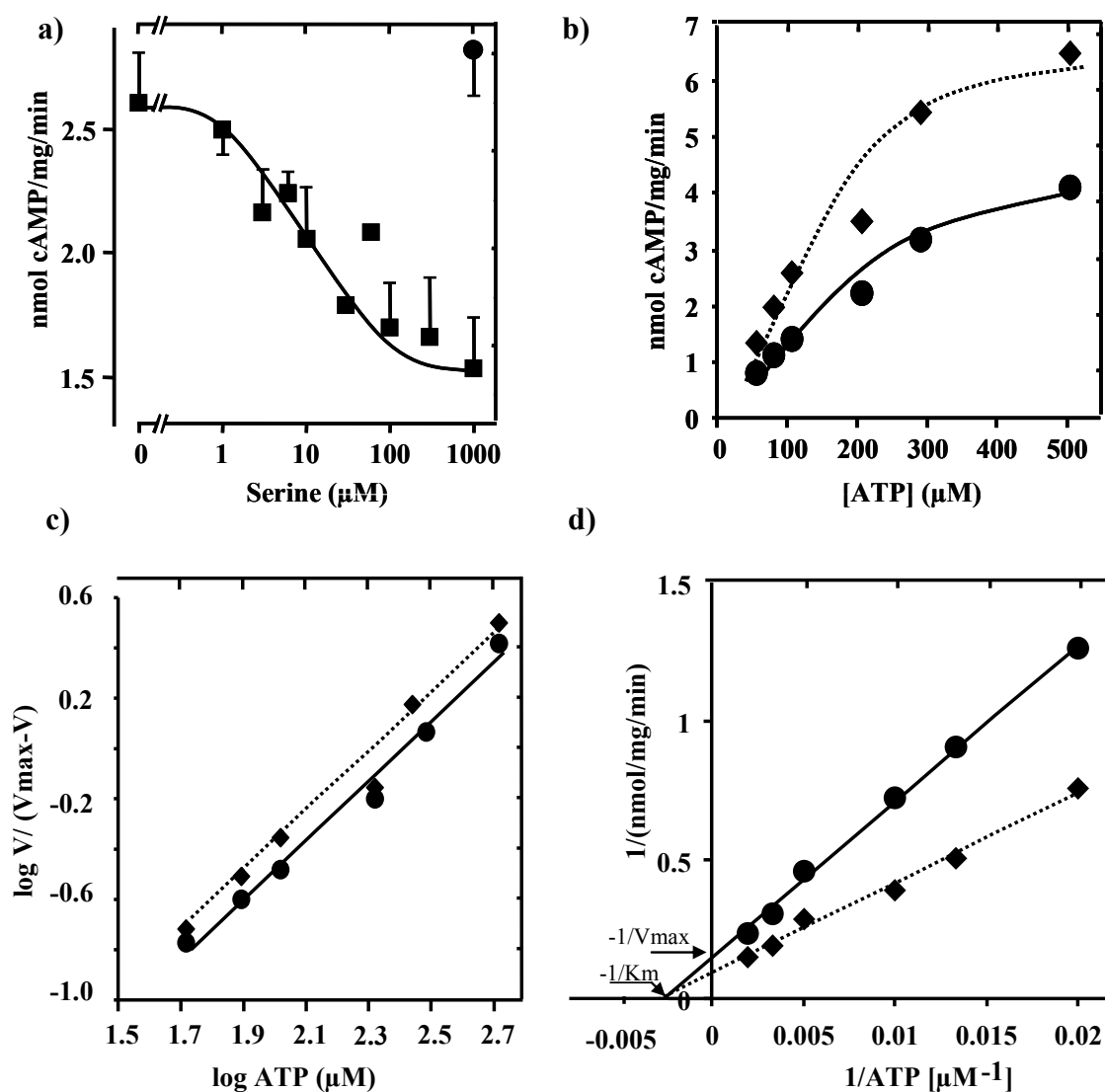
*Figure 3.31 **a)** Serine concentration response curve; circle: 1 mM aspartate, n = 4; **b)** Michaelis-Menten plot; **c)** Hill plot; **d)** Lineweaver-Burk plot, circles: with 1mM serine; diamonds: without serine. Assay conditions: 0.1 mM Tris/HCl pH 7.5, 37°C, 10 min, 20 μg protein.*

3.2.1.c Tsr-HAMP$_{Tar}$ -Rv3645 AC

Construct design, expression and membrane preparation

The chimera starts with Tsr receptor (M1-K215) followed by HAMP$_{Tar}$ (R214-T267) and Rv3645 CHD (L331-G549). The chimera was cloned into pQE30 and expressed in *E. coli.* BL21 (DE3) [pREP4]. Expression and membrane preparations were carried out similar to the chimera in 3.2.1 a. A Western blot was carried out with an anti-RGSHis$_6$ antibody which gave a single band of 54 kDa. The protein had a predicted pI of 5.8.
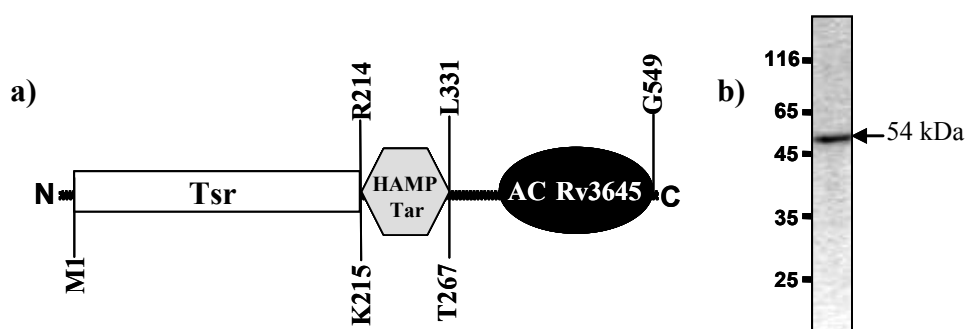
Figure 3.32: **a)** *Domain organization of the chimera;* **b)** *Western blot, 4 µg protein.*

Regulation by serine

On increasing the serine concentration cAMP production was inhibited. Inhibition was specific for serine as 1 mM aspartate used as a control had no effect on activity. The IC_{50} for serine was 4.2 µM ± 1 µM and the maximum inhibition was 73 ± 2% (Fig. 3.33 a).

Substrate kinetics

Substrate kinetics were carried out with 50-500 µM of ATP. Km and the Vmax of the reaction were calculated from Lineweaver-Burk plot (Fig. 3.33). In the absence of serine the Km of the chimera was 454 µM and Vmax was 19.4 nmol/mg/min while in presence of 1 mM serine the Km was 322 µM and Vmax was 2.5 nmol/mg/min. The Hill coefficient of the construct without serine was 1.2 ($R^2 = 0.98$) and with serine 1.2 ($R^2 = 0.87$) which indicates no cooperativity.
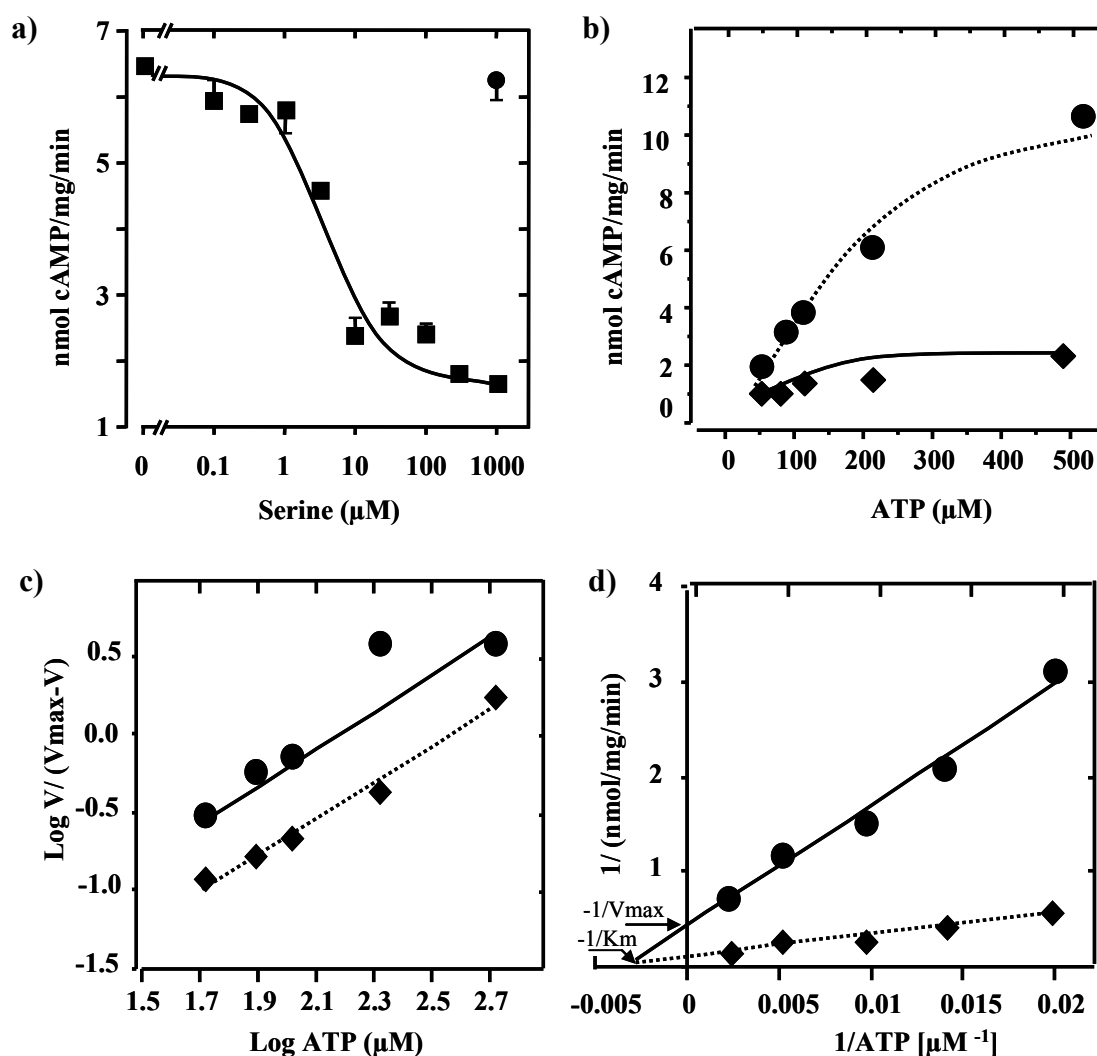
Figure 3.33: *a)* *Serine concentration response curve; circle: 1 mM aspartate, n = 6;* *b)* *Michaelis-Menten plot;* *c)* *Hill plot;* *d)* *Lineweaver-Burk plot; circles: with 1mM serine; diamonds: without serine. Assay conditions: 0.1mM Tris/HCl pH 7.5, 37°C, 10 min, 20 µg protein.*

3.2.2 Biochemical characterization of Tar/Rv3645 chimeras

3.2.2.a Tar-HAMP$_{3645}$-Rv3645AC

Construct design, expression and membrane preparation

The chimera starts with the Tar receptor domain (M1-R213) followed by HAMP$_{3645}$ (M278-R330) and the Rv3645 CHD (L331-G549). The chimera was cloned into pQE80 (Δ Xho1, Δ

Nco1) and expressed in *E. coli* BL21 (DE3) [pREP4]. Expression and membrane preparations were carried out similar to the chimera in 3.2.1 a. A Western blot was carried with an anti-RGSHis$_6$ antibody which gave a single band at the expected size of 54.7 kDa. The predicted pI of the protein was 6.
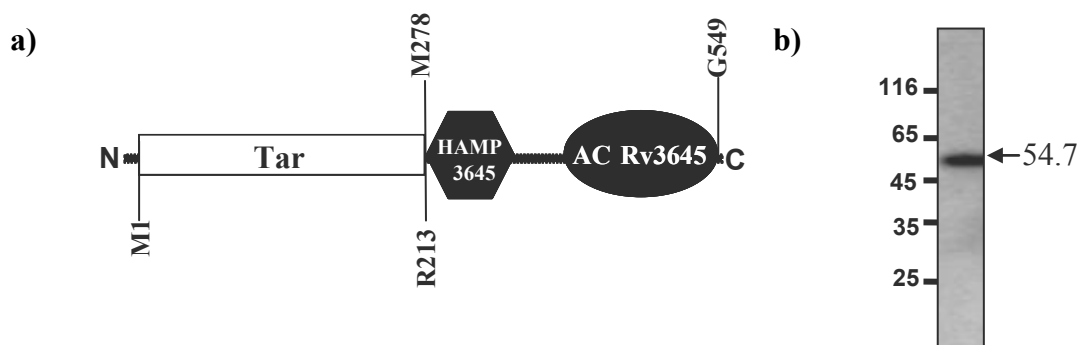
Figure 3.34: ***a)*** *Domain organization of the chimera;* ***b)*** *Western blot, 4 μg protein.*

Regulation by aspartate

On increasing the aspartate concentration AC activity was decreased. Inhibition was specific for aspartate, as 1mM serine had no effect The IC$_{50}$ for aspartate was 10 μM ± 1 μM and the maximum aspartate inhibition was 42 ± 5% (Fig. 3.35 a).

Substrate kinetics

Substrate kinetics were carried out with 50-500 μM of ATP. In the absence of aspartate the Km of the chimera was a 575 μM ATP and Vmax was 14.3 nmol/mg/min while in presence of aspartate the Km was 390 μM and Vmax was 9 nmol/mg/min. The Hill coefficient of the construct without aspartate was 1.3 (R^2 = 0.99) and with aspartate 1.3 (R^2 = 0.98) showing no clear positive cooperativity.
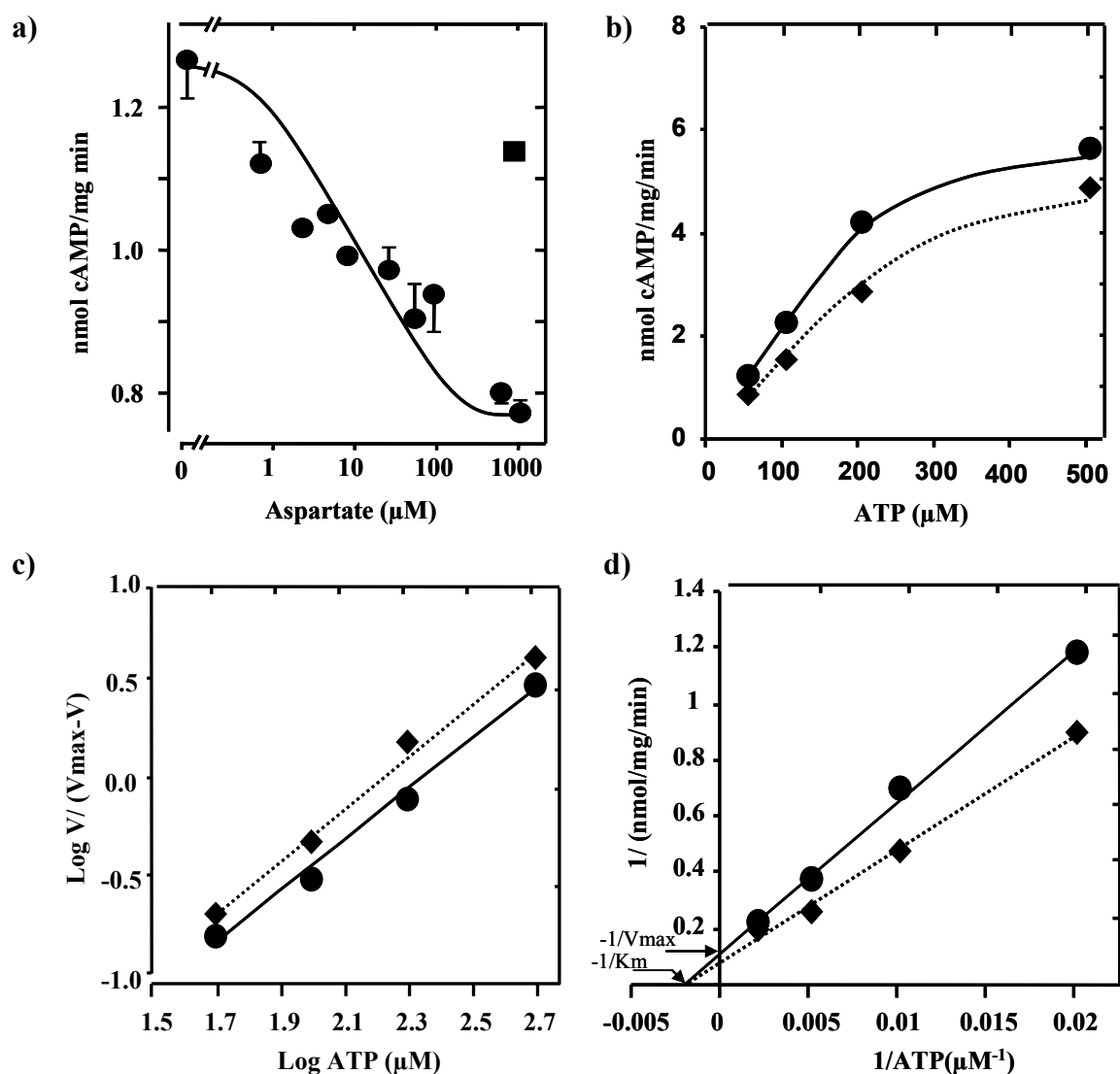
Figure 3.35: **a)** *Aspartate concentration response curve; square: 1 mM serine, n = 2;* **b)** *Michaelis-Menten plot;* **c)** *Hill plot;* **d)** *Lineweaver-Burk plot; circles: 1mM aspartate; diamonds: without aspartate. Assay conditions: 0.1 mM Tris/HCl pH 7.5, 37°C, 10 min, 20 µg protein.*

3.2.2.b Tar- HAMP$_{Tar}$-Rv3645 AC

Construct design, expression and membrane preparation

The chimera starts with Tar (M1-R213) followed by HAMP$_{Tar}$ (R214-T266) and Rv3645 CHD (L331-G549). The chimera was cloned into pQE30 and expressed in *E. coli* BL21

(DE3) [pREP4]. Expression and membrane preparations were carried out similar to the chimera in 3.2.1 a. A Western blot was carried out with an anti-RGSHis$_6$ antibody which gave a single band at the expected size of 54.3 kDa and the predicted pI of the protein was 6.7.

a)   b)
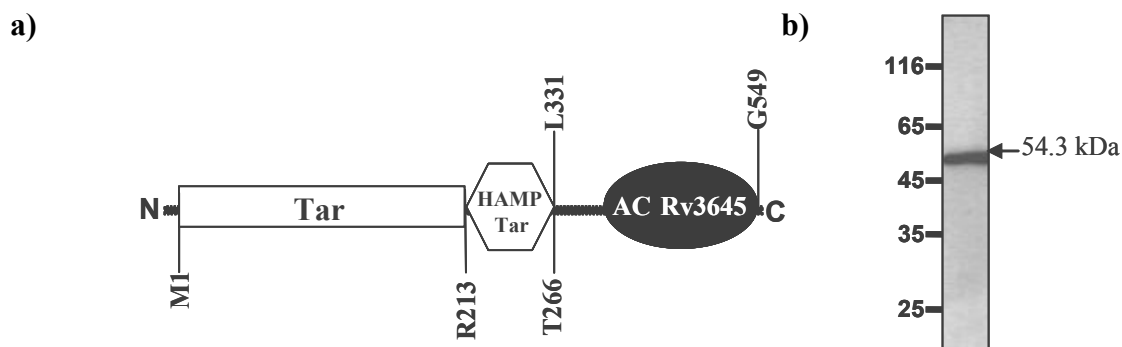
*Figure 3.36: **a)** Domain organization of the chimera; **b)** Western blot, 5µg protein.*

Regulation by aspartate

Aspartate up to 10 mM did not inhibit AC activity (Fig. 3.37).
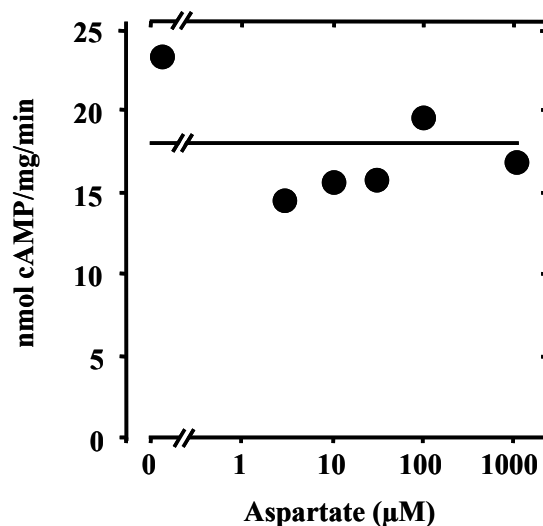
Figure 3.37: Aspartate concentration response curve; Assay conditions: 0.1 mM Tris/HCl pH 7.5, 37°C, 10 min, ATP 200 µM, 20 µg protein, n = 2.

3.2.2.c Tar-HAMP$_{Tsr}$ -Rv 3645 AC

Construct design, expression and membrane preparation

The chimera starts with Tar (M1-R214) followed by HAMP$_{Tsr}$ (A216-G268) and Rv3645 CHD (L331-G549). The chimera was cloned into pQE30 and expressed in *E. coli* BL21 (DE3) [pREP4]. Expression and membrane preparations were carried out similar to the chimera in 3.2.1 a. A Western blot was carried out with an anti RGSHis$_6$ antibody which gave a single band of 54 kDa. The predicted pI of the protein was 6.
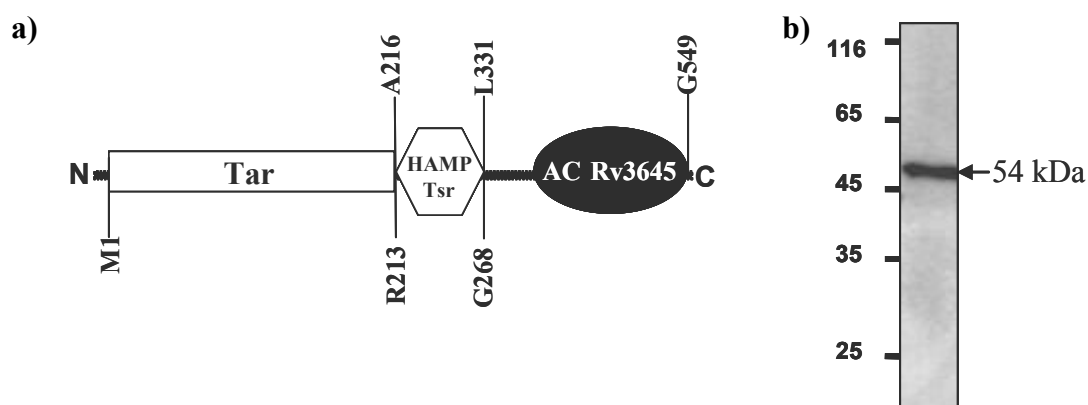
*Figure 3.38: **a)** Domain organization of the chimera; **b)** Western blot, 3 μg protein.*

Regulation by aspartate

Aspartate inhibited AC activity. The inhibition was specific for aspartate as 1 mM serine had no effect. The IC$_{50}$ for aspartate was 12 μM ± 2 μM and the maximum aspartate inhibition was 40 ± 8% (Fig. 3.39 a).

Substrate kinetics

Substrate kinetics were carried out with 50-100 μM ATP. Km and the Vmax of the reaction were calculated from Lineweaver-Burk plot. In the absence of aspartate the Km was 370 μM ATP and Vmax was 12 nmol/mg/min while in presence of aspartate the Km was 470 μM and Vmax was 8 nmol/mg/min. The Hill coefficient of the construct without aspartate was 0.9 ($R^2 = 0.90$) and with aspartate 1.2 ($R^2 = 0.99$) indicating no cooperativity.
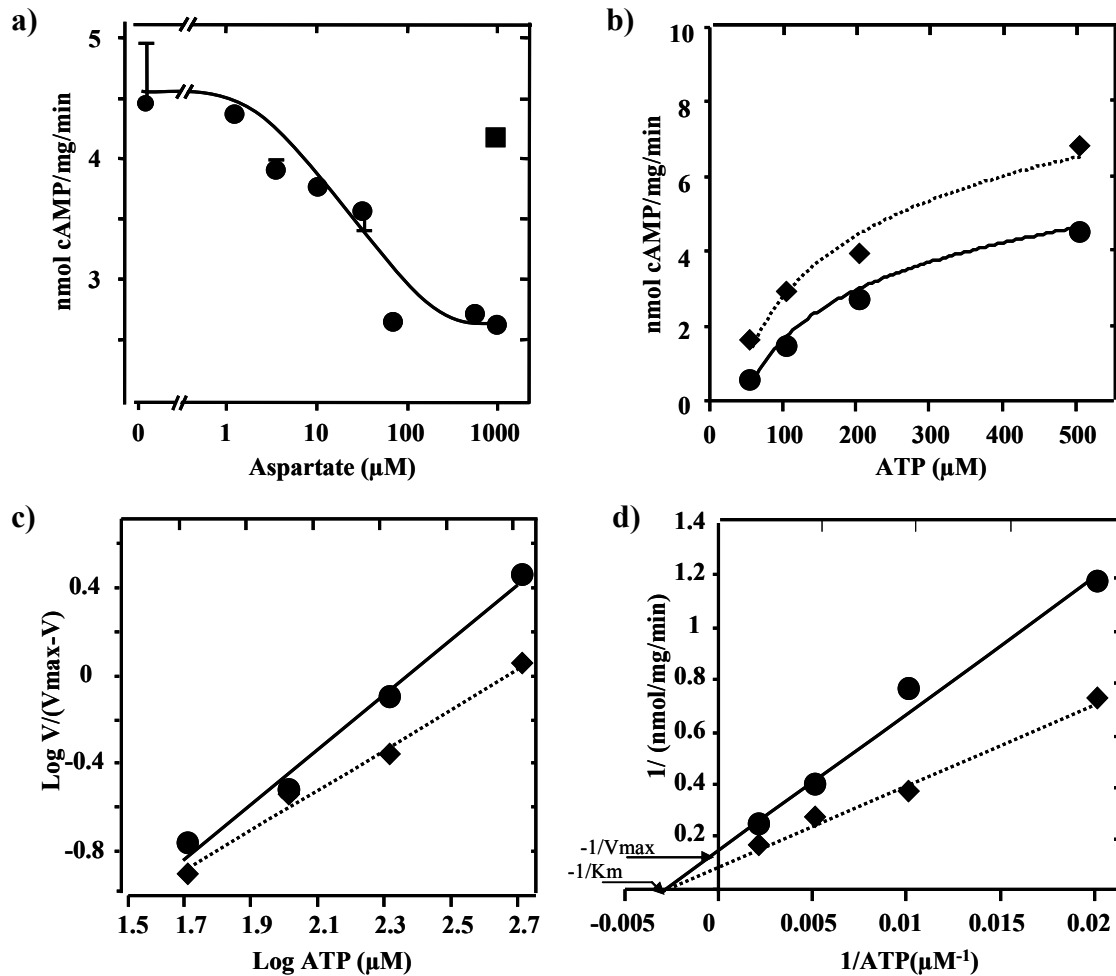
*Figure 3.39: **a)** Aspartate concentration response curve; square: 1mM serine; n = 2; **b)** Michaelis-Menten plot; **c)** Hill plot; **d)** Lineweaver-Burk plot; circles: with 1 mM aspartate; diamonds: without aspartate. Assay conditions: 0.1 mM Tris/HCl pH 7.5, 37°C, 10 min, 20 μg protein.*

3.3 Functional complementation of receptor point mutants confirming the receptor mediated signal regulation in receptor/AC chimeras

The results obtained from the chimeras above shows that ACs not only can sense intracellular signals as known before but can also sense extracellular signals. To confirm whether the signaling was due to activation of the receptor attached to its N-terminus and not due to nonspecific interaction of serine and aspartate with the chimeric proteins, two point mutations were inserted into the receptor part of the chimeric protein. Chemotaxis receptors Tsr and Tar can be inactivated by different single point mutations. It has been shown before that such

point mutants are unable to signal as homodimers but as soon as two of such complementary mutants heterodimerize, chemotaxis is restored (Fig. 3.40) (Ames et al., 2008; Gardina and Manson, 1996; Mowbray and Koshland, 1990).
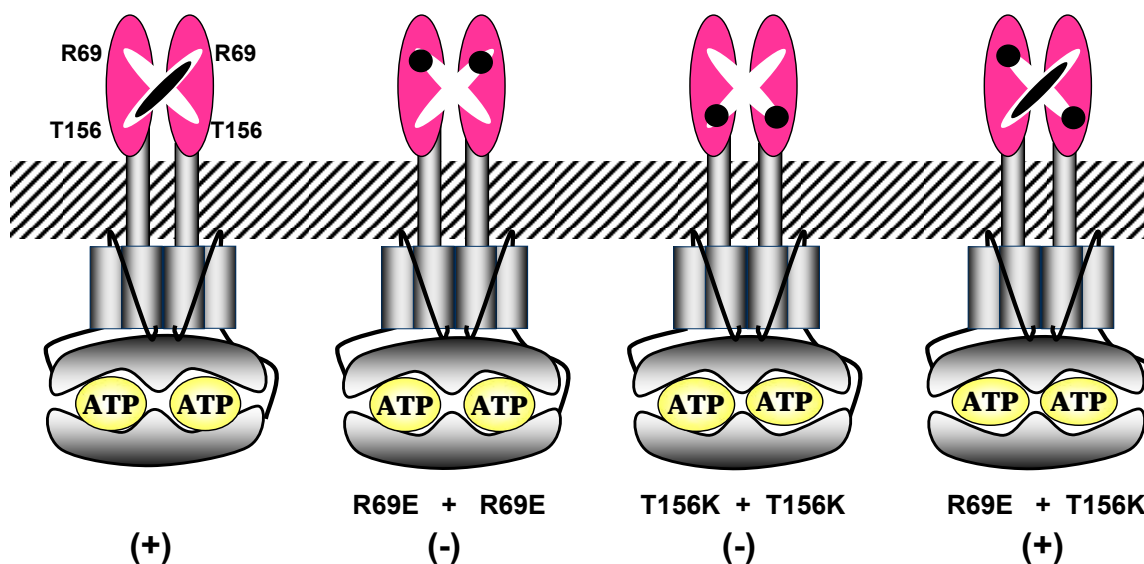
Figure 3.40: The rationale for receptor complementation is shown. The residues R69 and T156 in Tsr receptor are essential for ligand binding. Mutants R69E and T156K cannot bind serine and signal. Heterodimers i.e. (R69E + T156K) complement each other and signaling is restored (Gardina and Manson, 1996).

Tsr-HAMP$_{Tsr}$-Rv3645 AC chimera was used for this study. The individual mutants R69E and T156K were generated. When these two mutants were expressed separately, the proteins had AC activity but were not regulated. When the mutants were co-expressed together signaling was restored. Tsr mutants were cloned into pQE30 and expressed in *E. coli* BL21 (DE3) [pREP4]. The proteins were probed with an anti-RGS His$_6$ antibody, which showed single bands confirming the integrity of the proteins. For co-expression the mutants were cloned into pETDUET and expressed in *E. coli* BL21 (DE3) [pREP4]. pETDUET has two multiple cloning sites (MCS) hence two proteins can be expressed simultaneously in equal amounts. MCS I has an N-terminal histidine tag while MCS II has a C-terminal S-tag. Tsr (R69E) mutant was cloned into the MCS I site and Tsr (T156K) was cloned into the MCS II site. Both mutants were co-expressed (22°C, 0.1 mM IPTG, 4h). The R69E mutant was probed with an anti-His$_4$ antibody and T156K was probed with an anti S-tag antibody. Both Western blots gave a single band confirming the expression of both mutants simultaneously.

Similar experiments were done with Tar-HAMP$_{CyaG}$-CyaG AC chimera. Tar (R69E) and Tar (T154I) mutants were generated and cloned into pQE80 and expressed in *E. coli* BL21 (DE3) [pREP4]. The AC was active but there was no regulation in either mutant. On co-expression in pETDUET, signaling was restored. Expression of the single mutants was confirmed by an anti-RGSHis$_6$ antibody and after co-expression the mutants were probed with an anti-His$_4$ antibody and S-tag antibody.
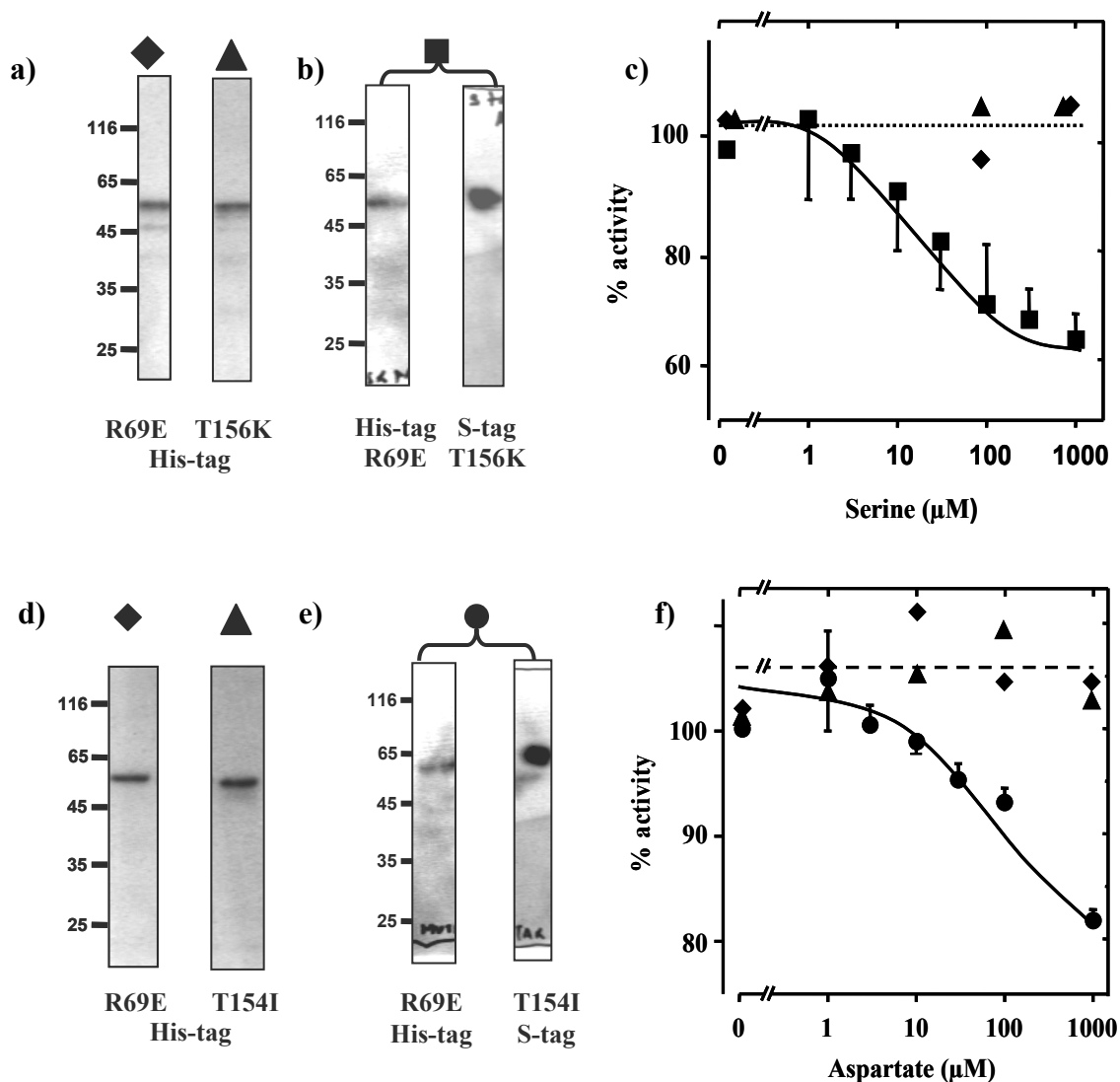
*Figure 3.41 Functional complementation of the inactivated Tsr and Tar receptor point mutants, **a)** Western blot of single mutants (Tsr), 5µg protein per lane; **b)** Western blot of co-expressed mutants, 10 µg protein per lane; **c)** Concentration response curve of the mutants; R69E, diamonds; T156K, triangles; Co-expressed mutants, squares; % inhibition 35 ± 5, IC$_{50}$ 15 ± 5 µM, (n = 4); **d)** Western blot of single mutants (Tar), 5µg protein per lane; **e)** Western blot of co-expressed mutants, 10µg protein per lane; **f)** Concentration response curve of the mutants; R69E, diamonds; T154I, triangles; Co-expressed mutant, squares; % inhibition 20 ± 4, IC$_{50}$ 20 ± 8 µM, (n= 4).*

Further controls were made to confirm receptor mediated signaling. CHD of both CyaG and Rv3645 AC were assayed with different amino acids along with serine and aspartate to see whether there is inactivation of the enzyme. No inhibition was seen with any of the amino acids used. Serine and aspartate did not inhibit the AC activity of CyaG and Rv3645 holoenzyme. The chimeras were also checked with D-aspartate. This was negative, confirming that signaling observed in the above chimeras is receptor mediated and not due to nonspecific interactions with the chimeras.
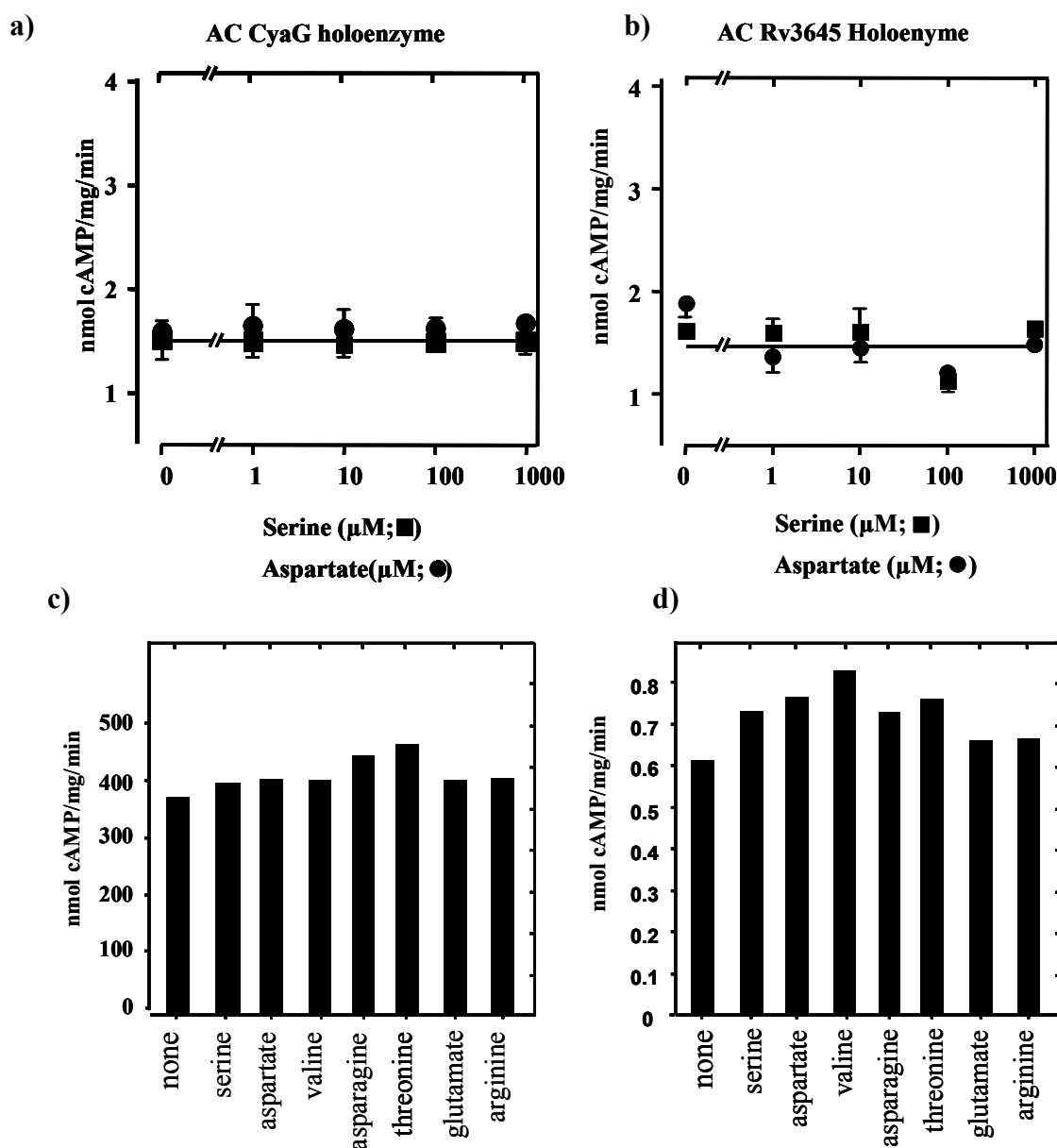
Figure 3.42: Ligand responses of Rv3645 and CyaG holoenzyme and their respective CHDs. ***a)*** *CyaG holoenzyme, serine and aspartate concentration responses;* ***b)*** *Rv3645 holoenzyme, serine and aspartate concentration responses;* ***c)*** *AC activity of CyaG CHD, 10 mM aa;* ***d)*** *AC activity of Rv3645 CHD, 10 mM aa.*

3.4 Transmembrane signaling by non HAMP containing Adenylyl cyclases

The reason why *E. coli* chemotaxis proteins were used to make AC chimeras was the fact that they share identical domain organisations as that of Rv3645 and CyaG ACs, hence it was intriguing to know whether ACs with different domain organizations could also interact with the MCPs in a similar fashion as observed in the case of Rv3645 and CyaG AC.

3.4.1 Mycobacterial Rv1264 chimeras

To investigate whether class IIIc ACs also have the capacity to sense signals of bacterial chemotaxis receptors, chimeras of Rv1264 and Tsr receptor were made. Rv1264 AC was chosen for this study because of the availability of biochemical and structural details of the AC (Linder et al., 2002; Tews et al., 2005). The domain organization of Rv1264 is different from Rv3645 AC and CyaG ACs studied before. It is a soluble AC which was reported to be a pH sensing module which regulates the CHD (Linder et al., 2002; Tews et al., 2005).

3.4.1.a Characterization of Tsr-HAMP$_{Tsr}$-Rv1264 AC

Construct design, expression and membrane preparation

The chimera starts with Tsr followed by HAMP$_{Tsr}$, a small stretch of Rv3645 linker and finally it ends with the Rv1264 CHD C-terminally (Fig. 3.43). Tsr-HAMP$_{Tsr}$-Rv3645 chimera and Rv1264 holoenzyme were aligned to find conserved regions and simultaneously to determine an appropriate site where both proteins could be connected. The chimera was cloned into pQE80 and expressed in *E. coli* BL21 (DE3) [pREP4] (0.1 mM IPTG, 22°C, 4 h). Expression and Membrane preparations were carried out as described in 2.8.2 and 2.8.4 and stored at -20°C.

a)

b)

```
          Rv 1264: QIVPLLGP MIQDM L FMQLRHM M ETEAVNAGERAAGKPLPGA RQ V T V AF ADLVG F TQL
Tsr-HAMP_Tsr-Rv3645: LRHMQGEL M RTVG L RDLFGRY V GEDVARRALERGTELGGQE RD V A V L F V DLVG S TQL
```

Tsr-HAMP$_{Tsr}$ Rv3645 Linker   Rv1264

LRHMQGELMRTVGLRDLFGRYVGEDVARRALERGTELGGGARQVTVAFADLVGFTQL

Tsr-HAMP$_{Tsr}$-Rv3645   Rv1264
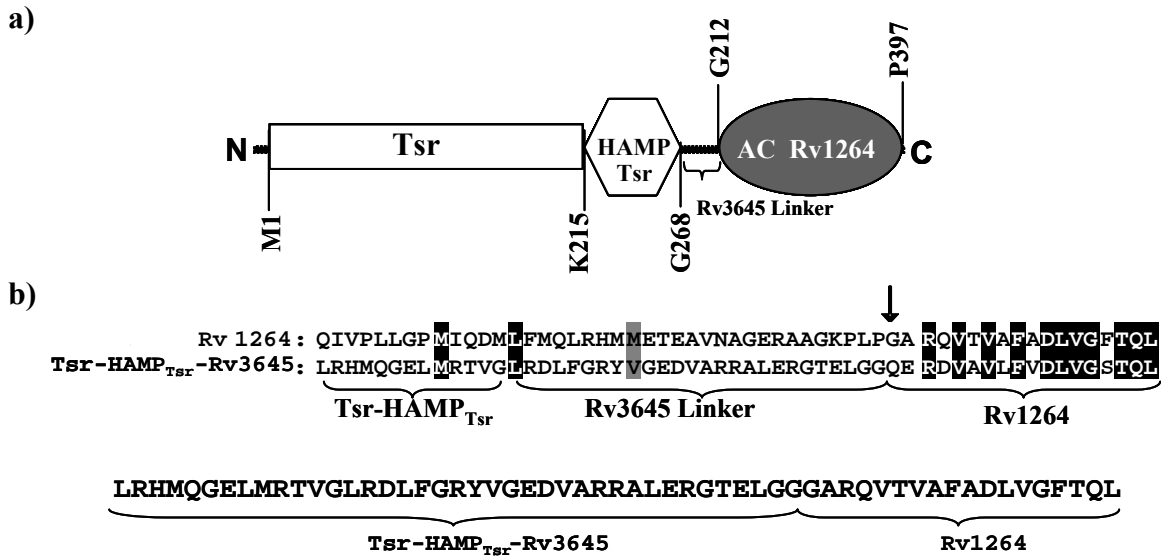
*Figure 3.43: **a)** Domain organization of the chimera; **b)** Alignment of Tsr-HAMP$_{Tsr}$-Rv3645 chimera and Rv1264 AC; residues in black are identical, grey residues are similar. The arrow marks the site where the proteins were connected. Below is the partial sequence of the chimera.*

Serine did not inhibit AC activity. The basal activity was 2.5 nmol/mg/min. A Western blot with an anti-RGSHis$_6$ antibody showed a single band of the expected size confirming the presence of undegraded protein. The protein had a predicted pI of 6.8.

*Figure 3.44: **a)** Western blot, 4µg of protein; **b)** Serine concentration response curve; Assay conditions: 0.1 mM Tris/HCl pH 7.5, 10 min, 37°C, 4 µg protein, 200 µM ATP.*

To check whether the protein retained its correct register, one or two alanines were introduced at the junction of Rv3645 linker and Rv1264 CHD. Both the mutants were cloned and expressed in a similar way as before. Serine concentration response curves for both

chimeras were established. The mutant chimeras were more active than Tsr-HAMP$_{Tsr}$-Rv1264 chimera. The chimera with two alanines had a basal activity of 100 nmol/mg/min and with one alanine 23 nmol/mg/min. Serine did not regulate. A Western blot was carried out with an anti-RGSHis$_6$ antibody which gave single bands without any degradation products. The predicted pI was 6.8 for both proteins.
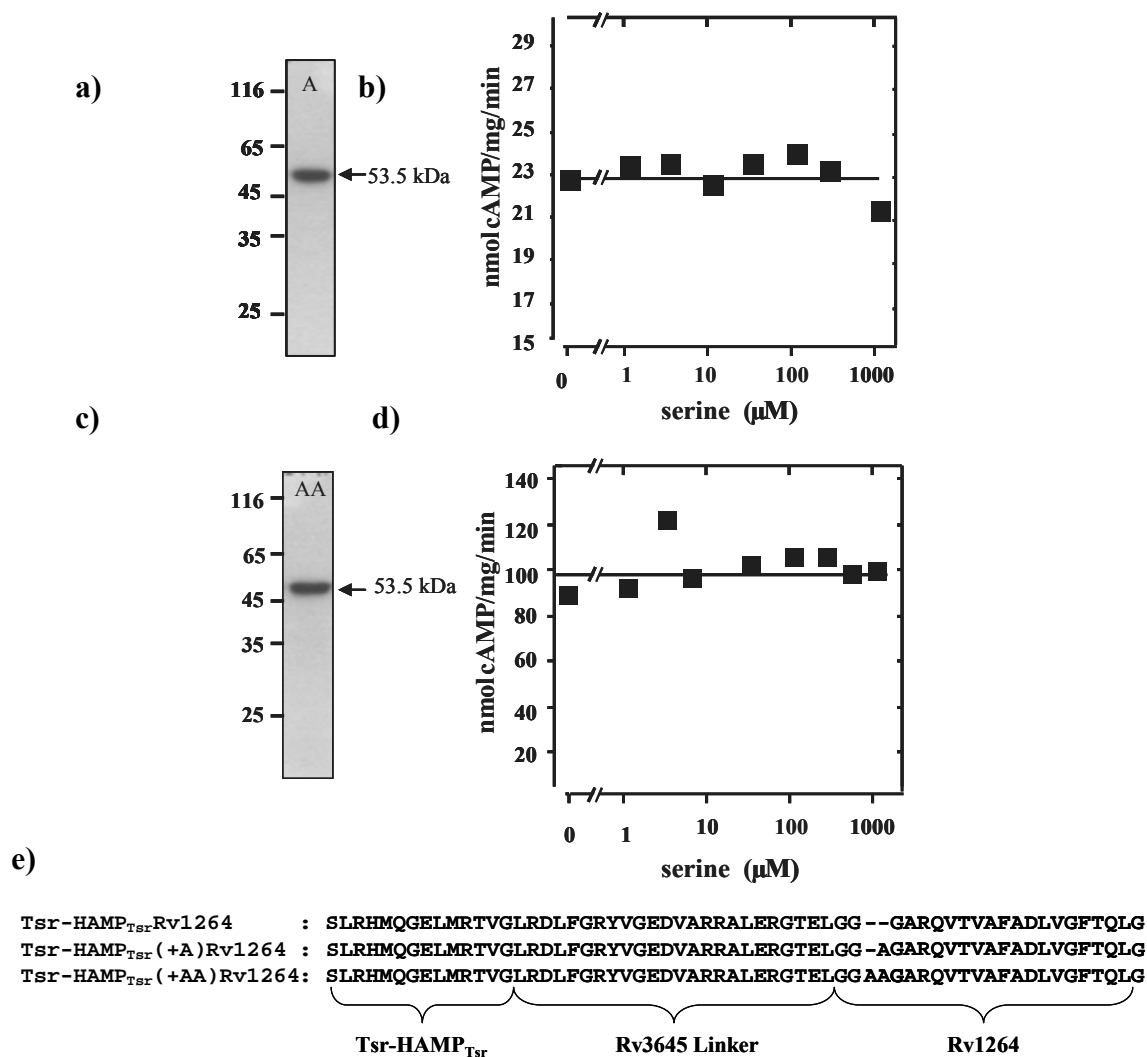
Figure 3.45: ***a)*** *Western of Tsr-HAMP$_{Tsr}$-(+A)-Rv1264 chimera, 4 µg of protein;* ***b)*** *Serine concentration response curve;* ***c)*** *Western of Tsr- HAMP$_{Tsr}$-(+AA)-Rv1264 chimera, 4µg of protein;* ***d)*** *Serine concentration response curve; Assay conditions: 0.1 mM Tris/HCl pH 7.5, 10 min, 37°C, 6 µg protein, 200 µM ATP;* ***e)*** *Alignment of Rv1264 chimeras.*

3.4.2 Cyanobacterial CyaB1 AC chimeras

Two ACs, CyaB1 and CyaB2, from *Anabaena* sp. PCC 7120 have tandem GAF domains, a PAS domain and a CHD. CyaB1 recognizes an intracellular cAMP signal via GAF domains

but the mechanism underlying the intra-molecular signal transfer is unknown. The tandem arrangement of GAF domains in mammalian phosphodiesterases (PDE2, -5, -6 and -10) is identical to that in CyaB1. A recent X-ray structure of phosphodiesterase 2A (PDE2A) proposes rotation of linkers present between the tandem GAF domains as well as between GAF and PDE2A catalytic domains for the activation of the enzyme on binding cGMP (Pandit et al., 2009). Replacing the tandem GAF domains of CyaB1 with those of rat PDE2 resulted in a chimera which could be activated by cGMP (Kanacher et al., 2002). This indicated that CyaB1 might share a similar mechanism of signal propagation like those of PDE2A. Rotation was also reported as a model for signal transfer in the case of HAMP domains (Hulko et al., 2006). Successful generation of AC and chemotaxis receptor chimeras with HAMP domains in section 3.2 suggested that the mechanism of intramolecular signal transmission might be similar in MCPs and ACs. To explore whether CyaB1 AC could also be connected to chemotaxis receptors along with the respective HAMP domains, Tsr/CyaB1 chimeras were made. The major question was whether such a chimera would be functional in a similar way as seen earlier with mycobacterial and cyanobacterial CyaG AC. If it could be regulated then we could speculate that CyaB1 employs a similar mechanism for intramolecular signalling as HAMP domains and phosphodiesterases.
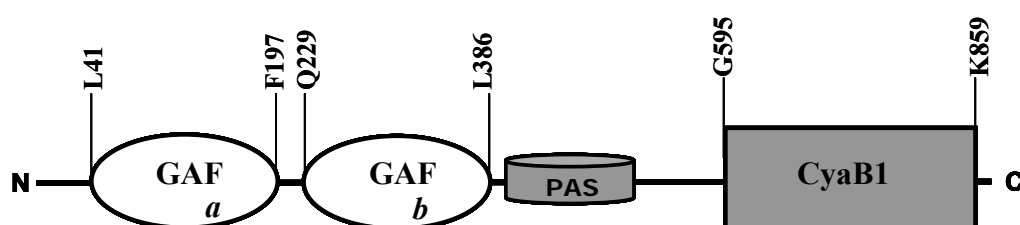
Figure 3.46: Domain organisation of CyaB1 AC from Anabaena.

3.4.2.a Characterization of Tsr-HAMP$_{Tsr}$-CyaB1 AC

Construct design, expression and membrane preparation

The chimera starts with Tsr followed by HAMP from Tsr (M1-G268), it is linked to CyaB1 CHD via the PAS domain (L386-K859). The chimera was cloned into pQE30 and expressed in *E. coli* BL21 (DE3) [pREP4] (0.06 mM, IPTG, 16°C, overnight). 10 mM sterile $MgCl_2$ was added while inducing the culture with IPTG. Membranes were prepared as described in section 2.8.4 and stored at -20°C.
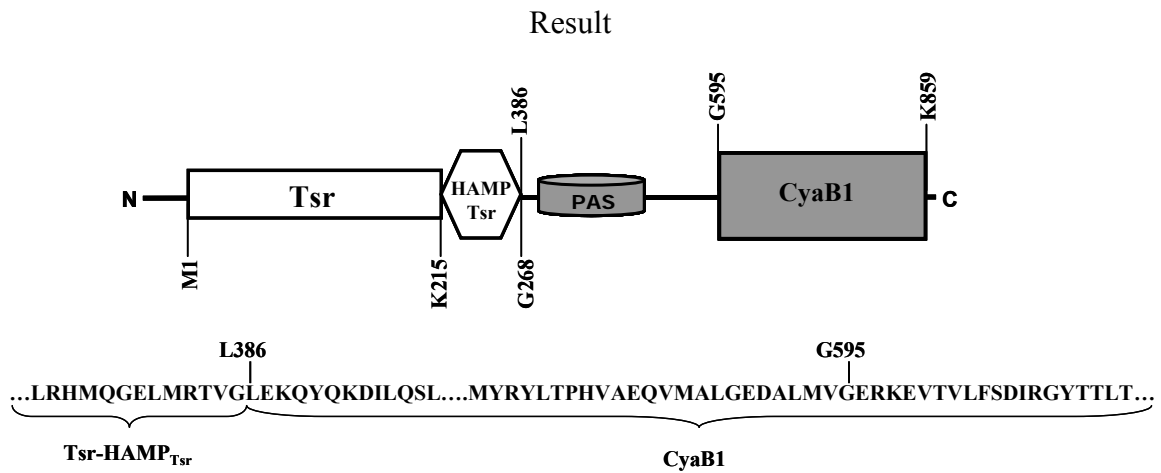
Figure 3.47: Domain organization of the chimera, below is the partial sequence of the chimera showing the point where both the proteins were linked. L386 site was used to link chimeras of PDEs/CyaB1 and CyaB2/CyaB1 chimeras before (Lab data). All these chimeras were active and optimally recombined hence this site was used to connect Tsr and CyaB1 AC.

Serine did not affect AC activity. Basal activity was 7 ± 5 nmol/mg/min. A Western blot with an anti-RGSHis$_6$ antibody gave a single band confirming the expression of the correct 85 kDa protein. The predicted pI was 6.
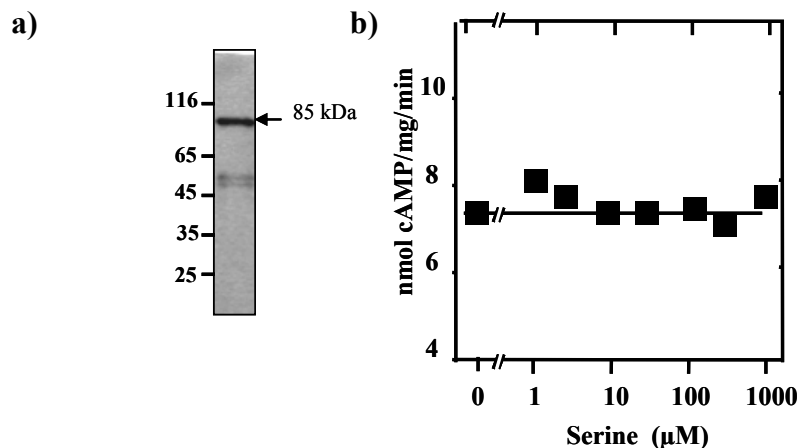
*Figure 3.48: **a)** Western blot of CyaB1 chimera, 4 µg protein; **b)** Serine concentration response curve; Assay conditions: 0.1 mM Tris/HCl pH 7.5, 10 min, 37°C, 12 µg protein, ATP 75 µM.*

3.4.2.b Tsr-HAMP$_{Tsr}$(ΔPAS)-CyaB1 AC

Tsr-HAMP$_{Tsr}$-CyaB1 AC chimera was not regulated. It might be possible that the linked domains were not in correct register or the signal was being sensed by the receptor but somehow not getting transferred efficiently by the HAMP through the PAS domain to the

CHD. Since in Rv3645 and CyaG chimeras the HAMP domain was directly linked to the CHD, a chimera was designed where the Tsr + HAMP$_{Tsr}$ was directly connected to the CyaB1 CHD. The expression and membrane preparation of the chimera was similar to the chimera in 3.3.2a. The predicted pI was 6.4.

...HMQGELMRTVGLRDLMYRYLTPHVAEQVMALGEDALMVGERKEVTVLFSDIRGYTTLT....

Tsr-HAMP$_{Tsr}$-Rv3645   CyaB1
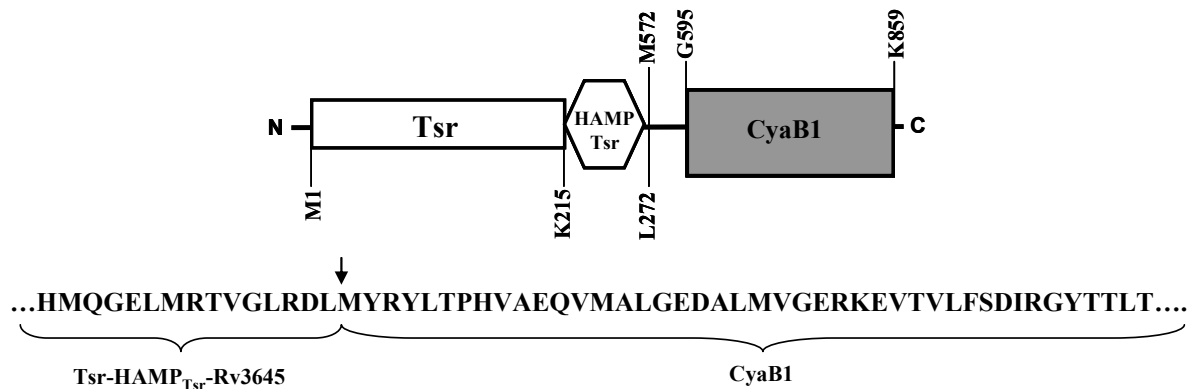
Figure 3.49: Domain organization of the chimera; below is the partial sequence of the chimera, arrow shows the point where the proteins were connected.

Serine regulated the AC activity. The inhibition was specific for serine as 1 mM aspartate had no effect. A Western blot with an anti-RGSHis$_6$ antibody gave a faint band of the correct size. The expression of the chimera compared to the chimera with PAS domain was low (figure 3.50 a). The IC$_{50}$ for serine was 12 ± 3 μM and maximum inhibition was $81 \pm 2\%$ (Fig. 3.50 b). The predicted pI was 6.4.
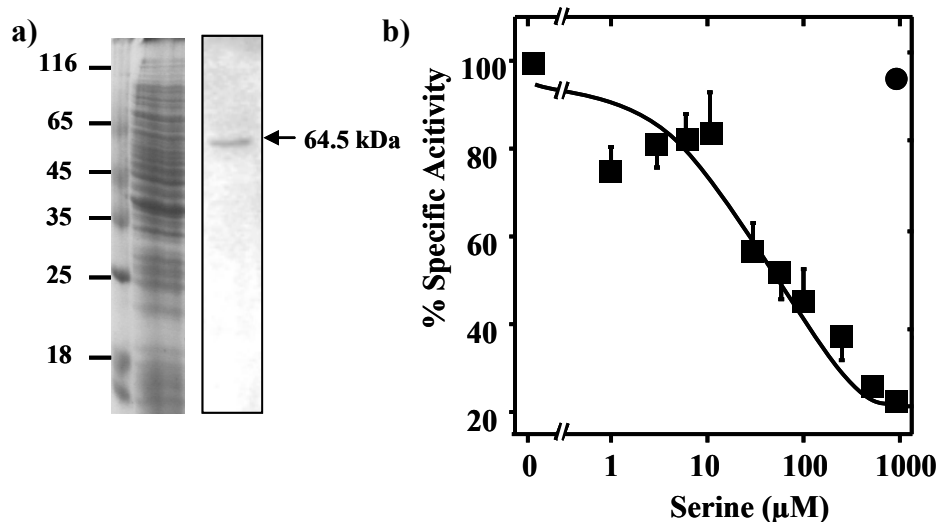
*Figure 3.50: **a)** 15% SDS PAGE and Western blot, 4μg protein; **b)** Serine concentration response curve; circle: 1mM aspartate. Assay conditions: 0.1 mM Tris/HCl, 10 min, 37°C, 12 μg protein, ATP 75 μM, n = 8. The activity of the chimera fluctuated for every expression hence the data was normalized for the statistical analyses. 100% activity corresponds to 0.12 ± 0.05 nmol/mg/min and maximum inhibition to 0.03 ± 0.01 nmol/mg/min.*

The optimum temperature of the chimera was 45°C. The activation energy of the chimera as derived from the Arrhenius plot was 37.6 kJ/mole. The optimum pH was pH 8.
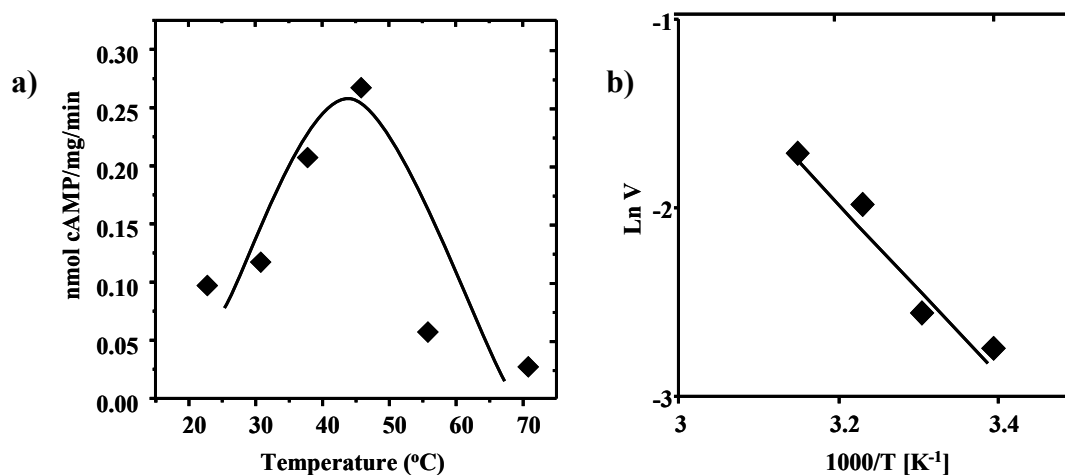
Figure 3.51: **a)** *Temperature dependence;* **b)** *Arrhenius plot; Assay conditions: 0.1 mM Tris/HCl pH 7.5, 10 min, 22-70°C, ATP 75 µM, 20 µg protein.*
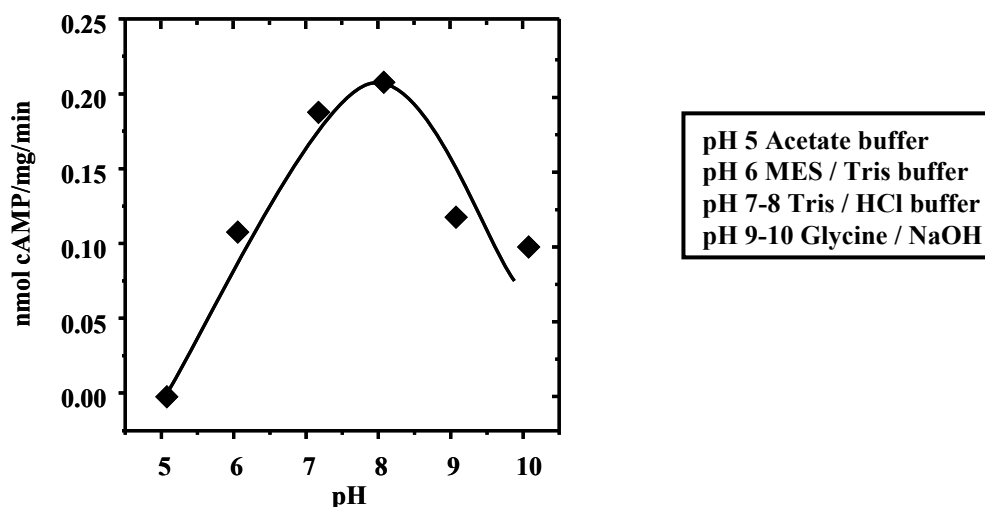
Figure 3.52: pH dependence; Assay conditions: 0.1 mM buffer pH 5-10, 10 min, 37°C, ATP 75 µM, 20 µg protein.

Km and the Vmax were calculated from the Lineweaver-Burk plot. In presence of serine the Km was 285 µM and Vmax was 0.77 nmol/mg/min while in absence of serine the Km was 528 µM and Vmax was 2.18 nmol/mg/min. The specific activity starts to level off at 200 µM concentration but 75 µM ATP was used for all CyaB1 chimeras because this ATP concentration was standardized before in the lab. The Hill coefficient in absence of serine

was 0.97 (R^2 = 0.875) and in presence of serine it was 0.964 (R^2 = 0.94) which shows no cooperativity.
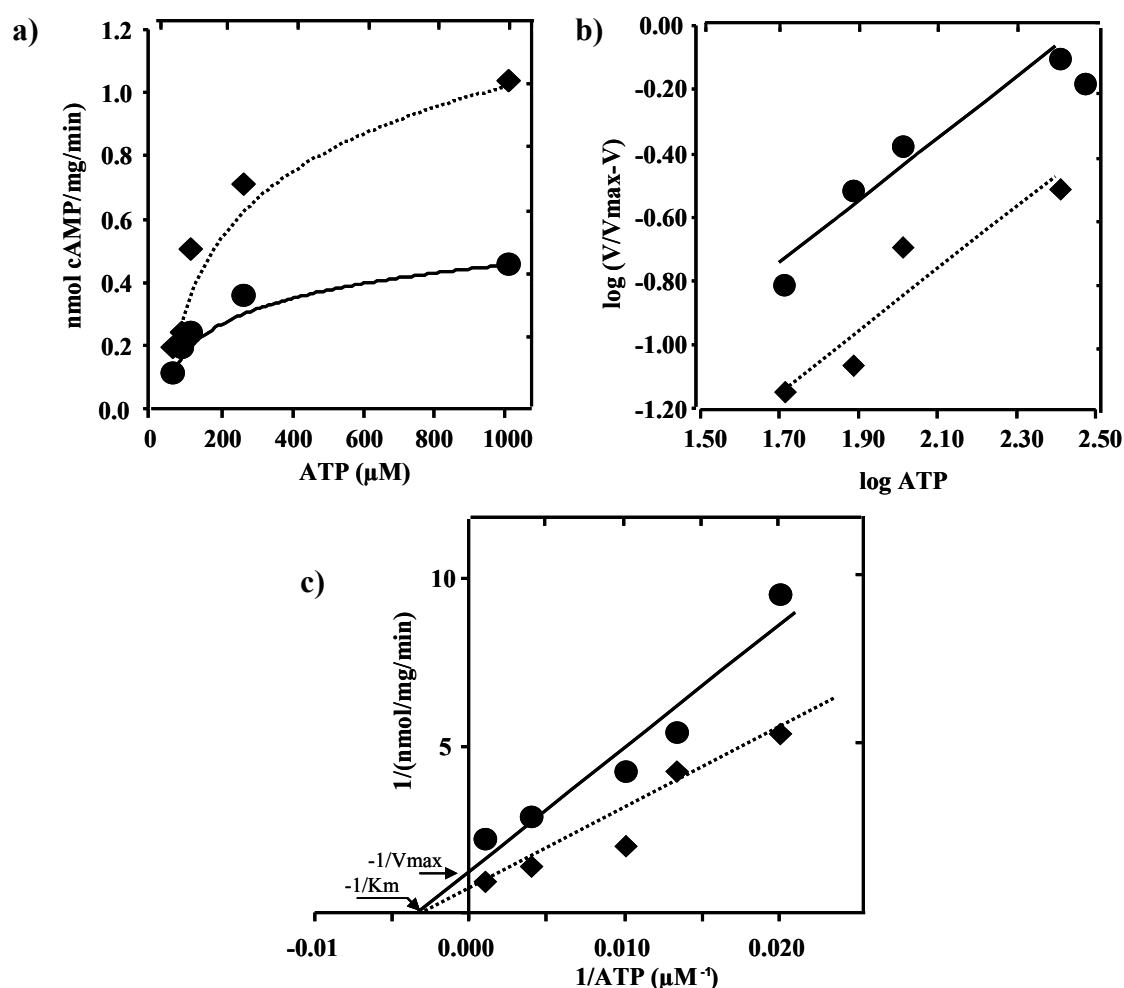
*Figure 3.53: Substrate kinetics, **a)** Michaelis-Menten plot; **b)** Hill plot; **c)** Lineweaver-Burk plot; circles: with serine; diamonds: without serine; Assay conditions: 0.1 mM Tris/HCl pH 7.5, 20 μg protein, 10 min, 37°C.*

3.4.3 Mycobacterial Rv1625c Chimeras

M. tuberculosis AC Rv1625c has a domain organization similar to mammalian ACs. It has six TM domains which are connected directly to its CHD. Due to its high similarity to mammalian ACs as well its successful purification for crystalization studies it is studied in some detail. Signaling properties of Rv1625c AC were explored by constructing similar chimeras with the Tsr receptor.

3.4.3.a Characterization of Tsr-HAMP$_{Tsr}$-Rv1625c AC

Construct design, expression and membrane preparation

Alignments of the Tsr-HAMP$_{Tsr}$-Rv3645 chimera and of the Rv1625c holoenzyme were made to find a conserved region suitable to link the two proteins. The construct has Tsr and HAMP$_{Tsr}$ domain followed by a short linker from Rv3645 AC and then the Rv1625c CHD. The chimera was cloned into pQE80 and expressed in *E. coli* BL21 (DE3) [pREP4] (22°C, 0.1 mM IPTG, 4 h). Membrane fractions were prepared as described in section 2.8.4 and stored at -80°C. The protein was expressed as evident from the SDS PAGE, but was found to be inactive in the AC assay (not shown). The protein had a predicted pI of 6.5.

*Figure 3.54: **a)** Domain organization of the chimera; below is the alignment of Tsr-HAMP$_{Tsr}$ -Rv3645 and Rv1625c, the arrow marks the point where both the proteins were connected; **b)** 12% SDS PAGE showing the expressed protein, 10 μg protein.*

Next the Tsr/Rv1625c chimera was redesigned by replacing the linker of Rv3645 with that of Rv1625c. Rv3645 and Rv1625c belong to different subclasses of AC, hence it might be possible that their linkers are incompatible. In addition other possibilities were checked such as replacing the N-terminal sequence of the chimera with the N-terminus of Rv1625c and replacing HAMP$_{Tsr}$ and Rv3645 linker of the chimera with the linker sequence and HAMP from CyaG which is a class IIIa AC as Rv1625c. Domain organizations of all the chimeras are shown (Fig. 3.55 a). Chimeras were expressed in *E. coli* BL21 (DE3) [pREP4] (22°C, 0.1 mM IPTG, 4 h). Membrane fractions were prepared and stored at -80°C. All chimeras were

active, but none of them were regulated by serine. The chimera wherein the linker as well as the N-terminal sequence was replaced with that of Rv1625c was most active (Fig. 3.55c)

a) i

L202 L253   V443

N — [Tsr] — HAMP Tsr — AC Rv1625c — C

M1   K215   G268 Rv1625c Linker

```
        215                  268 202                                              253
         |                    ||                                                   |
FAVWFGIKASLVAPMNRLI...LRHMQGEL MRTVGLRDTARAEAVMEAEHDRSEALLANMLPASIAERLKEPERNIIADKYDEASVLFADIVGFTERASST
```
Tsr-HAMPtsr   Rv1625c Linker   Rv1625c CHD

ii

M1 S40

N — 1625 — [Tsr] — HAMP Tsr — AC Rv1625c — C

Rv1625c Linker

```
  1         40      215                    268 202                                 253
  |          |       |                      ||                                      |
MAARK...QHYAESFAVWFGIKASLVAPMNRLI...LRHMQGEL MRTVGLRDTARAEAVMEAEHD..LKEPERNIIADKYDEASVLFADIVGFTERASST
```
Nt1625c   Tsr-HAMPtsr   Rv1625c Linker   Rv1625c CHD

iii

L202

N — [Tsr] — HAMP CyaG — AC Rv1625c — C

R370 A431   Rv1625c Linker

```
   370                  431 202                                              253
    |                    ||                                                   |
FAVWFGIKRWISEPILRLS...FNMMIQNLRDSFIALRDTARAEAVMEAEHDRSEALLANMLPASIAERLKEPERNIIADKYDEASVLFADIVGFTERASST
```
Tsr-HAMPCyaG   Rv1625c Linker   Rv1625c CHD

iv

E249

N — [Tsr] — HAMP CyaG — AC Rv1625c — C

R370 A431 E485

HAMPCyaG+Linker CyaG

```
   370              431                                               485 249
    |                |                                                 ||
FAVWFGIKRWISEP...FNMMIQNLRDSFIALENTNRELEQRVLERTAALLQEKERSEELLLNVLPKPIADQLKANKKAIASAIEEASVLFADIVGFTERAS
```
Tsr-HAMPCyaG   CyaG Linker   Rv1625c CHD

b)

```
i.   Tsr-H(tsr)Rv1625c        : VMIVVLAVIFAVWFGIKASLVAPMNRLIDSIRHIAGG--------DLVKPIEVDGSNEMGQLAESLRHMQGE
ii.  Nt1625-Tsr-H(tsr)Rv1625  : VMIVVLAVIFAVWFGIKASLVAPMNRLIDSIRHIAGG--------DLVKPIEVDGSNEMGQLAESLRHMQGE
iii. Tsr-H(Ap)-L(1625)Rv1625c : VMIVVLAVIFAVWFGIKRWISEPILRLSEASSAIASGARNATASAELNQKVKVEKIRELGMLSESFNMMIQN
iv.  Tsr-H(Ap)-L(Ap)Rv1625c   : VMIVVLAVIFAVWFGIKRWISEPILRLSEASSAIASGARNATASAELNQKVKVEKIRELGMLSESFNMMIQN
```

```
i.   Tsr-H(tsr)Rv1625c        : LMRI-VGLRDTAR--------AEAVMEAEHDRSEALLANMLPASIAERLKEPERNIIADKYDEASVLFADIVG
ii.  Nt1625-Tsr-H(tsr)Rv1625  : LMRI-VGLRDTAR--------AEAVMEAEHDRSEALLANMLPASIAERLKEPERNIIADKYDEASVLFADIVG
iii. Tsr-H(Ap)-L(1625)Rv1625c : LRDSFIALRDTAR--------AEAVMEAEHDRSEALLANMLPASIAERLKEPERNIIADKYDEASVLFADIVG
iv.  Tsr-H(Ap)-L(Ap)Rv1625c   : LRDSFIALENTNRELEQRVLERTAALLQEKERSEELLLNVLPKPIADQLKAN-KKAIASAIEEASVLFADIVG
```

*Figure 3.55: **a)** Domain organization of Tsr/Rv1625c chimeras; **i.** Tsr-H (Tsr)-Rv1625c, **ii.** Nt1625-Tsr-H (Tsr)-Rv1625c, **iii.** Tsr-H (Ap)-L (Ap)-Rv1625c, **iv.** Tsr-H (Ap)-L (1625)- Rv1625c; **b)** Alignment of all chimeras.*

b)
c)

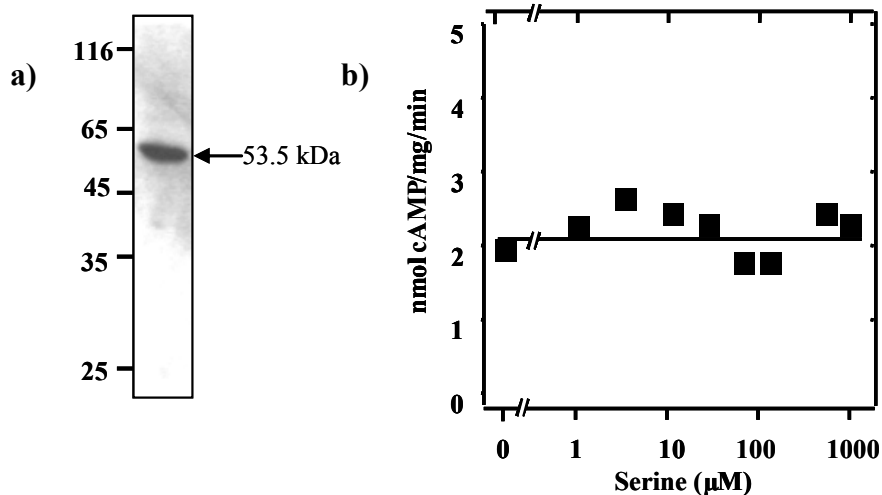*Figure 3.55:* **b)** *Western blot, 2 μg protein;* **c)** *Serine concentration responses; Assay conditions: 0.1 mM Tris/HCl pH 7.5, ATP 200 μM, 20 μg protein, 10 min, 37°C, n = 2.*

3.4.3.b Characterization Tsr-Rv1625c AC chimeras

Rv1625c has a linker of 51 aa which connects the CHD to the membrane anchors. Replacing the linker of Rv1625c with that of Rv3645 makes the AC completely inactive hence it seems that the linker region has certain functional significance of its own rather than just connecting the two domains. An alignment of Rv1625c was made with mycobacterial membrane bound ACs and CyaG as well as with the membrane bound mammalian ACs. A small stretch of these linkers was conserved among all. In mammalian ACs there are two linkers, only the second linker showed this conserved segment. This conserved segment was usually present in class IIIa and IIIb ACs. The Rv1625c holoenzyme sequence was used to investigate the potential presence of a coiled-coil like structure by using the programme [COILS], [http://www.ch.embnet.org/software/COILS_form.html] (Lupas et al., 1991). The programme showed the presence of a coiled-coil around aa positions 218-250, which is in the conserved linker region. To check the importance of this linker region, several chimeras were designed wherein the Rv1625c along with its linker was connected directly to the Tsr/Tar receptor without a HAMP domain to see whether this linker has any function of its own.

Result
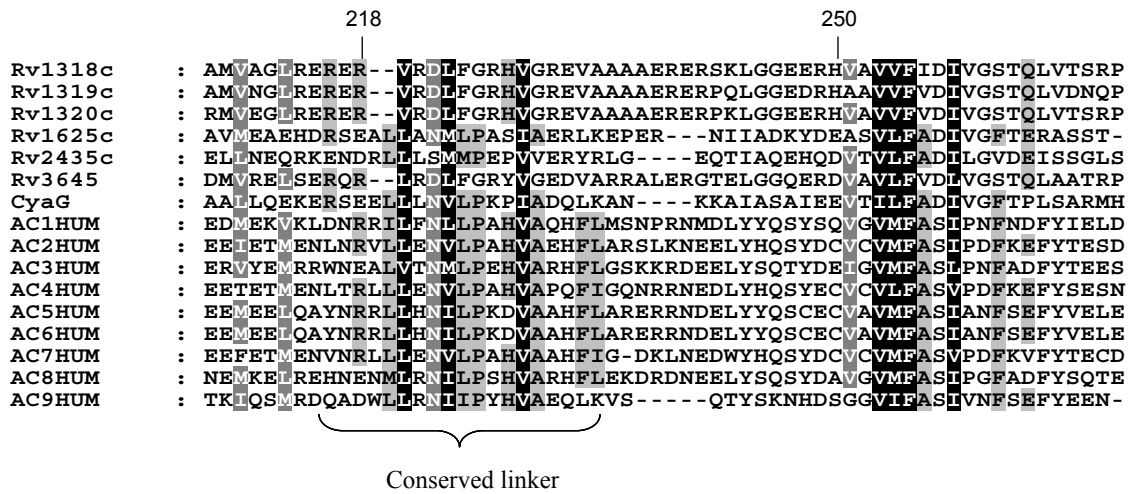
Figure 3.56: Alignment of Class III ACs, part of the alignment showing the conserved linker.

All chimeras (Fig. 3.57) were expressed in *E. coli* Bl21 (DE3) [pREP4] (22°C, 4 h, 0.1 mM IPTG). Membranes were prepared and stored at -80°C. A Western blot was carried out with an anti-RGSHis$_6$ antibody which showed single bands confirming the presence of undegraded protein (Fig. 3.58 a). All Tsr/Tar constructs had differences in their activities but none was inhibited by serine. The Tar chimera was inhibited by 100 mM aspartate (data not shown). CHD of Rv1625c was used as a control to check whether 100 mM aspartate was inhibitory and indeed it was. 10 mM of serine or aspartate did not affect the catalytic activity of the Rv1625c AC. The chimeras had a predicted pI in the range of 6.1-6.5.
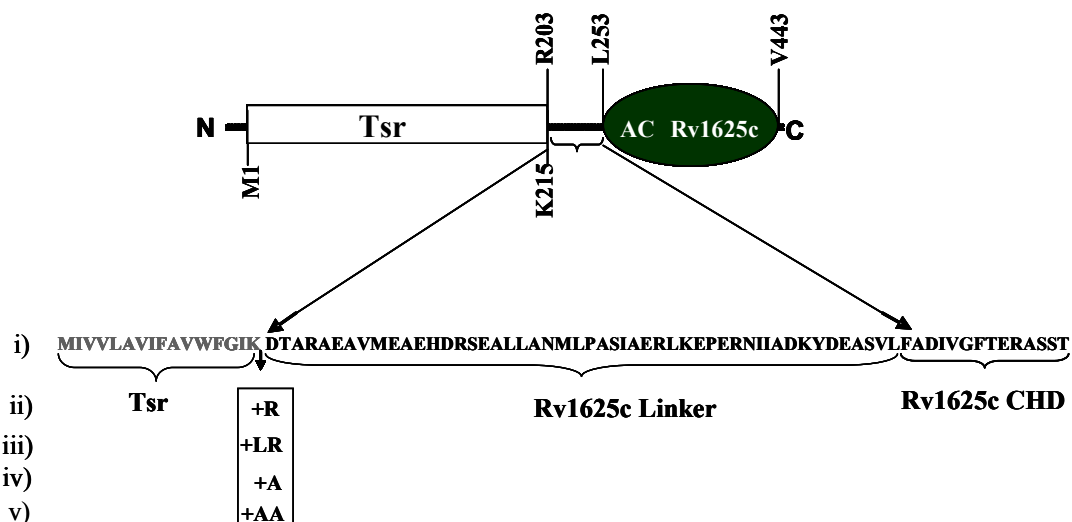
Figure 3.57: Domain organization of Rv1625c chimeras. ***i)*** *Tsr-Rv1625c;* ***ii)*** *Tsr (+R) Rv1625c;* ***iii)*** *Tsr (+LR) Rv1625c;* ***iv)*** *Tsr (+A) Rv1625c;* ***v)*** *Tsr (+AA) Rv1625c.*
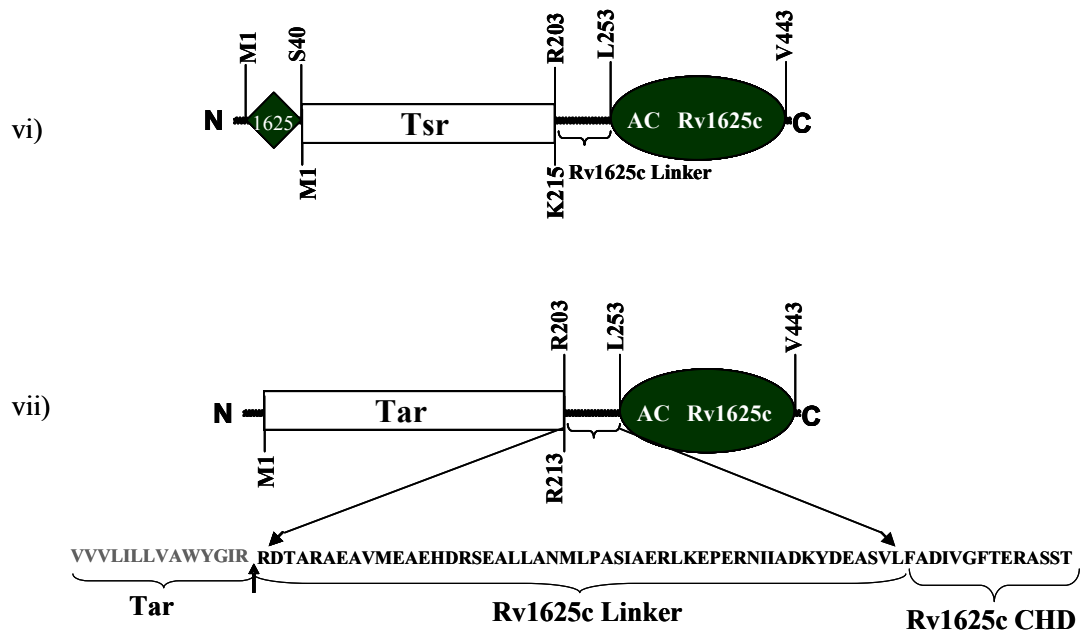
*Figure 3.57: Domain organization of Rv1625c chimeras. **vi)** Nt1625-Tsr-Rv1625c; **vii)** Tar-Rv1625c; The sequence of the chimera is shown below, arrow marks the point where two proteins were linked, residues in grey are from Tsr and Tar while those in black are from Rv1625c AC.*
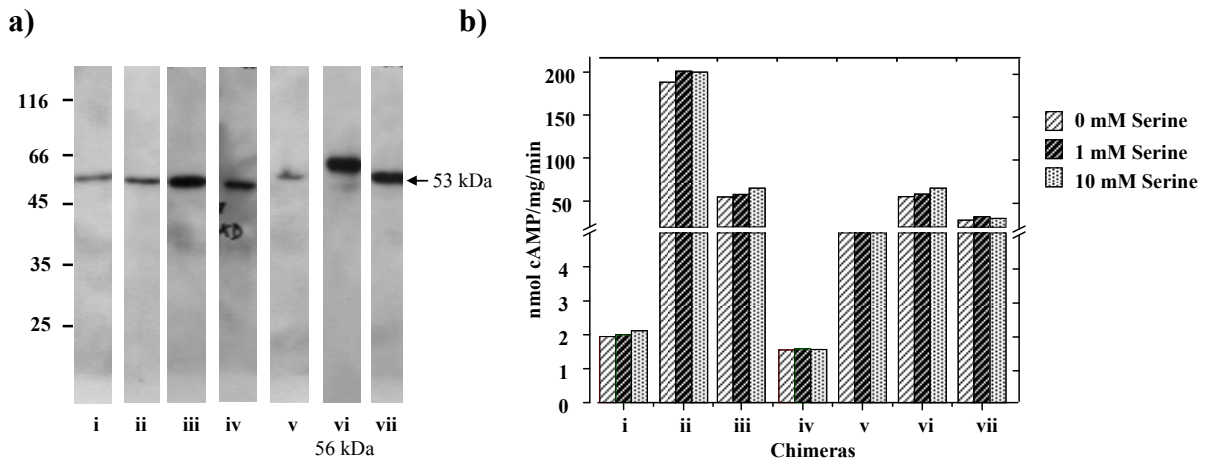
*Figure 3.58: **a)** Western blots, 4 μg protein, **b)** Serine responses; Assay conditions: 0.1 mM Tris/HCl pH 7.5, ATP 200 μM, 20 μg protein, 10 min, 37°C.*

3.4.3.c Tsr-TM5/6-Rv1625c AC chimera

Rv1625c has six predicted TMs. When the TM5/6 of Rv1625c was aligned with known HAMP domains, it displayed a conserved pattern of hydrophobic residues at a and d positions of the heptad repeat which is an important feature of HAMP domains (Figure 3.59 a). The question was whether TM5/6 is actually a HAMP? To investigate this possibility, Rv1625c along with its TM5/6 domains was connected to Tsr receptor. The expression of the chimera was checked by a Western blot with an anti-RGSHis$_6$ antibody which gave a single band of the expected size (figure 3.60 b). Serine concentration response curves were negative. The basal specific activity was 5 nmol/mg/min. The predicted pI was 5.3.
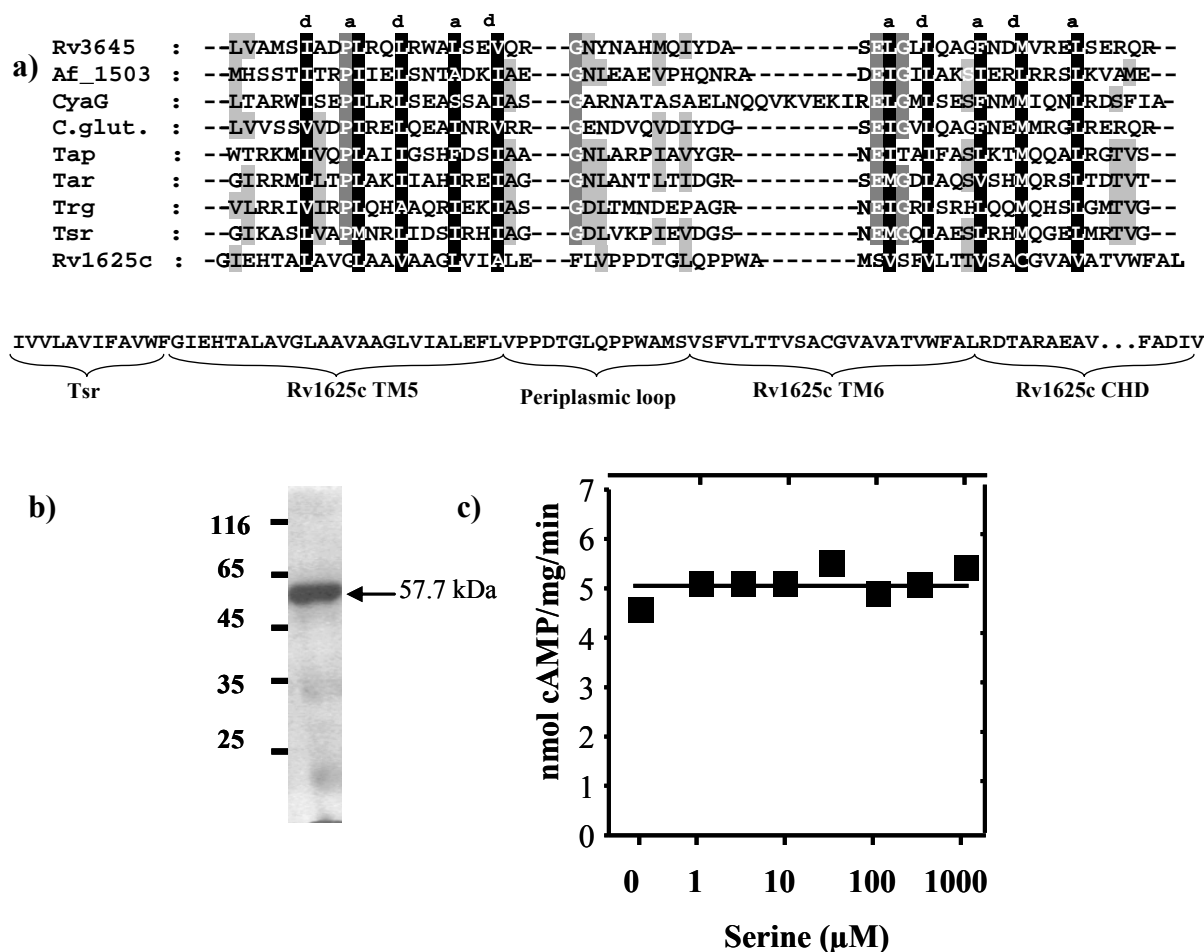
*Figure 3.60 **a)** Alignment of predicted HAMP domains along with TM5/6 of Rv1625c AC, hydrophobic residues at the positions a and d are highlighted with black, below is the partial sequence of the chimera showing the point where the two proteins were linked; **b)** Western blot, 4 µg of protein; **c)** Serine concentration response curve; Assay conditions: 0.1 mM Tris/HCl, pH 7.5, 10 min, 37°C, ATP 200 µM, 20 µg protein.*

3.5 Swapping of Transmembrane domains of Tsr with that of Tar and Af1503 in Tsr/Rv3645AC chimera

Transmembrane spans are the integral part of transmembrane receptor proteins as they play a critical role in signal transduction. In case of Tsr and Tar chemotaxis receptors binding of the respective ligands generates a 1 to 2 Å vertical displacement of TM2 towards the cytoplasm. This piston like movement is converted into a conformational change in the cytoplasmic domain of the receptor (Chervitz and Falke, 1995; Isaac et al., 2002; Ottemann et al., 1999). To investigate whether specific sequences are required in TM1 and TM2 of Tsr receptor for transmembrane signaling, TMs of the Tsr-HAMP$_{Tsr}$-Rv3645 chimera were swapped with those of Tar and Af1503. In case of Tsr receptor, it has been shown before that on replacing its TM2 with that of Tar receptor results in an active and functional chimera confirming that the specific sequence in TM2 of Tsr is not essential for intramolecular interaction between the two TMs and hence in signal transmission (Tatsuno et al., 1994). The similarity between TM1 of Tsr and Tar is 78% while in TM2 it is 54%, it might be possible that the sequences involved in intramolecular interaction and signal transmission falls in this conserved region and hence the function of Tsr receptor was not compromised when replacing its TMs with that of Tar. To explore this possibility Af1503 TMs were used because the similarity between its TMs and TMs of Tsr is only 30-40%. Af1503 is a protein from archeobacteria *Archaeoglobus fulgidus*. It has two TMs and a HAMP domain at the exit of TM2 like those in Tsr and Tar.

3.5.1 Tsr (Tar TM1, TM2) HAMP$_{Tsr}$-Rv3645 AC

TMs of Tsr and Tar were aligned to find conserved regions suitable to connect the domains. The chimera was cloned into pETDUET and expressed in *E. coli* BL21 (DE3) [pREP4] (22°C, 0.1 mM IPTG, 4 h). Membrane fractions were prepared and stored at -80°C. A Western blot was carried out with an anti- RGSHis$_6$ antibody which showed a single band at 52.4 kDa as well as second protein band at 30 kDa which might be a degradation product. The predicted pI of the protein was 5. The chimera interacted specifically with serine as a ligand, serine inhibited the AC activity. The maximum inhibition was 55 ± 8 % and IC$_{50}$ was 3 ± 0.3 μM. The basal activity was 6.7 ± 0.3 nmol cAMP/mg/min. 1 mM aspartate did not affect AC activity.

```
Tsr_TM1 : MLKRIKIVTSLLLVLAVFGLLQLTSGGLFFNALKNDK
Tar_TM1 : MINRIRVVTLLVMVLGVFALLQLISGSLFFSSLHHSQ

Tsr_TM2_: SYSQAMWILVGVMIVVLAVIFAVWFGIKASLVAP
Tar_TM2 : DYRFAQWQLAVIALVVVLILLVAWYGIRRMLLTP
```

```
MLKRIKVVTLLVMVLGVFALLQL...SSLKNDK...SYSFAQWQLAVIALVVVLILLVAWYGIKASLVAP..
```

Tsr   Tar TM1   Tsr (ligand binding domain)   Tar TM2   HAMP$_{Tsr}$
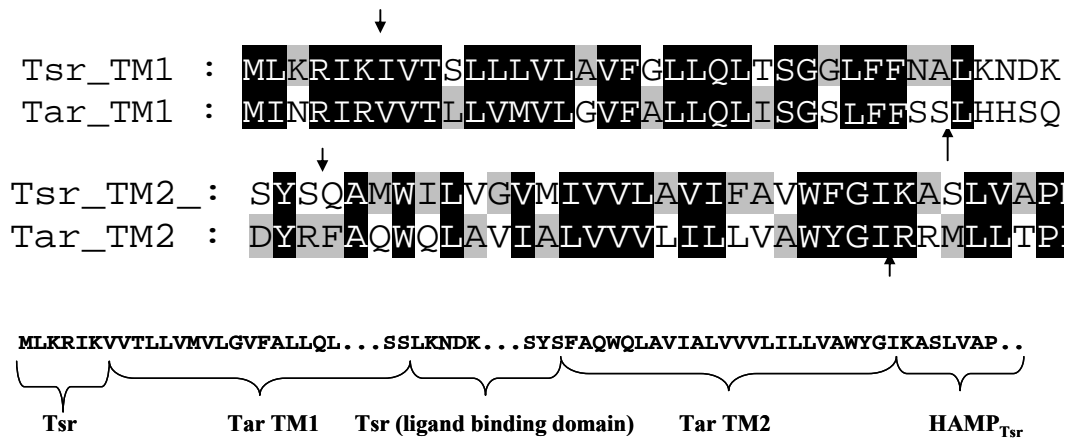
Figure 3.61: Alignment of Tsr and Tar TMs, Similarity in TM1 of Tsr and Tar was 78% and in TM2, it was 54%, residues in black are conserved; arrow indicates the point where the domains were exchanged.
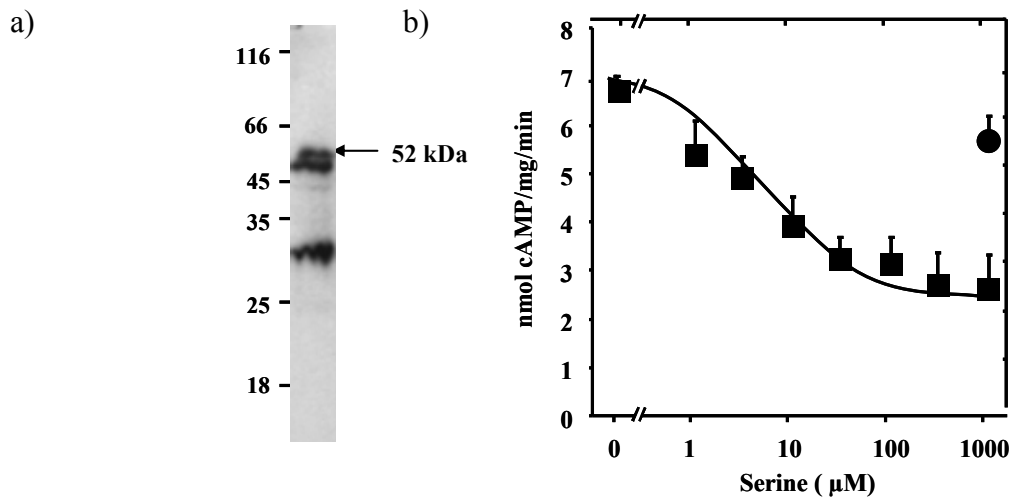
*Figure 3.62: **a)** Western blot, 5 μg protein; **b)** Serine concentration response curve; circle: 1mM aspartate; Assay conditions: 0.1 mM Tris/HCl pH 7.5, 10 min, 37°C, 200 μM ATP, 3 μg protein.*

3.5.2 Tsr (Af1503 TM1,TM2) HAMP$_{Tsr}$-Rv3645 AC

TM1 and TM2 domains of Tsr were replaced with TM domains of Af1503 protein. TMs of Tsr and Af1503 were aligned to find suitable regions for an exchange. The chimera was cloned into pETDUET and expressed in *E. coli* BL21 (DE3) [pREP4] (22°C, 0.1 mM IPTG, 4 h). Membrane fractions were prepared and stored at -80°C. A Western blot carried out with an anti-RGSHis$_6$ antibody showed an undegraded band at 52 kDa (Fig. 3.63 a). The predicted pI of the protein was 4.9. Serine did not regulate. Basal specific activity was 5.5 nmol

cAMP/mg/min. The chimera was redesigned by exchanging the TM domains at different sites as seen in the alignment (Fig. 3.64 a). The chimera was expressed at similar conditions as before but the expression was dismal compared to the first construct as evident from the Western blot (Fig. 3.64 b). The activity of the protein was low because most of the protein was degraded (0.8 nmol/mg/min). The chimera was not regulated by serine.

a)

```
Tsr-TM1    : MLKR----IKIVTSLLLVLAVFGLLQLTSGGLFFNALKNDKENFTVLQTIR
Af1503-TM1: MKLTPQIVLIVVVASLVPLSVLGYLTIAGMTSSAEEAKQGVTTVSQEYLTK

Tsr-TM2    : VSDNNASYSQAMWILVGVMIVVLAVIFAVWFGI--KASLVAPMNRLIDSI
Af1503-TM2: ISKSVEGAIQQVYYALG-IAAAIAIVFVIVLAVFTTSTITRPIIELSNTA
```

MLKRIKVVVASLVPLSVLGYLTIAGMTSSAEEAKN....NASYSQVYYALGIAAAIAIVFVIVIAVFTKASLV..

Tsr **Af1503 TM1** **Tsr (ligand binding domain)** **Af1503 TM2** **HAMP$_{Tsr}$**

b)

c)

*Figure 3.63: **a)** Alignment of Tsr and Af1503 TMs. Similarity in TM1 of Tsr and Af1503 was 26% and TM2 38%, residues in black are conserved; arrow indicates the point where the exchanges were made; **b)** Western blot, 5 µg protein; **c)** Serine concentration response curve; Assay conditions: 0.1 mM Tris/HCl pH 7.5, 10 min, 37°C, 200 µM ATP, 1 µg protein.*

a)

```
Tsr_TM1   :  MLKRIKIVTSLLIVLAVFGLLQLTSGGLFFNALKNDK
Af1503_TM1 : MKLTPQIVLIVVVASLVPLSVLGYLTIAGMTSSAEEA
```

MKLTPQIVLIVVVASLVPSVLGYLTIAGMTSSKNDK...

Af1503 TM1   Tsr

b)

116
66
45   ← 52 kDa
35
25

c)

nmol cAMP/mg/min

1.6
1.2
0.8
0.4
0

0   1   10   100   1000

Serine (μM)
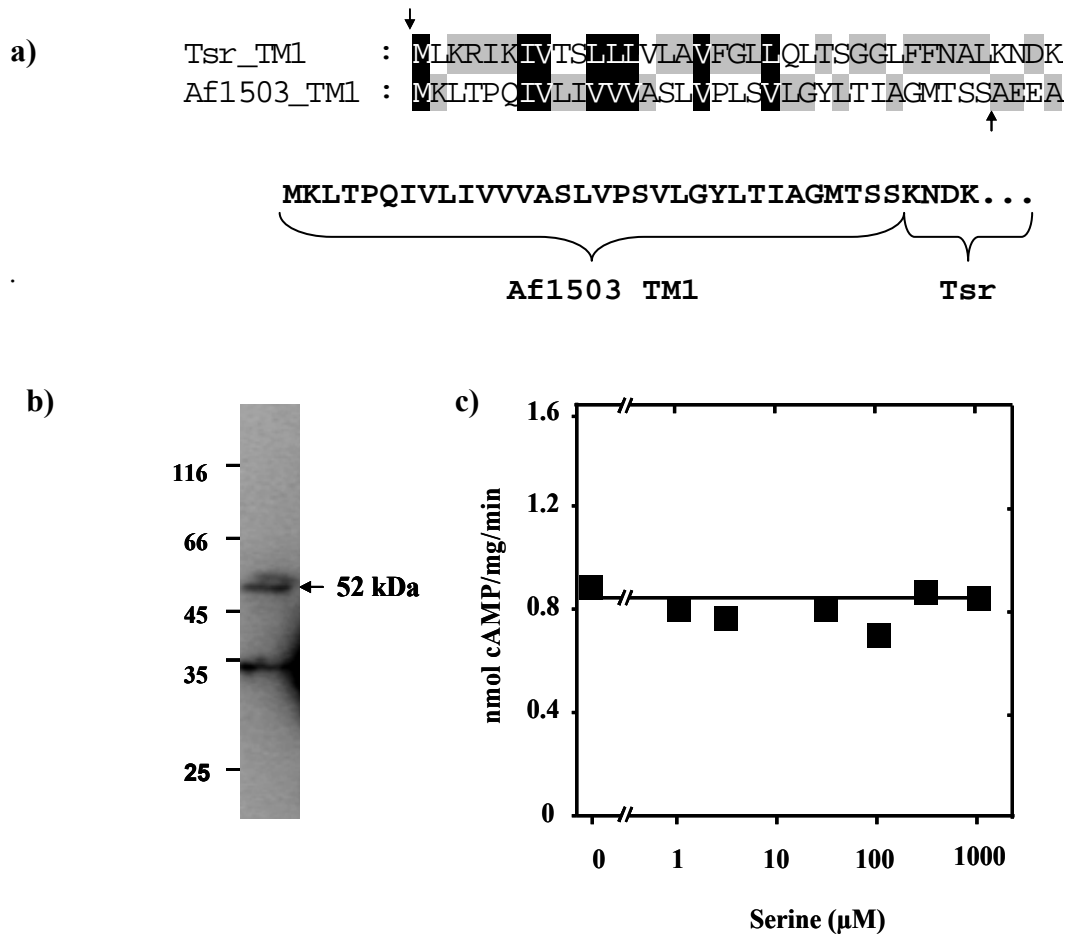
*Figure 3.64: **a)** Alignment of TM1 domains from Tsr and Af1503; residues in black are conserved; arrow indicates the point where the domains were exchanged. Below is the partial sequence of the chimera showing the exchanged domains; **b)** Western blot, 5 μg protein; **c)** Serine concentration response curve; Assay conditions: 0.1 mM Tris/HCl pH 7.5, 10 min, 37°C, 200 μM ATP, serine 0-1 mM, 1 μg protein.*

3.6 Swapping the Periplasmic loop of Rv3645 and Rv1625c AC with the ligand binding domain of Serine Receptor

On substituting six TM helices along with three periplasmic loops of Rv3645 AC with the Tsr receptor having two TM helices and a single periplasmic loop resulted in a chimera which was not only active but could be regulated by serine, as reported in the result section 3.2. The result lead to questions like whether these many TMs are really important for

Rv3645 AC? If yes then what is their functional significance, if no, then is there just a small unit of the membrane which is important for function? To explore this possibility, removal of TM domains consecutively was essential. This could be achieved only when a known ligand for Rv3645 holoenzyme would be available so that signaling of the AC could be observed. Instead of searching for a ligand for Rv3645 AC, the last one of its periplasmic loops was exchanged with the ligand binding domain of Tsr. The main goal was to get a chimera of Rv3645 AC which could sense the serine signal. A similar chimera was made with Rv1625c holoenzyme as well.

3.6.1 TM (1-5)-Tsr-TM6-HAMP($_{Rv3645}$)-Rv3645 AC

Rv3645 holoenzyme has six TMs and three periplasmic loops. The third periplasmic loop of the holoenzyme was exchanged with that of the ligand binding domain of Tsr. The alignments of TM1 of Tsr with TM5 of Rv3645 AC as well as TM2 of Tsr with TM6 of Rv3645 AC were made to locate the appropriate sites for the exchanges. The chimera was cloned into pQE30 and expressed in *E. coli* BL21 (DE3) [pREP4]. The expression and membrane preparations were identical to all former Rv3645 chimeras. A Western blot with an anti-RGSHis$_6$ antibody showed a single band at the expected size of 77 kDa. The predicted pI was 6.2. Serine did not regulate. Basal activity was 2 nmol/mg/min.
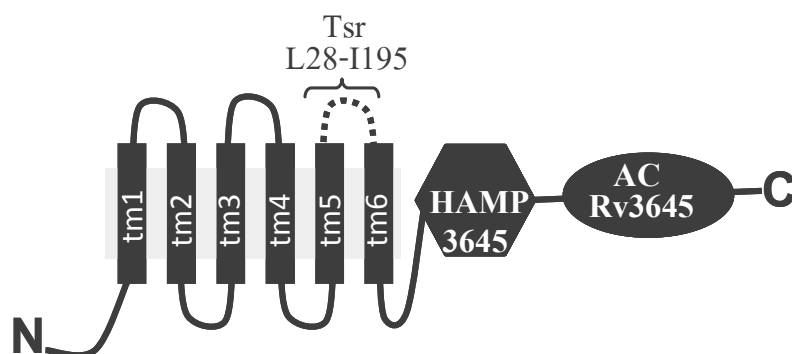
Figure 3.65: Domain organization of chimera. It has six TM spans (tm1-tm6), HAMP and CHD from Rv3645 AC. Dotted loop is the ligand binding domain from Tsr.

a)

```
TM1-Tsr      :  IVTSLLLVLAVFGLLQLTSGGLFFNALKNDKENFTVLQTIR
TM5-Rv3645:  LRLMLAWIPSTGVPLLAIVLAVAADKIALLHATPEALFNPI
```

```
TM2-Tsr      :  AMWLVGVMIVVLAVIFAVWFGIKASIVAPMNRLIDSIRHIAGGDLVK
TM6-Rv3645  :  LFNIILMMALAALGIGSVSTLLVAMSIADPLRQLRWALSEVQRGNYNA
```

L28   I195

```
...LRLMLAWIPSTGVPLLAIVLA LFFNAL ..........AMWI ILMMALAALG....
```

TM5 Rv3645   **Tsr (ligand binding domain)**   **TM6 Rv3645**

b)   **c)**
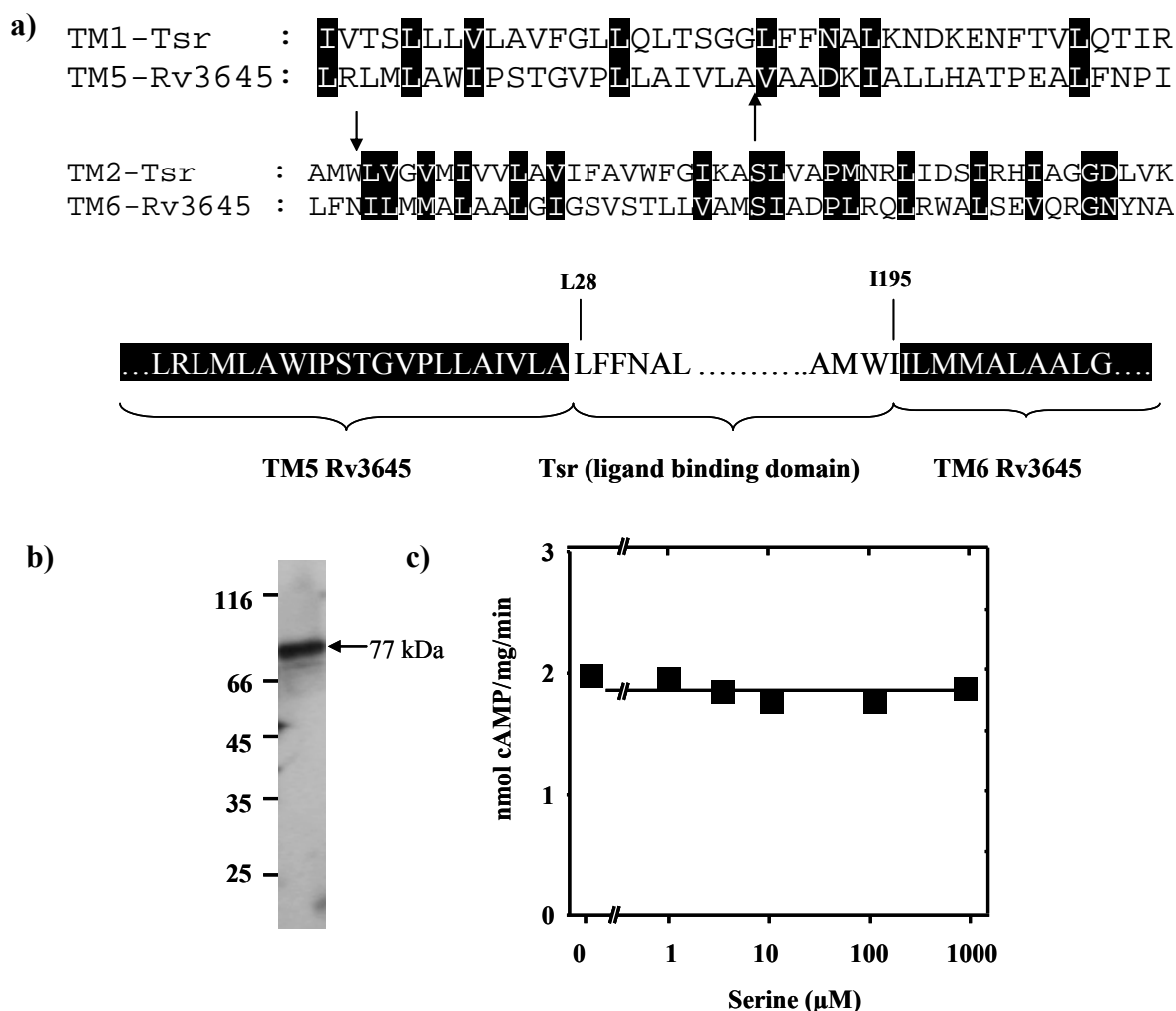
Figure 3.66: **a)** *Alignment of TM1 of Tsr with TM5 of Rv3645 and TM2 of Tsr with TM6 of Rv3645; arrows show the point where domains were exchanged. Below is the partial sequence of the chimera showing the exchanged domains, residues marked in black are from Rv3645;* **b)** *Western blot, 2 μg protein;* **c)** *Serine concentration response curve; Assay conditions: 0.1 mM Tris/HCl pH 7.5, 10 min, 37°C, 200 μM ATP, 10 μg protein.*

3.6.2 TM (1-5)-Tsr-TM6-Rv1625c AC

The chimera was constructed in the similar way as for Rv3645 AC. The chimera started with the first five TMs of Rv1625c, the third periplasmic loop was replaced with the ligand binding domain from Tsr and then continued with TM6 of Rv1625c including its CHD (Fig 3.67 a). Alignments of TM1 of Tsr with TM5 of Rv1625c as well as TM2 of Tsr with TM6 of Rv1625c were made to determine the point where the domains could be exchanged. The chimera was cloned into pQE80 (Δ XhoI, Δ NcoI) and expressed in *E. coli* BL21 (DE3)

[pREP4]. Expression and membrane preparations were as described above for Rv1625c chimeras. Western blots with an anti-RGSHis$_6$ antibody showed a single band at 66 kDa. The predicted pI of the protein was 6.3. Serine did not regulate.

*Figure 3.67: **a)** Domain organization of the chimera. It has six TM domains (tm1-tm6) and the CHD from Rv1625c AC. The dotted loop is the ligand binding domain from Tsr; **b)** Alignment of TM1 of Tsr with TM5 of Rv1625c and TM2 of Tsr with TM6 of Rv1625c, arrow shows the point where domains were exchanged. Below is the partial sequence of the chimera showing the exchanged domains, residues in black are from Rv1625c; **c)** Western blot, 2 μg protein; **d)** Serine concentration response curve; Assay conditions: 0.1 mM Tris/HCl pH 7.5, 10 min, 37°C, 200 μM ATP, 10 μg protein.*

3.7 Transmembrane deletion constructs to get soluble and functional chimeras

The ease with which the HAMP domains could be swapped between the chimeric proteins which yielded both active and functional chimeras in the first part of the results suggested that the HAMP domains in general have a similar mechanism of signal regulation. The recent structure of an archeal HAMP domain from Af1503 suggested helix rotation as the mode of signal transmission (Hulko et al., 2006). A major question which remains to be answered is whether the same mechanism might operate in all TM proteins with HAMP domains. To understand the mechanism of HAMP mediated signal transmission in chemotaxis receptor/AC chimeras a need exists to have a soluble protein for structural analysis. Since efforts to solubilize the chimeras failed, transmembrane deletion constructs were designed to get soluble chimeras which might perhaps retain ligand sensing ability.

3.7.1 Tsr-(ΔTM1&TM2)-HAMP$_{Tsr}$-Rv3645 AC

The first attempt was to delete TM1 and TM2 from the Tsr-HAMP$_{Tsr}$-Rv3645 chimera while leaving the ligand binding loop intact. 17 residues (L11- G27) from TM1 and 18 residues (M193-V210) from TM2 were deleted. The chimera was cloned into pETDUET and expressed in *E. coli* BL21 (DE3) [pREP4] (22°C, 0.1 mM IPTG, 4 h). The protein was successfully purified according to the procedure described in section 2.8.3. (Fig. 3.68 b). A Western blot with an anti-RGSHis$_6$ antibody showed a single band at the expected size of 51 kDa, confirming the stability of the protein in absence of TM domains. Predicted pI of the protein was 5.6.

a)

TM1   **IVTSLLLVLAVFGLLQLTSGGL**

                         ΔTM1

TM2   **MWILVGVMIVVLAVIFAVWFGI**

                  ΔTM2

b)

Figure 3.68: **a)** *Sequence of TM1 of Tsr receptor (ΔTM1 refers to sequence which was deleted) and sequence of TM2 (ΔTM2 refers to sequence which was deleted);* **b)** *12% SDS PAGE (10 µg protein) and Western blot; lanes are marked with letters which stand for: L, load (protein lysate before Ni-NTA interaction); P, pellet (inclusion bodies after 18,000 g spin); F, flowthru (protein lysate after Ni-NTA interaction); E1 and E2, eluate (purified protein); M, marker; W, Western, 0.5 µg protein.*

Serine did not regulate. Basal specific activity was approximately 1 nmol/mg/min which was far below the wild type protein (18 ± 7 nmol/mg/min).

Figure 3.69: Concentration response curve of Tsr-(ΔTM1&TM2)-HAMP$_{Tsr}$-Rv3645 chimera; filled squares: L-serine; empty squares: D-serine; Assay conditions: 0.1 mM Tris/HCl pH 7.5, 10 min, 37°C, 200 µM ATP, 20 µg protein.

The above chimera could not sense a serine signal. This may be due to the fact that the HAMP domains are connected directly to TM2 of Tsr. It might be that the aa residues present at the TM2 and HAMP junction are critical for proper orientation of the HAMP domain for signaling. New chimeras were designed wherein the aa residues were put back sequentially from the C-terminus of the TM2 to find out the minimum amount of aa needed to get a

regulatable yet soluble chimera. An additional deletion of L28 was made in TM1 so that total residues deleted are 18. Three chimeras [named TsrΔTm1C'Rv3645Mt1, Mt2 and Mt3] were cloned into pETDUET. TsrΔTm1C'Rv3645Mt3 was recloned into pQE80 because of instability in pETDUET. All chimeras were expressed and purified in a similar way as described in section 2.8.2 and 2.8.3. SDS PAGE and Western blots carried out with an anti-RGSHis$_6$ antibody showed the presence of purified protein at the expected size. Ligand sensing capacity of the purified proteins was checked with 1 mM and 10 mM serine. Serine did not affect AC activity of the chimeric proteins.

a)

	TM1	TM2
Tsr-HAMP$_{Tsr}$-Rv3645	...IVTSLLLVLAVFGLLQLTSGGLFF............	AMWILVGVMIVVLAVIFAVWFGI....
Tsr- (ΔTM1,TM2)-Rv3645	...IVTS--------------------------------LFF............	A---------------------------------WFGI....
Tsr- (ΔTM1)-C'Rv3645Mt1	...IVTS--------------------------------FF............	A--------------------------------VWFGI....
Tsr- (ΔTM1)-C'Rv3645Mt2	...IVTS--------------------------------FF............	A-------------------------------AVWFGI...
Tsr- (ΔTM1)-C'Rv3645Mt3	...IVTS--------------------------------FF............	A-----------------------------FAVWFGI....

b)
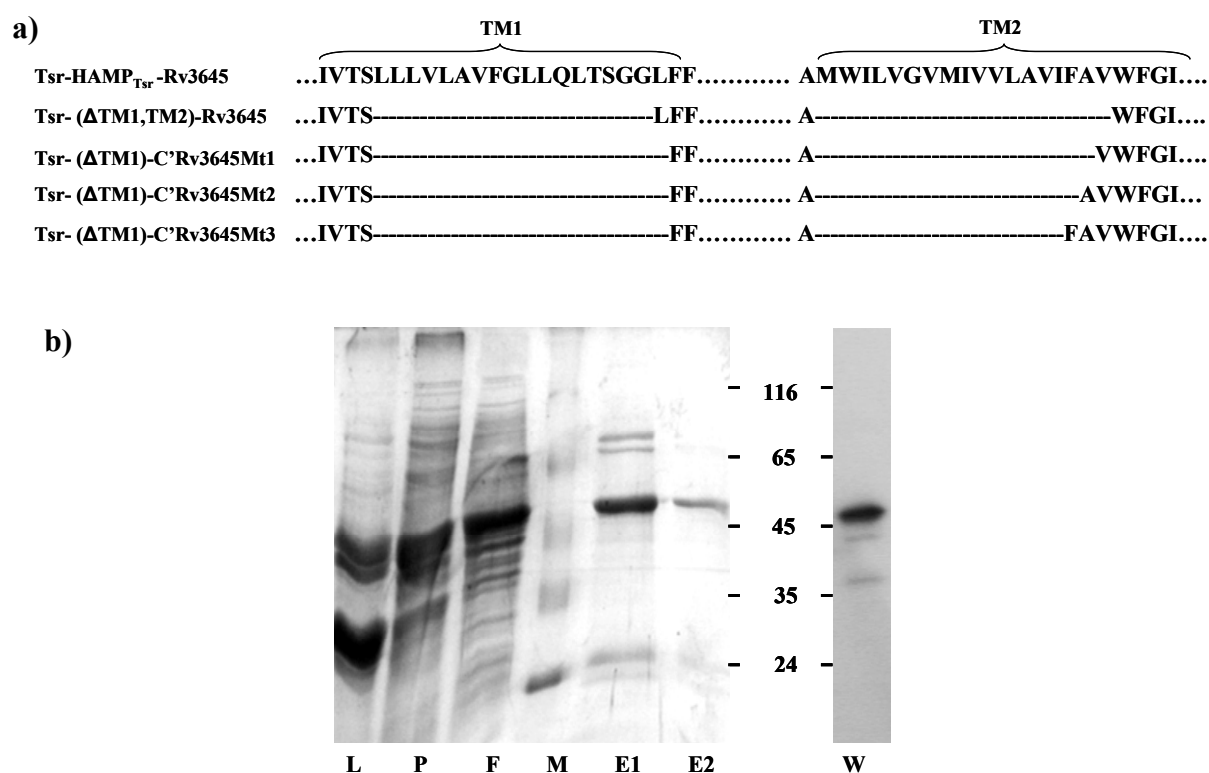
Figure 3.70: **a)** *Alignment of TM chimeras. The solid line marks residues which were deleted from TM1 and TM2, respectively. The dotted line shows the continuation of the protein sequence;* **b)** *Purification and Western of TsrΔTm1C'Rv 3645Mt1; lanes are marked with letters which stand for: M, marker; L, load; P, pellet; F, flowthru; E and E2 eluates; W, Western blot with 0.5µg protein, SDS PAGE with 10 µg protein, M. wt. 51 kDa.*
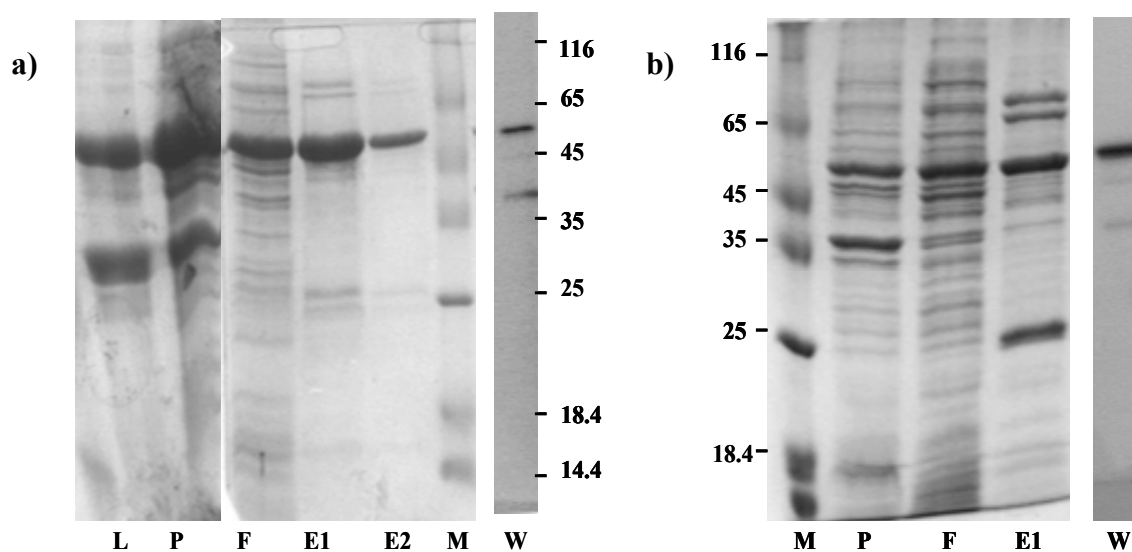
*Figure 3.71 **a)** Purification and Western of TsrΔTm1C'Rv3645Mt2; **b)** Purification and Western of TsrΔTm1C'Rv3645Mt3; lanes are marked with letters which stand for: M, marker; L, load; P, pellet; F, flowthru; E and E2 eluates; W, Western blot with 0.5µg protein, SDS PAGE with 10 µg protein, M. wt. 51 kDa.*
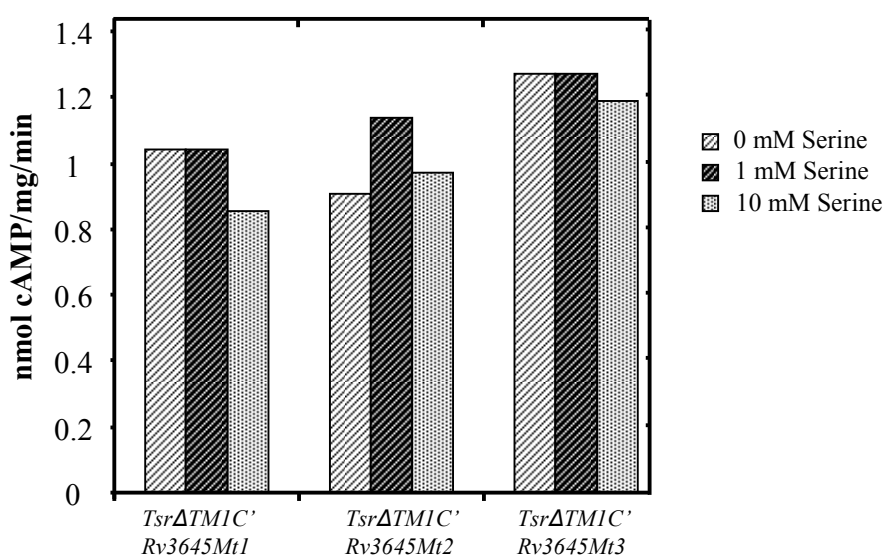
Figure 3.72: Serine concentration responses; Assay conditions: 0.1 mM Tris/HCl pH 7.5, 10 min, 37°C, 200 µM ATP, 20 µg protein, n = 2.

Prediction of domain boundaries by several softwares available online are not stringent hence it is difficult to predict exact borders. TM domains ideally consist of 18 residues but this

number varies. Considering the TM2 domain to start at V197 a new set of chimeras was designed with C-terminal and N-terminal aa additions (Fig. 3.73). In 9 chimeras the number of aa removed C-terminally from TM2 was decreased (TsrΔTm1C'Rv3645Mt4-Mt12) and in 6 chimeras more N-terminal aa were retained (TsrΔTm1N'Rv3645Mt1-Mt6) (Fig. 3.76). All were cloned in pETDUET. Half of the cultures was used for protein purification while the other half was used to prepare membrane fractions to determine whether the protein was present in the soluble or in the membrane fractions. In chimeras TsrΔTm1C'Rv3645Mt4-Mt8, protein could be purified successfully hence the membranes were not purified (Fig. 3.74 a-e). In case of TsrΔTm1C'Rv3645Mt9 and Mt10 chimeras, the protein was partly soluble but a substantial fraction partitioned into the membrane. AC activity was higher in the membrane fractions as compared to soluble proteins. Concerning the chimera TsrΔTm1C'Rv3645Mt11, a very faint band of purified protein was visible on SDS PAGE as well as on a Western blot. TsrΔTm1C'Rv3645Mt12, the Western blot with membrane fractions gave a clear band at the expected size confirming that the protein was in the membrane. No soluble protein was detected. None of the chimeras showed any regulation with serine (1 mM and 10 mM).

```
                                        TM1                                    TM2
                            ⌢⌢⌢⌢⌢⌢⌢⌢⌢⌢⌢⌢⌢⌢⌢⌢⌢⌢⌢⌢⌢                  ⌢⌢⌢⌢⌢⌢⌢⌢⌢⌢⌢⌢⌢⌢⌢⌢⌢⌢⌢⌢⌢⌢
Tsr-HAMP_Tsr-Rv3645         ...IVTSLLLVLAVFGLLQLTSGGLFF.......... AMWILVGVMIVVLAVIFAVWFGI....

Tsr-(ΔTM1)C'Rv3645Mt4       ...IVTS----------------------------------FF.......... AMWIL--------------------------VWFGI....

Tsr-(ΔTM1)C'Rv3645Mt5       ...IVTS----------------------------------FF.......... AMWIL----------------------AVWFGI...

Tsr-(ΔTM1)C'Rv3645Mt6       ...IVTS----------------------------------FF.......... AMWIL-------------------FAVWFGI...

Tsr-(ΔTM1)C'Rv3645Mt7       ...IVTS----------------------------------FF.......... AMWIL-----------------IFAVWFGI...

Tsr-(ΔTM1)C'Rv3645Mt8       ...IVTS----------------------------------FF.......... AMWIL----------------VIFAVWFGI...

Tsr-(ΔTM1)C'Rv3645Mt9       ...IVTS----------------------------------FF.......... AMWIL--------------AVIFAVWFGI...

Tsr-(ΔTM1)C'Rv3645Mt10      ...IVTS----------------------------------FF.......... AMWIL--------------LAVIFAVWFGI...

Tsr-(ΔTM1)C'Rv3645Mt11      ...IVTS----------------------------------FF.......... AMWIL-----------VLAVIFAVWFGI...

Tsr-(ΔTM1)C'Rv3645Mt12      ...IVTS----------------------------------FF.......... AMWIL-----------VVLAVIFAVWFGI...
```

Figure 3.73: Alignment of all the chimeras with aa addition, solid line marks the residues which were deleted from TM1 and TM2, dotted line shows the continuation of the protein sequence.
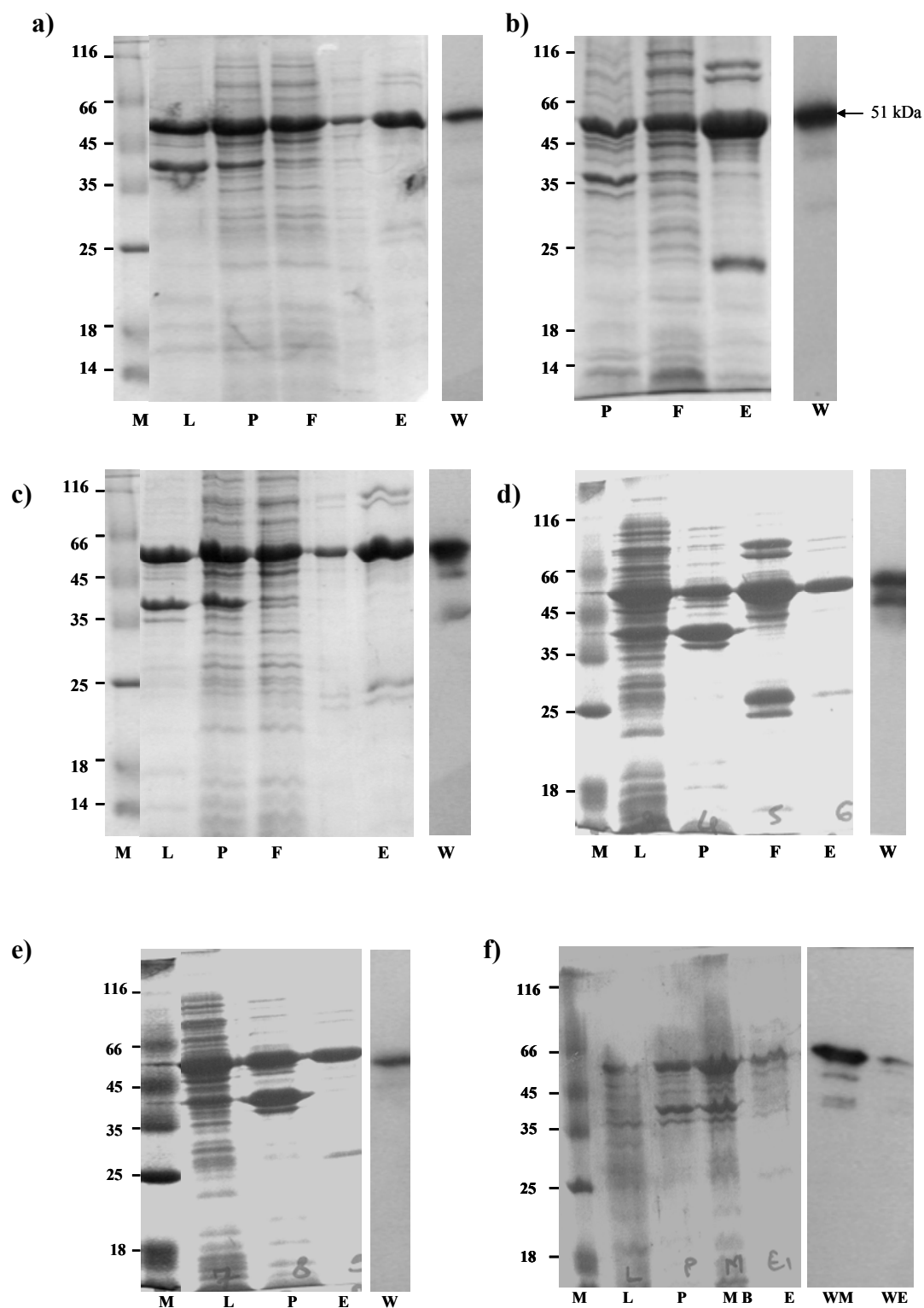
Figure 3.74: 15% SDS PAGE (10 µg protein) and Western blots (with anti-RGSHis$_6$ antibody, 0.5µg protein); **a)** *Purification of TsrΔTm1C'Rv3645Mt4;* **b)** *TsrΔTm1C'Rv3645Mt5;* **c)** *TsrΔTm1C'Rv3645Mt6;* **d)** *TsrΔTm1C'Rv3645Mt7;* **e)** *TsrΔTm1C'Rv3645Mt8;* **f)** *TsrΔTm1C'Rv3645Mt9; lanes are marked with letters which stand for: M, marker; L, load; P, pellet; F, flowthru; MB, membrane fraction; E, eluate; W, Western; WM, Western with membrane fraction; WE, Western with eluate, M. wt. of all chimeras was around 51 kDa.*

Result
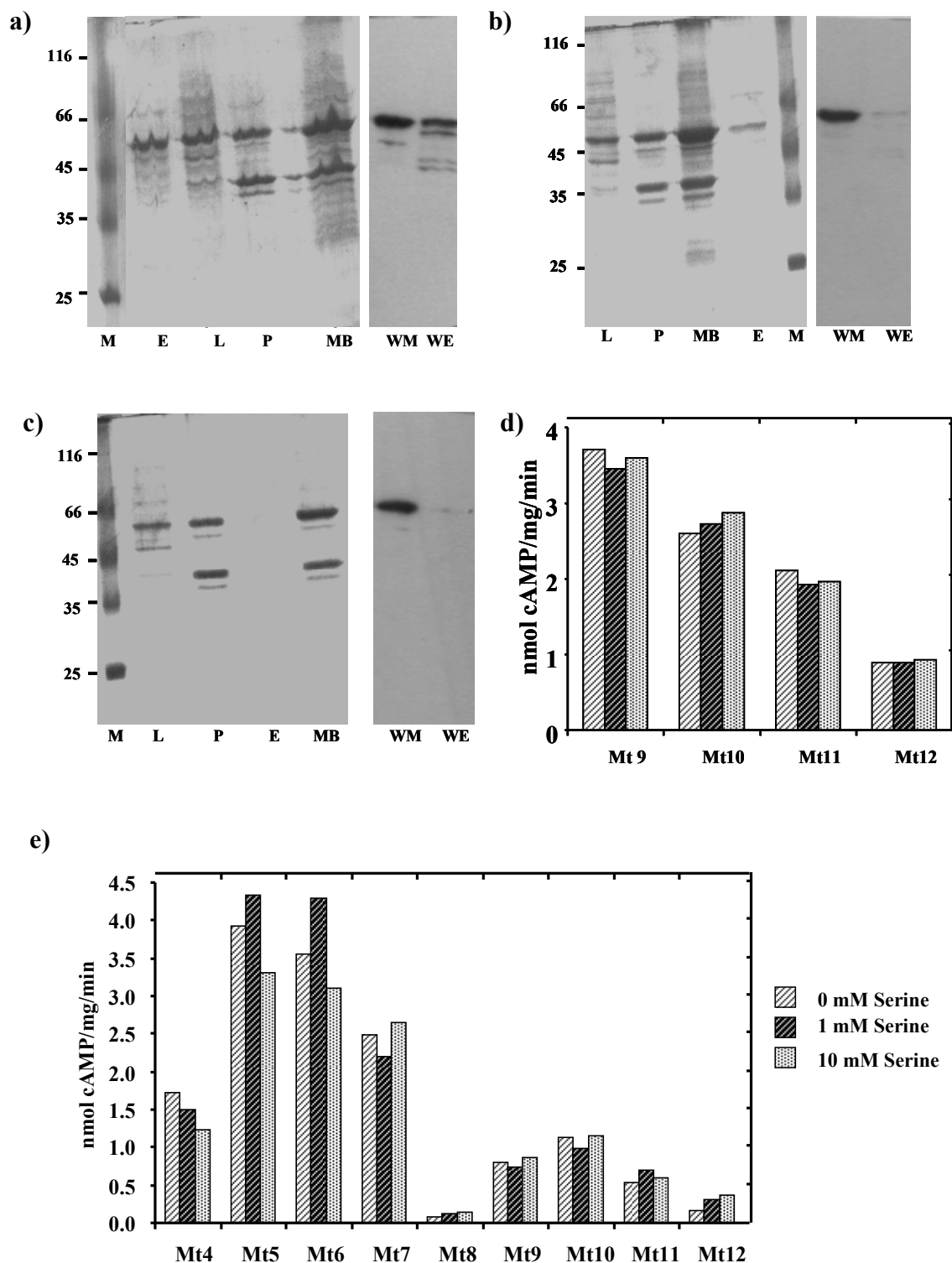
*Figure 3.75: 15% SDS PAGE (10 μg of protein) and Western blots (with anti-RGSHis$_6$ antibody, 0.5μg of protein); **a)** Purification of TsrΔTm1C'Rv3645Mt10; **b)** TsrΔTm1C'Rv3645Mt11; **c)** TsrΔTm1C'Rv3645Mt12; lanes are marked with letters which stand for: M, marker; L, load; P, pellet; F, flowthru; MB, membrane fraction; E, eluate; WM, Western with membrane fraction; WE, Western with eluate; **d)** AC activity with membrane fractions of chimeras from TsrΔTm1C'Rv3645Mt9-Mt12; **e)** AC activity with soluble purified fractions from TsrΔTm1C'Rv3645Mt4-Mt12. Assay conditions: 200 μM ATP, 20 min, 10 μg of protein, 37°C, Tris/HCl pH 7.5, n= 2.*

110

All chimeras in which more N-terminal aa were retained in TM2 were purified and membrane fractions were prepared as above. From the SDS PAGE it is evident that these proteins are present in the membrane (Fig. 3.77 a, b and c). AC assays were carried out with both the membrane and soluble fractions of the protein. Basal specific activity was negligible for the soluble protein (0.1-0.5 ± 1 nmol cAMP/mg/min). The basal specific activity of the membrane fraction was in the range of 0.5-3 ± 2 nmol/mg/min. No regulation of cAMP formation by serine at (1 mM and 10 mM) was observed.

a)

	TM1		TM2	
Tsr-HAMP$_{Tsr}$ - Rv3645	...IVTSLLLVLAVFGLLQLTSGGLFF	....	AMWILVGVMIVVLAVIFAVWFGI....	
Tsr- (ΔTM1)N'Rv3645Mt1	...IVTS------------------------------------ FF	....	AMWILVG -----------------------WFGI...	
Tsr- (ΔTM1)N'Rv3645Mt2	...IVTS------------------------------------ FF	....	AMWILVGV---------------------WFGI...	
Tsr- (ΔTM1)N'Rv3645Mt3	...IVTS------------------------------------ FF	....	AMWILVGVM------------------WFGI...	
Tsr- (ΔTM1)N'Rv3645Mt4	...IVTS------------------------------------ FF	....	AMWILVGVMI-----------------WFGI...	
Tsr- (ΔTM1)N'Rv3645Mt5	...IVTS------------------------------------ FF	....	AMWILVGVMIV----------------WFGI...	
Tsr- (ΔTM1)N'Rv3645Mt6	...IVTS------------------------------------ FF	....	AMWILVGVMIVV--------------WFGI...	
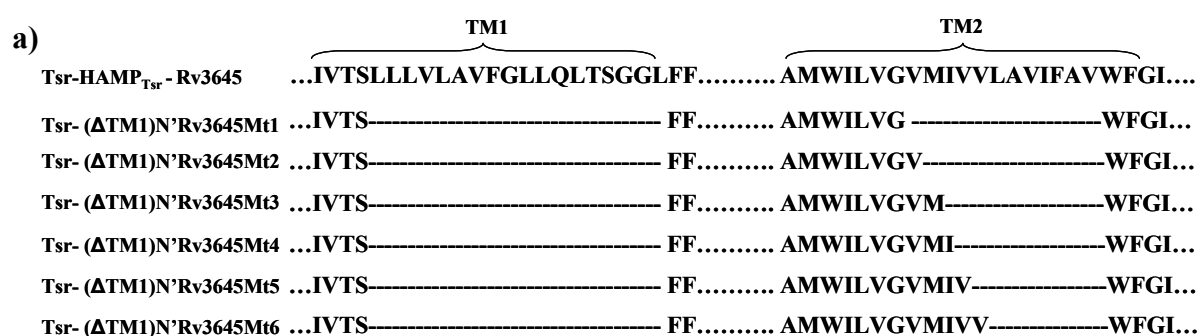
Figure 3.76: Alignment of all chimeras with aa additions; solid line marks the residues which were deleted from TM1 and TM2, dotted line shows the continuation of the protein sequence.
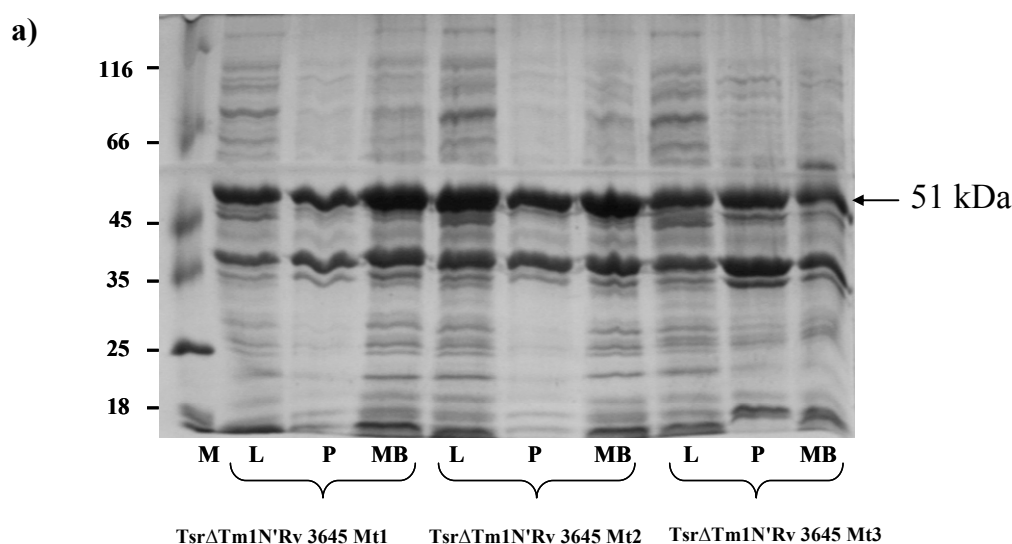
*Figure 3.77: **a)** 12% SDS PAGE of chimeras TsrΔTm1N'Rv3645Mt1-3.*

b)

51.7 kDa →

116
66
45
35
25
18

L   P   MB   L   P   MB   L   MB   M

TsrΔTm1N'Rv3645 Mt4   TsrΔTm1N'Rv3645 Mt5   TsrΔTm1N'Rv3645 Mt6

c)

M   Mt1 Mt2 Mt3 Mt4 Mt5 Mt6
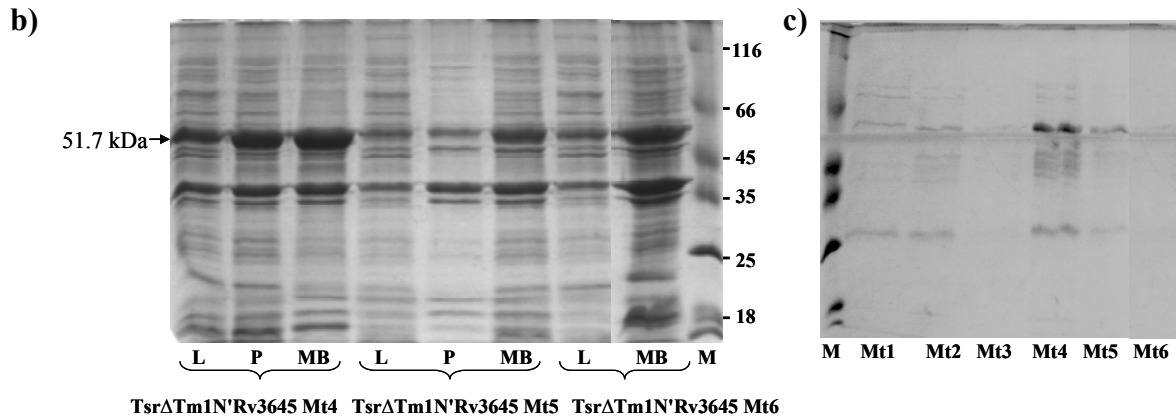
Figure 3.77: **b)** *12% SDS PAGE of chimeras TsrΔTm1N'Rv3645Mt1-3, 5μg protein; lanes are marked with letters which stand for: M, marker; L, load; P, pellet; MB, membrane fraction;* **c)** *12% SDS PAGE of the soluble fractions of the chimeras (Mt1-Mt6), 10μg protein.*

a)

b)

0 mM Serine   1 mM Serine   10 mM Serine
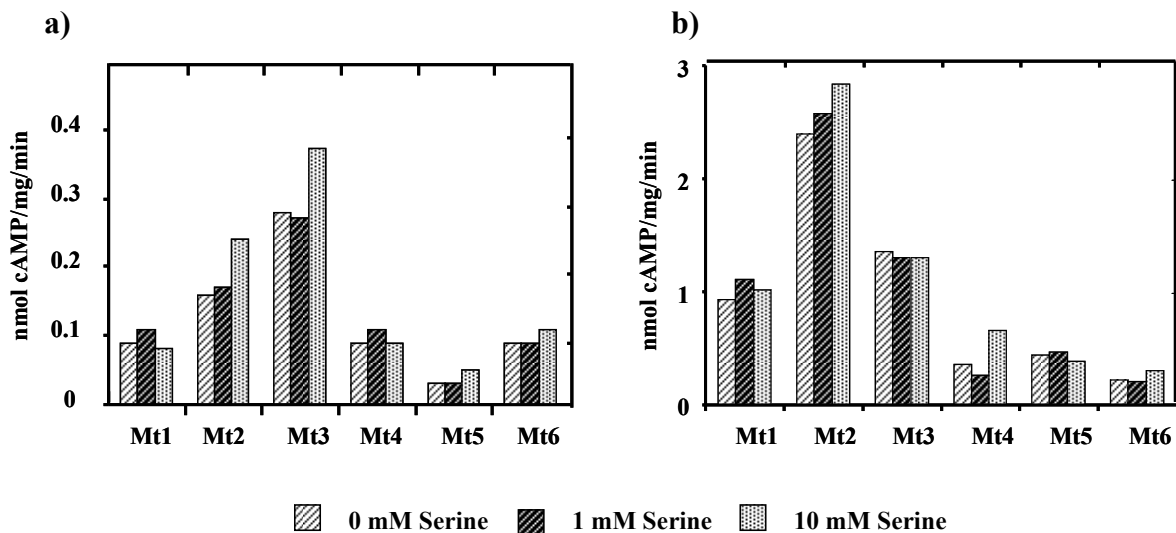
Figure 3.78:   AC activity of TsrΔTM1N'Rv3645Mt1-Mt6; **a)** *TsrΔTm1N'Rv3645Mt1-Mt6 soluble proteins,* **b)** *TsrΔTm1N'Rv3645Mt1-Mt6 membrane fractions, n = 2.*

3.7.2 Tsr-(ΔTM1)-HAMP$_{Tsr}$-Rv3645 AC

The entire TM2 sequence is important for efficient signaling. Before concluding that both TMs of Tsr are equally important for signaling and dimerization, a chimera wherein only TM1 was deleted while TM2 remained intact was cloned. In this chimera 18 residues (L11-

L28) were deleted. The chimera was named as Tsr-(ΔTm1)-HAMP$_{Tsr}$-Rv3645. It was cloned into pQE80, expression and purification procedures were the same as used for all Rv3645 chimeras. Protein was present in the membrane fraction as well as in the soluble, purified fraction in comparable amounts. Western blots with an anti-RGSHis$_6$ antibody showed the expected band at 52 kDa with some degradation products. AC assay was done with both fractions, protein in the membrane fraction had higher activity as compared to the soluble protein but none was regulated by 1 mM or 10 mM serine. The results suggested that both membrane domains were important for signaling.
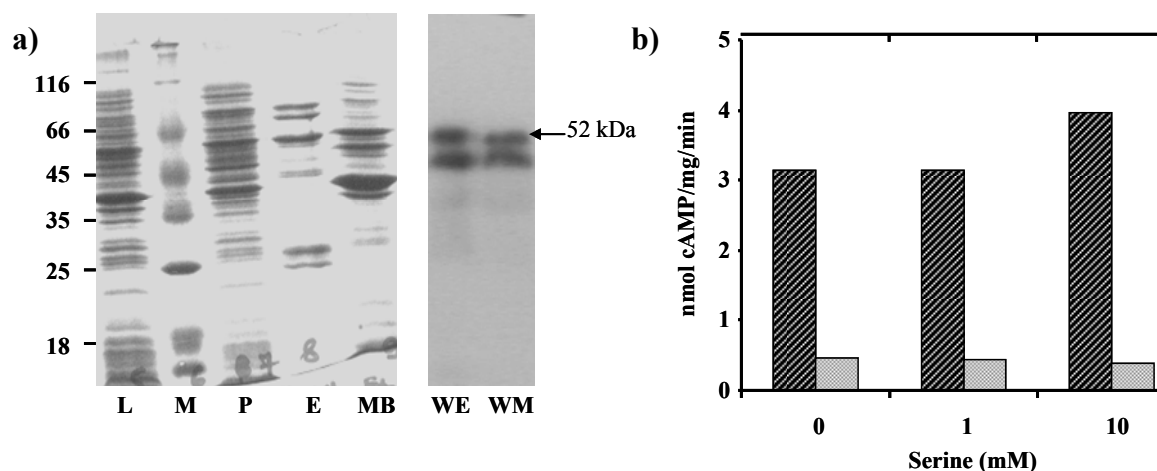
Figure 3.79: ***a)*** *15% SDS PAGE (5µg protein) and Western blot (0.5µg protein); lanes are marked with letters which stand for: M, marker; L, load; P, pellet; E, eluate; MB, membrane fraction; WE, Western with eluate; WM, Western with membrane fraction;* ***b)*** *AC activity of the chimera with membrane fraction (black patterned column) and soluble fraction (grey patterned column). Assay conditions: 200 µM ATP, 20 min, 10 µg of protein, 37°C, Tris/HCl pH 7.5, n = 2.*

3.7.3 Tsr-(mut)-Rv3645 and Tsr-(LL-RR)-Rv3645 AC

Chimeras with TM deletions were not regulated confirming the importance of the TM spans in signaling. Instead of removing the whole TM domains specific point mutations which would make the TMs less hydrophobic were incorporated into TM1 and TM2 of Tsr to investigate whether these mutations help in getting a soluble and regulated protein. The mutations were designed according to mutational studies done on Tsr and Tar membrane anchors (J. S. Parkinson, personal communication). They mutated every residue sequentially in the Tsr membrane to study the effect and role of each residue in receptor function. According to their analysis only those residues which had least deleterious effect on Tsr

function were mutated in the following chimeras (Fig. 3.80 a). In the second chimera only mutations L12R and L13R were inserted which according to the analysis blocks membrane insertion (Fig. 3.80 b). Both chimeras were first cloned into pETDUET3 and recloned into pQE80 later as the protein was unstable in pETDUET3. Proteins were expressed and purified with the same protocol as used for the mutant chimeras before. Both mutant proteins were still in the membrane as evident from the SDS PAGE and Western blot (Fig. 3.80 c). None of the mutants was regulated by serine.
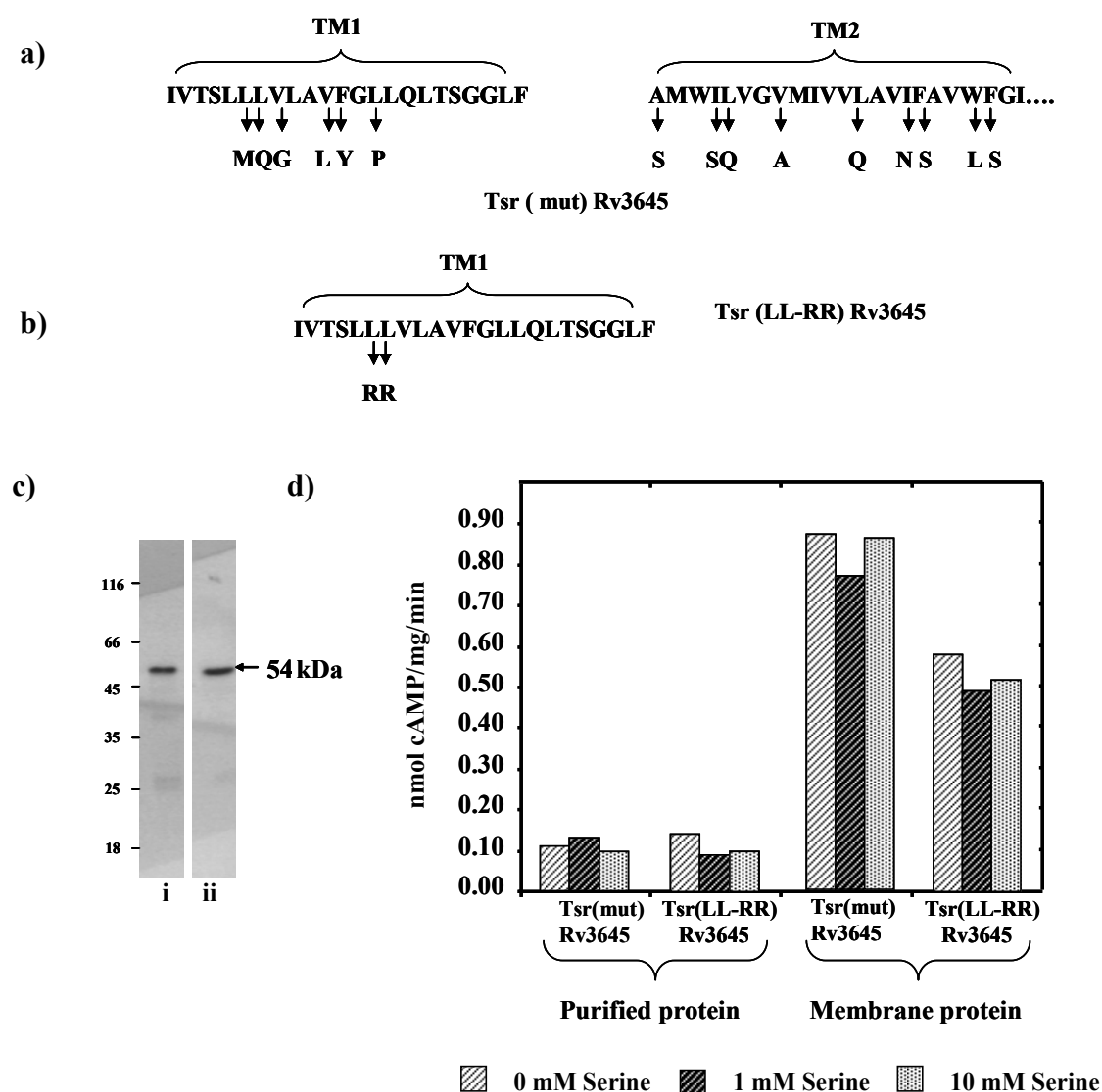
*Figure 3.80 **a)** Sequence of TM1 and TM2 of Tsr showing the mutations introduced in chimera Tsr-(mut)-Rv3645; **b)** Sequence of TM1 showing the mutations introduced in chimera Tsr-(LL-RR)-Rv3645; **c)** Western blot of membrane fractions with anti-RGSHis₆ antibody, **i)** Tsr-(mut)-Rv3645, **ii)** Tsr-(LL-RR)-Rv3645; 1μg protein, (pI 5.6 and 5.8 respectively); **d)** AC activity with serine. Assay conditions: 200 μM ATP, 20 min, 10 μg of protein, 37°C, Tris/HCl pH 7.5, n = 2.*

3.8 Exploring ligands for *Arthrospira* cyclase CyaG holoenzyme

Successful generation of chimeras of receptor and CyaG (section 3.1) confirmed that the AC can sense extracellular signals. This further strengthened the hypothesis that the TM along with the periplasmic loop of CyaG may have receptor properties like those of MCPs. Since the CyaG domain organization resembled more closely the MCPs than other ACs, CyaG was used for a potential ligand search. An extensive domain analysis of CyaG was made through the NCBI conserved domain search database before starting the search for the ligands (Marchler-Bauer et al., 2009). The NCBI search revealed two more domains i.e. HNOBA domain and CACHE domain which were not revealed by SMART domain search analysis. The HNOBA domain overlaps with HAMP domain and the linker region of CyaG AC. The HNOBA domain is always found associated with a HNOB domain hence called as HNOB associated domain. HNOB stands for Heme-NO binding. It is predicted to function as a heme dependent sensor for gaseous ligands and transduce diverse signals in bacteria and animals (Iyer et al., 2003). In the first round of analysis the nitrogen containing compounds such as $NaNO_2$, KNO_3, guanosine, adenosine and SNOC along with heme were tested to see whether they have any effect on AC activity of the chimera.

3.8.1 *Effect of $NaNO_2$ on AC activity of CyaG AC*

On increasing the $NaNO_2$ concentration to 10 mM there was no change in specific activity. The assay was carried out with the membrane fraction of CyaG. 10 mM KNO_3 was also used. To exclude the possibility of non-specific interactions with the CyaG CHD, concentration response curves were made with CyaG CHD as a control. The AC assay was repeated with the protein lysate just after french press to ensure that all accessory proteins possibly needed for receptor mediated interaction were available. $NaNO_2$ and KNO_3 neither affected AC activity of the holoenzyme (membrane fraction and lysate) nor the AC activity of the CyaG CHD.
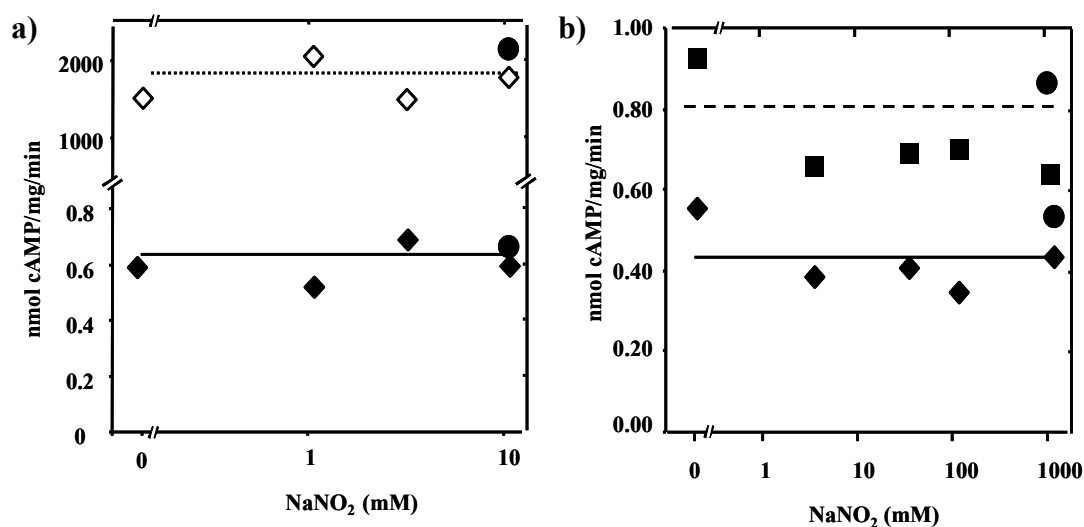
*Figure 3.81: Concentration response curve with NaNO₂; **a)** AC activity with membrane fraction and CHD; filled diamonds: membrane fraction; circles: 10 mM KNO₃; empty diamonds: purified CHD; **b)** AC activity with membrane fraction and lysate; squares: protein lysate. Assay conditions: 750 µM ATP, 20 min, 10 µg of protein, 37°C, Tris/HCl pH 7.5, n = 2.*

3.8.2 *Effect of Guanosine and Adenosine on activity of CyaG AC*

Concentration response curves with guanosine and adenosine (up to 0-10 mM) were established with membrane fractions as well as the CyaG CHD as a control. Guanosine and adenosine decreased AC activity. The inhibition was not specific as it was also observed with CyaG CHD. The extent of inhibition was greater with guanosine than with adenosine.

3.8.3 *Effect of SNOC and heme on AC activity*

Since the HNOB domain supposedly has heme-NO binding property, the effect of NO on the AC was investigated. SNOC or S-nitrosocysteine is an NO donor. SNOC was prepared fresh before the assay because its half life is just several minutes. SNOC was prepared by mixing 400 µl of NaNO₂ salt (stock 125 mmol/l) and 8.8 mg cysteine-HCl together and then 50 µl HCl (1M) was added. The solution turns red. After 2 min 50 µl of NaOH (1M) is added. A 1:100 dilution of this solution gives 1mM SNOC. Two concentrations of SNOC, 0.5 mM and 1 mM SNOC, were used. SNOC along with 0.5 µM and 1 µM heme was tested. Neither the addition of SNOC and heme alone nor when they were added together affected the AC activity.
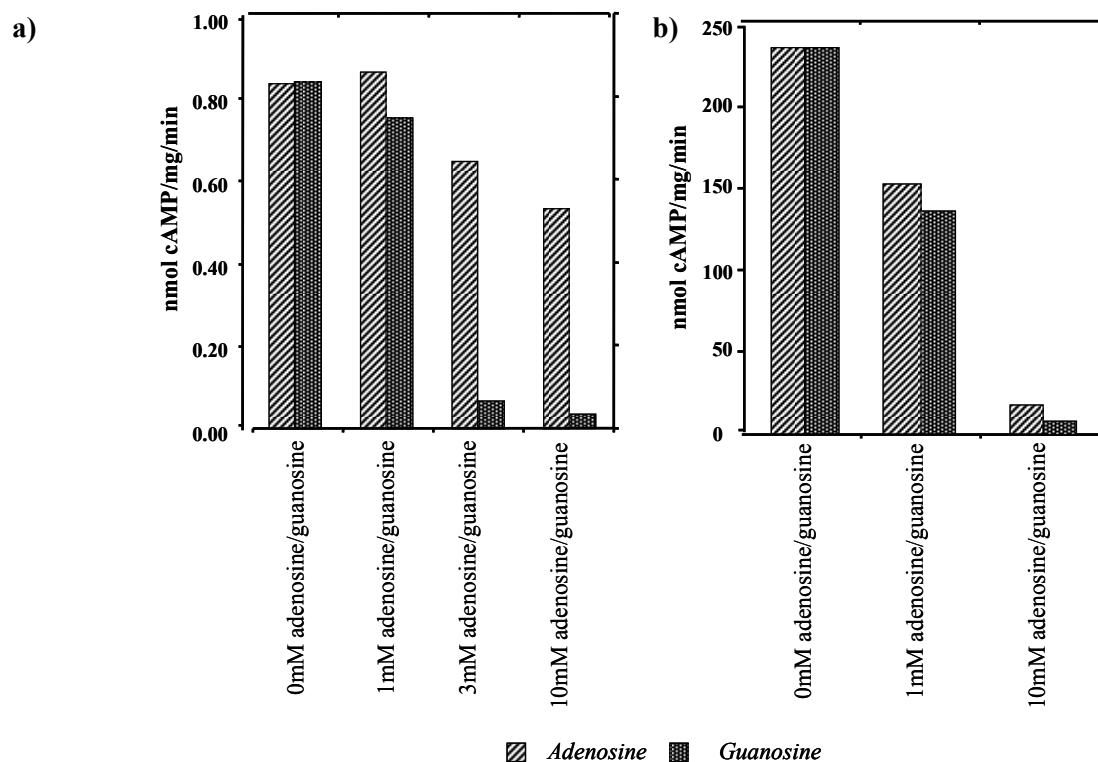
Figure 3.82: Concentration responses with guanosine and adenosine; **a)** *AC activity with membrane fraction;* **b)** *AC activity with purified CHD; Assay conditions: 750 μM ATP, 10 min, 20 μg of protein, 37°C, Tris/HCl pH 7.5, n = 2.*
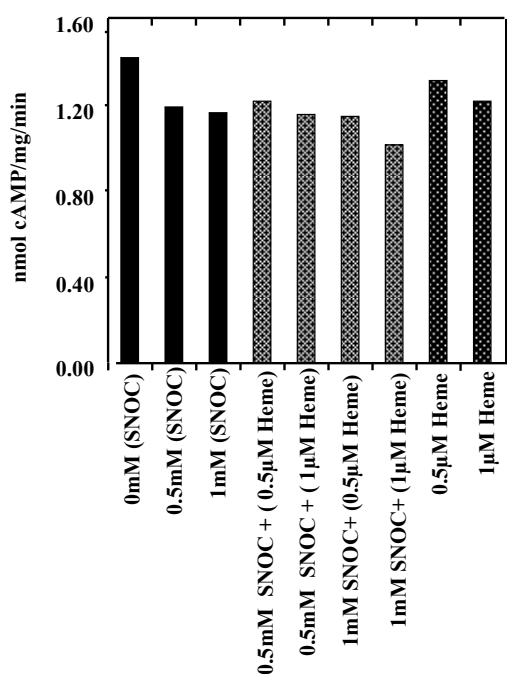
Figure 3.83: AC activity of the chimera with SNOC and heme; Assay conditions: 750 μM ATP, 20 min, 10 μg of protein, 37°C, Tris/HCl pH 7.5, n = 2.

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4. DISCUSSION

ACs catalyze the formation of the universal second messenger cAMP from ATP which regulates a large variety of intracellular processes in all organisms. Nine out of ten isoforms of mammalian ACs are membrane anchored having twelve predicted TM spanning α-helices (Krupinski et al., 1989). The function of these TM spans, apart from membrane localization, is unknown despite their topological analogy to transporters (Krupinski et al., 1989). Many ACs have also been found in prokaryotes which have membrane anchors of unknown function. There are four mycobacterial ACs (namely, Rv3645, Rv1319, Rv1318 and Rv1320) and CyaB from *C. glutamicum* which are anchored to the membrane by six predicted TM spans as well as CyaG from *A. platensis* which has two predicted TMs. The latter 6 ACs also have a HAMP domain which connects the TM region to a cytoplasmic CHD (Linder et al., 2004). HAMP domains are proposed to mediate signaling between extracellular sensory input domains and cytoplasmic output domains, thus they are often found immediately after the exit of the last TM domain (Hazelbauer et al., 2008). The four mentioned membrane bound mycobacterial ACs as well as CyaB and CyaG share the same molecular architecture i.e. the HAMP domain is located immediately after the exit of the last TM span and connects to the CHD, indicating that at least in these instances the membrane anchor may actually be a receptor. Bacterial MCPs also share this tripartite domain organization. They have a 2TM sensory domain with a large periplasmic segment which serves as a ligand binding receptor, a HAMP domain (signal propagation and conversion) and a chemotactic output domain (Ames and Parkinson, 1994; Boyd et al., 1983; Hazelbauer et al., 2008). Chimeras between bacterial ACs and MCPs were made here wherein the sensory domain of bacterial MCPs was connected to the CHD of the bacterial ACs with the HAMP originating either from the AC or from the MCP. The idea was to investigate whether the AC could sense extracellular ligands via N-terminally attached receptors. Thus in this study, two hitherto unrelated cellular signaling systems have been brought together which appear to be mechanistically connected by the HAMP signal converter in the midst of a periplasmic sensing and cytoplasmic output domain (Kanchan et al., 2010).

4.1 HAMP mediated signal transmission by different subclasses of bacterial ACs

4.1.1 *HAMP mediated Signal Transmission by Class III a AC*

CyaG from *A. platensis* is a class IIIa AC. The domain organization of this AC is identical to *E. coli* chemotaxis receptors in having two TM domains, TM1 and TM2, enclosing a large periplasmic loop of unknown function, a HAMP domain and an output domain. Chimeras were made wherein the membrane anchor along with the periplasmic loop of CyaG were replaced by the membrane anchor along with the periplasmic loop of Tsr, Tar, Trg and Tap chemoreceptors. The HAMP domain originated either from the receptor or from CyaG. The Tsr-HAMP$_{CyaG}$-CyaG chimera responded specifically to serine with an inhibition of cAMP production (Fig. 3.5). The IC$_{50}$ was in the micromolar range which is the same as seen for chemotaxis receptors (Mesibov and Adler, 1972). Tsr-HAMP$_{Tsr}$-CyaG chimera did not show signal regulation. This may be because of its very high activity which was 270 nmol/mg/min (Fig. 3.12). A mutation, I288S, was introduced into the HAMP of the chimera to reduce its activity because a similar mutation in case of Af1503 is known to turn HAMP to an OFF state. The mutation reduced the activity of the chimera by 98% with no signal regulation (Fig. 3.13 a). Another mutation, I291A, was inserted to counteract the effect of the I288S mutation. There was a slight increase in the activity but no regulation (Fig. 3.13 b). Introduction of large residues at position 291 in case of Af1503 makes the x-da (ON) state less favourable (Hulko et al., 2006). Possibly, an I291F mutant might have a weaker inactivating effect than I288S and might result in signaling. Further, CyaG was connected to the Tar receptor. The results were similar to those of Tsr/CyaG chimeras. The chimera which had the HAMP from CyaG showed an efficient and specific response to aspartate (Fig. 3.15) but the construct wherein the HAMP was from Tar did not show any regulation (Fig. 3.20). Similar constructs were made with Trg as well as Tap receptors, but none of the chimeras could sense their respective ligands (Fig. 3.22). Trg and Tap receptors need an extra periplasmic ligand binding protein for signaling (Abouhamad et al., 1991; Hazelbauer and Adler, 1971; Kalckar, 1971; Manson et al., 1986; Miller et al., 1980) hence AC assay for these chimeras were repeated along with their respective periplasmic binding proteins. GBP specific for Trg and DBP specific for Tap receptor was purified and used in the assay, but no signal regulation was observed (Fig. 3.23, 3.26). This may be due to the fact that the concentrations of GBP and DBP and their respective chimeric receptor proteins were not optimal. Another reason could

be that apart from periplasmic binding proteins they may require additional unknown accessory protein for signal regulation. Since membrane preparations of the Trg and Tap receptor chimeras have been used for the AC assays, these proteins might have been lost in the *in vitro* analysis. *In vivo* analysis by MacConkey assay and β-galactosidase assay gave contradicting results. A MacConkey assay with the Trg-HAMP$_{CyaG}$-CyaG chimera showed an activation of the AC in presence of 1M galactose while the β-galactosidase assay showed 35% inhibition in AC activity (Fig. 3.24). A β-galactosidase assay is a more direct measurement of the amount of cAMP produced i.e. more reliable than the MacConkey assay which is a qualitative assay. May be the activation seen on the MacConkey was from a nonspecific interaction of galactose with the maltose operon. It has been reported that the MalT is induced not only by maltodextrin but also by non-maltodextrin carbon sources, thus for instance degradation of galactose can cause mal gene induction (Schlegel et al., 2002). *In vivo* experiments with Trg-HAMP$_{Trg}$-CyaG chimera and Tap chimeras gave negative results. All the chimeras except the Tap receptor chimera, showed signal regulation on being linked to the CHD via HAMP$_{CyaG}$ domain and not when being linked to respective receptor HAMP domains. CyaG AC has relatively longer linker between the HAMP and the first catalytic residue, i.e. 63 amino acid as compared to 38 in Rv3645 AC. It seems that the CyaG AC is tuned to its linker along with its HAMP such that it is specific to its own HAMP domain hence it does not show any signal regulation on being linked with other HAMP domains. All the chimeras with receptor HAMP domains have unusually high activity, hence it is clear that misfolding of the protein could not be the reason for it not being regulated. May be that the connection between the HAMP and the CyaG linker is not in register or may be the AC is unhooked from its regulatory scaffold and freely folded into an active dimer.

4.1.2 *HAMP mediated Signal Transmission by Class III b AC*

Mycobacterial Rv3645 AC has threonine instead of aspartate as substrate specifying residue in the catalytic core which is specific for class IIIb isoforms. Similar constructs were made with Rv3645 AC as for CyaG chimeras. Six TM domains present in the AC were replaced with the sensory domain of Tsr and Tar receptors. In case of Tsr/Rv3645 chimeras signal regulation was seen whether the HAMP domain was from receptor or from the AC itself. The percentage inhibition with HAMP$_{Tsr}$ domain was higher compared to the chimera which had the HAMP domain from the AC (compare Fig. 3.28 a and 3.31 a). This may be due to the fact

that the extent of inhibition depends upon several factors such as the binding energies between receptor and ligand, the type of HAMP domain as well as the type of output domain. Since in all chimeras the basic design is the same but they are still different from each other in having different receptor ligand pairs, different HAMP domains and different output domains the extent of inhibition as well as the response varies between different chimeras. The inhibition in case of Tsr-HAMP$_{Tar}$-Rv3645 chimera which had the HAMP domain from Tar was comparable to the chimera having the HAMP domain from Tsr (compare Fig. 3.28 a and 3.33 a). Further chimeras with the Tar receptor and Rv3645 AC were made which behaved differently from Tsr/Rv3645 chimeras. The Tar-HAMP$_{Tar}$-Rv3645 chimera did not show regulation (Fig. 3.37) but Tar-HAMP$_{3645}$-Rv3645 showed a specific and potent response to aspartate (Fig. 3.35 a). Tar-HAMP$_{Tsr}$-Rv3645 also showed regulation (40% inhibition) (Fig. 3.39 a). These results with different chimeras suggest that the bacterial MCPs and ACs have a common mechanism of signal conversion via the HAMP domain. The results also suggest that HAMP is an independent signaling unit which can be swapped between different sensory and output modules in bacteria. Previously, hybrid sensors have been made in which the sensor module and HAMP linker of one protein communicates with the output module of another protein and most of these were functional. Such hybrids have been made between different *E. coli* MCPs (Feng et al., 1997; Krikos et al., 1985; Weerasuriya et al., 1998), *Bacillus subtilis* MCPs (Kristich et al., 2003), the MCPs Tar or Trg and the sensor kinase EnvZ (Baumgartner et al., 1994; Utsumi et al., 1989), and the sensor kinase NarX and the MCP Tar (Ward et al., 2002). The apparent ease with which such functional hybrids have been generated using an arbitrary fusion point suggests that the HAMP domain is a commonly used signal-transducing structure and that the HAMP domain from one protein can communicate with heterologous input and output modules (Appleman et al., 2003). However, in a few instances these hybrids were not functional or their response could not be interpreted. Similar observations were made with the chimeras discussed here. Though the HAMP domain could be swapped between constructs, in some instances the chimera did not show any response, hence it seems that though having a conserved structure and mode of action, the HAMP domains cannot be viewed as freely interchangeable module. This fact may accounts for the presence of the huge number of HAMP domains in the database (>12,000 HAMP domains are in the EMBL SMART database).

4.1.3 *Functional complementation of Tsr and Tar mutants*

Receptor mediated signaling seen in receptor/AC chimeras was confirmed by introducing R69E and T156K mutations in Tsr and R69E and T154I mutations in Tar chimeras. Individual mutants of Tsr and Tar chimeras were unresponsive to their respective ligands but on co-expressing the mutants together the serine and aspartate signaling was restored respectively (Fig 3.41 c, f). This proved that the signaling was receptor mediated. The point mutations were designed according to those used successfully in chemotaxis assays (Ames et al., 2008; Gardina and Manson, 1996; Mowbray and Koshland, 1990). The extent of inhibition in the Tsr and Tar mutant chimeras was reduced as compared to the wild type chimeras. This difference in the rate of inhibition was expected because during co-expression the probability for the formation of inactive homodimeras is two while it is one for active heterodimers.

 Several other controls were made to confirm the receptor mediated regulation of these chimeras. Tsr and Tar interacts only with L-serine and L-aspartate, respectively, hence AC assays carried out with D-serine and D-aspartate as a control did not affect activity (data not shown). CyaG and Rv3645 holoenzyme as well as their purified CHDs were tested with L-serine and L-aspartate which gave negative results (Fig. 3.42). The control experiments confirmed that the ligand binds to the periplasmic domain of the chemotaxis receptors and probably the mechanism of signal conversion is also the same as in the MCPs. In chemoreceptors, serine and aspartate mediate an attractant response. Upon binding the attractant there is a movement in the TM helix, which in turn shift the HAMP domain to a more expanded conformation leading to reduced kinase activity which corresponds to a "kinase-off" state while in absence of attractant the HAMP is in a more compact conformation leading to an increase in kinase activity which corresponds to a "kinase-on" state (Falke and Hazelbauer, 2001; Hazelbauer et al., 2008; Khursigara et al., 2008; Parkinson, 1993). The experiments here show a similar situation i.e. an increase in serine or aspartate concentration shifts the AC to an off state and vice versa, this explains why an inhibition of cAMP production is seen on ligand binding in almost all the receptor/AC chimeras.

4.2 Transmembrane signaling by non HAMP containing ACs

4.2.1 *Mycobacterial Rv1264 AC chimeras*

Generation of successful and functional chimeras with Rv3645 AC as well as CyaG AC which belong to class IIIb and class IIIa ACs, respectively, confirmed that the receptor mediated regulation was not restricted to any specific subclass of AC. Rv1264 being a class IIIc AC was connected to Tsr receptor with the HAMP of Tsr to investigate whether class IIIc ACs are compatible to receptor mediated regulation. Rv1264 was different from Rv3645 and CyaG in being a soluble AC without a TM anchor and a HAMP domain hence it was interesting to find out whether such an AC could sense the extracellular signal via the N-terminally connected receptor. Tsr-HAMP$_{Tsr}$-Rv1264 chimera was not regulated by serine (Fig. 3.44 b). Alanines were inserted at the junction where the receptor and the AC were linked to check whether the construct was in correct register. The chimera with two alanines was 50 times more active than the wild type but none of the chimeras could sense the serine signal (Fig. 3.45 b, d). Possible reasons may be that the chimera was still not in correct register or may be it needs its own linker. Tsr-HAMP$_{Tsr}$-Rv1264 AC chimera had a linker from Rv3645. From the crystal structure of Rv1264 it is clear that the linker region of the AC is very important as it plays a major role in maintaining the two state conformation i.e. active and inhibited state of Rv1264. Interaction between the N-terminal regulatory domain and the CHD in Rv1264 AC is lost on mutating the residues in the linker region (Linder et al., 2002; Tews et al., 2005). Probably by including the linker from Rv1264 in the Tsr-HAMP$_{Tsr}$-Rv1264 AC chimera could bring about regulation of the AC via an external HAMP domain.

4.2.2 *Cyanobacterial CyaB1 AC chimeras*

CyaB1 does not share the tripartite domain organization as observed in bacterial MCPs and Rv3645 and CyaG ACs. Its structure is completely different in having cytoplasmic tandem GAF domains, a PAS domain and a C-terminal CHD (class IIIb) (Kanacher et al., 2002; Katayama and Ohmori, 1997). Initially CyaB1 along with its PAS domain was connected to Tsr and HAMP$_{Tsr}$, but the chimera was not regulated (Fig. 3.48 b). Surprisingly on removing the PAS domain and connecting the Tsr and HAMP$_{Tsr}$ domain directly to the CyaB1 CHD, there was regulation i.e. on increasing the serine concentration there was decrease in the amount of cAMP produced (Fig. 3.50 b). This functional chimera suggested that ACs in

general have similar mechanisms of regulation. CyaB1 though devoid of TMs and a HAMP domain, could be connected to Tsr and sense a serine signal. The HAMP domain brings about signal transmission only when it has a threshold amount of energy which is provided by the binding energies of the receptor ligand pair. Apart from binding energy, signal transmission also depends on the type of HAMP domain and as well as the output domain hence for every chimera the threshold energy needed by the HAMP domain is different. It could be possible that this threshold energy needed for the chimera with the PAS domain was much higher, hence no signal regulation was observed. On removing the PAS domain the threshold energy decreased and the HAMP domain could efficiently transfer the signal received from the receptor.

4.2.3 *Mycobacterial Rv1625c AC chimeras*

Rv1625c AC has a domain organization identical to mammalian ACs. Rv1625c AC chimera was inactive when connected to Tsr and HAMP$_{Tsr}$ with the linker from Rv3645 AC (data not shown). Both the ACs are of different subclasses, may be their linkers are incompatible to each other. Linker compatibility was checked by replacing the linker of Rv3645 with that of Rv1625c as well as replacing it with the linker from CyaG. All chimeras were active confirming that AC Rv1625c could successfully form the dimeric catalytic center with its own linker as well as with linkers from the same subclass of ACs. The activity was enhanced on replacing the N-terminus of Tsr-H(Tsr)-Rv1625c chimera with that of Rv1625c N-terminus. Although all chimeras were active none of them was regulated (Fig. 3.55 c). Possible reasons could be that the constructs were out of register or the energy required for observing regulation was below the threshold.

Rv1625c CHD is connected to its membrane anchor directly via a linker. It is observed that on connecting Rv3645 AC directly to the Tsr receptor without a HAMP domain resulted in an unregulated chimera (data from J. Linder, not shown) indicating the importance of HAMP domains in signaling. Absence of any predicted signal converter domains between the TMs and the CHD in Rv1625c put forth an obvious question as to how the signal regulation is achieved in this cyclase. There have been speculations that the mammalian ACs linkers have some coiled-coil like properties hence the Rv1625c linker was aligned with mammalian ACs and some of the bacterial ACs to see whether there were any similarities. A small region of this linker was conserved in all the ACs aligned (Fig. 3.56). Further Rv1625c showed the

presence of a coiled-coil like structure in the linker region (R218-R240) when analyzing its sequence through a program called [COILS].

[http://www.ch.embnet.org/software/COILS_form.html] (Lupas et al., 1991).
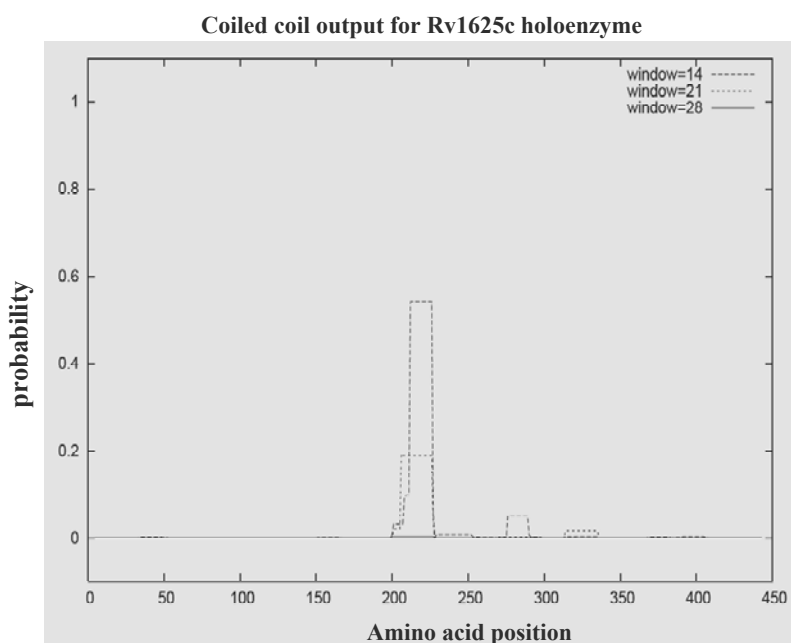
Figure 4.1: Sequence of Rv1625c showing the probability of coiled-coils in the region (R218-R240). Sequence analysed by [COILS] (Lupas et al., 1991). Window 14, 21 and 28 represent the heptad repeat frame. Window 14 is two heptads long, window 21 is 3 heptads long and window 28 is 4 heptads long. The coiled-coil is more prominent in window 14 with probability approximately 0.6 while no coiled-coil was predicted in window 28.

Functional significance of this linker was explored by designing several chimeras wherein Rv1625c along with its linker was directly connected to Tsr as well as to Tar without a HAMP domain. Several permutations and combinations were made in Tsr/Rv1625 chimera to confirm the correct position where both proteins could be linked (Fig. 3.57). The chimera where the CHD of Rv1625c started with an arginine (Tsr (+R) Rv1625c) was the most active (200 nmol/mg/min) (Fig. 3.58 b). The activity reduced on replacing this arginine with either one or two alanines (Fig. 3.58 b). Other chimeras wherein the N-terminus of Tsr-Rv1625c was replaced with the N-terminus of Rv1625c and a Tar/Rv1625c chimera were four-fold less active than the Tsr (+R) Rv1625c AC chimera (Fig. 3.58 b). None of the chimeras showed any regulation by serine/aspartate as a ligand (Fig. 3.58 b). It seems that though the

linker was important as it modulates the AC by either increasing or decreasing its activity, it could not transmit the signal.

TM5 and TM6 of Rv1625c were aligned with the HAMP domains of MCPs and ACs. The alignment showed a similar pattern of hydrophobic residues as found in HAMPs (Fig. 3.6 a). To explore whether the TM5 and TM6 of RV1625c function as a HAMP domain, Rv1625c was connected to Tsr via its TM5 and TM6 without the presence of HAMP of Tsr. The protein was expressed and active but it did not show regulation (Fig. 3.60 c). The results do not tell us whether the TM5 and TM6 is a TM sequence as predicted or a HAMP domain. Since HAMP domains are coiled-coil (CC) structures, Rv1625c holoenzyme sequence when analyzed for the presence of CCs, CC structure was found only in the region R218-R240, which is the Rv1625 linker region (Fig. 4.1). No CC structure was found in the region of TM5 and TM6, indicating that TM5 and TM6 were predicted TM helices and not coiled-coils.

4.3. Swapping of Transmembrane and Ligand binding domains in Tsr/Rv3645 chimeras and Rv3645/Rv1625c holoenzyme respectively

Transmembrane signal regulation in chimera Tsr-HAMP$_{Tsr}$-Rv3645 was studied in more detail by replacing its TMs with that of Tar and Af1503 TMs. The idea was to investigate whether there was need for specific sequences for inter- and intrahelical interactions between the TMs and the ligand binding domain for signal propagation. As reported before, when TM2 and the cytoplasmic domain of Tsr was replaced by the corresponding domain from Tar and vice versa, this resulted in a functional hybrid with apparent ligand binding affinities similar to wild type suggesting that specific sequences were not required in TM2 of Tsr or Tar for respective signal transmission (Tatsuno et al., 1994). The similarities between TM1 and TM2 of Tsr and Tar is high (Fig. 3.61), probably the sequences crucial for signal propagation lie in this conserved region and hence the ligand binding affinities of the chimeras were not compromised. This possibility was confirmed by replacing both TMs of Tsr-HAMP$_{Tsr}$-Rv3645 chimera with those of Tar as well as with the TMs from a completely unrelated protein, i.e. Af1503 protein. The chimera with the Tar TMs recognized serine as a ligand and showed 60% inhibition in cAMP production (Fig. 3.62 b) while the chimera with Af1503 TMs was unresponsive to serine (3.63 c, 3.64 b). Af1503 TMs share just 20-30%

similarities with TMs of Tsr (Fig. 3.63 a), hence it seems that the specific sequences required for signal propagation are not present in the conserved region and, thus, regulation is impossible. The same argument applies to Tar TMs. Similarity is high which is 78% in TM1 and 58% in TM2 on comparing it with TM1 and TM2 of Tsr, respectively (Fig. 3.61). Obviously, the sequences needed for signal propagation are conserved, thus, we see regulation. This observation could be explored further by swapping just the ligand binding domains of Tsr and Tar or between other receptors to see whether the ligand binding domain could still sense the ligand which would confirm whether specific sequences in TMs are needed for signal transmission.

Rv3645 and Rv1625c holoenzyme have three periplasmic loops of unknown function and six TM spans. The 3rd periplasmic loop was replaced in each protein with the ligand binding domain from Tsr (Fig. 3.65, 3.67 a). Both chimeras were active but could not signal (Fig. 3.66 c, 3.67 d). This indicates that specific sequences and hence specific TM spans are required for ligand sensing and signal propagation. It has been shown before that single point mutations in TM1 and TM2 of Tar and Tsr can make them unresponsive to their respective ligands and hence no chemotaxis is observed (Ames et al., 2008; Gardina and Manson, 1996; Mowbray and Koshland, 1990). It seems that the sequences in the TM spans are not only involved in inter- and intrahelical interactions which govern the signal propagation but are also involved in the dimerization of the protein and hence any change in the sequences of these membrane spans also affect ligand binding affinities.

4.4 Transmembrane deletion constructs to get soluble and functional chimeras

The structural analysis of the Af1503 HAMP domain indicated rotation as the mechanism of signal transmission (Hulko et al., 2006). Since Af1503 HAMP does not connect known input and output modules it is difficult to predict that different HAMP domains transmit signals by a similar mechanisms. To understand the mechanism of signal regulation via HAMP domain there is a need to have structural details of HAMP domains together with their native receptors. The Tsr-HAMP$_{Tsr}$-Rv3645 AC chimera has a HAMP domain which is connected N-terminally to the chemotaxis receptor Tsr and C-terminally to an AC domain hence it

provides a novel system to analyze HAMP mediated signaling quantitavely *in vitro* compared with scoring swimming behaviour *in vivo*. The chimera was solubilized by 1% CHAPS but the purification efforts failed because the protein lost much of its activity (J. Linder, personal communication). Purification efforts were also made with Tsr-HAMP$_{CyaG}$-CyaG AC chimera (data not shown). Yet the presence of TMs made the purification of these chimeras impossible. The first approach towards a soluble, signaling construct was to remove the TM spans from the Tsr-HAMP$_{Tsr}$-Rv3645 AC chimera (Fig. 3.68 a). A similar study had been done before wherein the TM spans from Tar were removed. This resulted in a soluble protein which bound aspartate and was methylated thus confirming that a receptor without membrane spans may relay the signal to the output domain (Ottemann and Koshland, 1997). Residues from (L11-G27) from TM1 and (M193-V210) from TM2 of the Tsr-HAMP$_{Tsr}$-Rv3645 AC chimera were deleted. The last four residues from TM2 were not deleted. It includes two aromatic aa, tryptophan and phenylalanine, which correspond to tryptophan and tyrosine in TM2 in Tar. It has been reported that these two aromatic residues play an important role in maintaining the baseline signaling state and ligand sensitivity in Tar (Draheim et al., 2005). The chimeric protein was purified successfully (Fig. 3.68 b) but AC activity of the chimera was reduced from 18 to 1 nmol/mg/min. In addition it was not regulated by serine (Fig. 3.69). It could be possible that the chimera was out of register or the presence of TMs was important for signaling. Several additional chimeras were made wherein deleted aa were put back sequentially from the C- and N-terminal end of TM2 to explore the minimum number of residues needed in TM2 to obtain a soluble and regulated chimera (see the alignments, Fig. 3.7 a, 3.73 and 3.76). The TsrΔTm1C'Rv3645Mt1-Mt8 chimeras could be purified successfully (see, Fig. 3.70 b, 3.71 and 3.74 a-e). The TsrΔTm1C'Rv3645Mt9-Mt12 as well as TsrΔTm1N'Rv3645Mt1-Mt6 chimeras could not be purified as they were in the membrane (Fig. 3.74 f, 3.75 a-c and 3.77). None of the chimeras was affected by up to 10 mM of serine (Fig. 3.75 d, e and 3.78). Similarly the chimera wherein TM1 was deleted while TM2 was retained also was not regulated (Fig. 3.79 b). In all deletion chimeras modifications were carried out only in TM2 while TM1 was deleted throughout. Therefore it may be that TM1 is required for signaling. It has been reported that mutating an alanine to lysine (A19K) as well as creating a deletion of seven amino acids in TM1 of Tar results in loss of aspartate chemotaxis (Oosawa and Simon, 1986). The same may be true for Tsr because Tsr and Tar are evolutionarily and functionally conserved. To explore the importance of TM2 in signal transmission there is a need to have a chimera where TM1 remains intact while TM2 is

deleted. More flexibility has been seen in case of TM2 of Tsr because replacing it with TM2 of Tar resulted in active and functional chimeras (Tatsuno et al., 1994) .

The chimeras with deleted TMs did not respond to serine hence point mutations were inserted in both TM1 and TM2 of Tsr to make them less hydrophobic to get a soluble protein. The residues which were conserved throughout chemoreceptors were not mutated. Residues which had least deleterious effects on the Tsr function were mutated (Fig. 3.80 a) (J.S. Parkinson, personal communication). Tsr-(mut)-Rv3645 as well as Tsr-(LL-RR)-Rv3645 AC chimeras (see, Fig. 3.80 a, b) could not be purified and their AC activity was low compared to wild type and was not regulated (compare Fig. 3.80 d and 3.28 a). It could be possible that the structure of Tsr is destabilized by replacing the hydrophobic residues by polar and charged residues in TM1 and TM2. A strong polar residue, or multiple weakly polar residues have been shown to cause formation of strong and potentially deleterious interhelical hydrogen bonds depending upon the localization of those residues within the TM sequence (Dawson et al., 2003). Similarly introduction of two arginine residues are known to drive the protein out of the membrane but it could also disrupt packing and dimerization of the helices (Sal-Man and Shai, 2005). These studies indicate that the TM domains of the chimera cannot be deleted as probably they are playing an integral role in signal transduction.

4.5 Biochemical characterization of CyaG and exploring its ligands

The CyaG holoenzyme and CyaG CHD with and without HAMP have been characterized in section 3.1.1. Basal activity of CyaG CHD was 800 nmol/mg/min. CyaG CHD along with the HAMP domain could not be purified. There were several non specific bands in the purified protein fraction and also the activity was reduced drastically (0.4 nmol/mg/min). The result agrees with earlier observation with mycobacterial HAMP containing ACs that the HAMP domain somehow modulates cyclase activity (Linder et al., 2004). Purification efforts for the CyaG holoenzyme were unsuccessful. The presence of TM and HAMP domain made the purification impossible. There is a need to standardize the solubilization and purification of the protein by screening different detergents. The domain organization of CyaG is more closely related to chemotaxis receptors, thus it was used further for exploring potential ligands. A conserved domain search was made using the NCBI database. While a domain analysis by SMART yielded only the TM, HAMP and AC domain, the NCBI search

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indicated the presence of additional domains such as HNOBA and CACHE domains (Fig. 4.2). The HNOBA domain is found associated with HNOB which is predicted to bind NO in presence of heme (Iyer et al., 2003). Thus various nitrogen containing compounds were examined whether they have effect on AC activity. $NaNO_2$, KNO_3 and SNOC alone as well as in presence of heme did not affect AC activity of the CyaG holoenzyme as well as its CHD (Fig. 3.81, 3.83). Adenosine and guanosine have an inhibitory effect on the holoenzyme as well as the CHD (Fig. 3.82). This means that the interaction is non specific i.e. they are interacting with the AC catalytic domain. It has been reported before in case of mammalian adenylyl cyclases that they can be inhibited by P-site inhibitors which interacts with the catalytic domain at a site distinct from 5' ATP binding pocket (Laurent et al., 1996). Adenosine and guanosine fall into this category. Efforts continue with other compounds. Another domain found within the AC was the CACHE domain. These are present as extracellular or periplasmic domains and are predicted to recognize small molecules in a wide range of proteins, including the animal dihydropyridine-sensitive voltage-gated Ca^{2+} channel; α_2, δ-subunit, and various bacterial chemotaxis receptors (Anantharaman and Aravind, 2000). It was first found in the voltage gated Ca^{2+} channels and various chemotaxis receptors hence named as CACHE (Anantharaman and Aravind, 2000; Anantharaman et al., 2001). These domains are abundant in bacteria. Extracellular CACHE domains are often fused to catalytic domains of histidine kinases, phosphatases, diguanylate cyclases and ACs. In CyaG the CACHE domain is predicted to be present in the periplasmic loop, suggesting that the periplasmic loop of CyaG may bind a ligand.
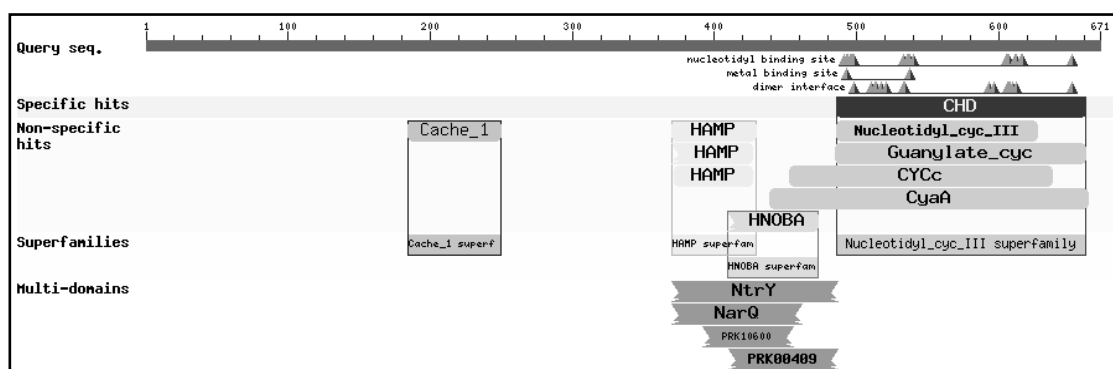

Figure 4.2: Conserved domain search of CyaG by a NCBI search.

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CyaG_(A.pl : WSEIYGYFSQALVVSANRPINNLLGVASSDLTLKRISEFLGTLEIGKTGQIVERNGYLV
gi74962226 : WTQLYMETGTGPIVTLSLPILKLAGVVAIDISIKEFTKHLPTSSEQMYGYIVDNNGMLI
gi75426209 : VTEPFVDATGEQILAMSLPVRQLLGVAAGEWKLETLTAILNSLKFDGAGYLVSDACKIL
gi75426210 : LTEPYMEPIHELVLTIASPARQPFGVVGGDLSLQTVVKIINSLDFGGMGYLVSGDCKIL
gi81552616 : VLTPARKSDSQWVSVTQELVANLGVLRLDISYETLEAYLNQLQLGQQGFIINENHEFV
gi81872883 : WTEAYIDSGLVLMTTVAMPVFILLGVVGTDVPVKEILKTIPKYKLGIHGYAITNNGYIL
gi23993866 : WTEPYKDVTGDMIVTASKAILKVIGVASYDIKLSAIQSMVNKQKVPYKGFLADASCNLL
gi25175729 : ITDPYKTATNTMVVTIAQQTKDGSGVIAINMTIENILKTTKKVNIGTQGYIMTKDKKVV
gi1164909  : WTNVYLDAELGLVITGTLPVFLILGVMGVDVSLEDIKRLTPRFTLCPNGYAIDPNGYVL
gi74869443 : WTHAFTDKRPRLMISVGVPAFRLLGVAGTDVPVEDIDKLTLPYKLGVNGYVVSNNGYVL
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Figure 4.3: Alignment of CyaG periplasmic loop with predicted CACHE domains, alignment taken from NCBI domain search database; **gi74962226** Hypothetical protein tag-180 from C. elegans, **gi75426209** Chemotactic transducer from P. aeruginosa, **gi75426210** Chemotactic transducer from P. aeruginosa, **gi81552616** a putative Histidine kinase from Streptococcus, **gi81872883** Voltage-dependent calcium channel from Mus musculus, **gi239938660** Methyl-accepting chemotaxis protein mcpC from Bacillus subtilis, **gi251757296** Methyl-accepting chemotaxis protein mcpA from bacillus subtilis, **gi1164909** Voltage-dependent calcium channel from Oryctolagus cuniculus (Rabbit), **gi74869443** CG12455-PA, isoform A from Drosophila melanogaster.

5. Open questions and outlook

Generation of successful chimeras of Tsr and Tar with Rv3645 and CyaG AC made us to postulate a functional significance of the membrane anchors of Rv3645 and CyaG AC as receptors. For confirmation there is a need to have a ligand for these membrane anchors. CyaG AC has a CACHE domain located in the periplasmic loop, since CACHE domain is suggested to bind small molecules it indicates that the periplasmic domain of CyaG AC may be a receptor. This possibility needs to explored. The mechanism of signal transmission via the HAMP domain is still enigmatic. There is a need to have a soluble chimera for structural analysis so that HAMP mediated signal transmission could be studied in more detail. Linkers between the AC and the N-terminal HAMP domain (or between the TM spans and the CHD, which have no HAMP domain) seem to be important as they affect the activity of the ACs. The linker from Rv1264 is known to play an important role in maintaining the two state conformation i.e inhibited and activated state of Rv1264 AC, yet nothing is known about the functional significance of Rv3645, CyaG and Rv1625c AC linkers. A small region of these linkers is conserved in bacterial ACs as well as in mammalian ACs. Interestingly, the linker from CyaG is 25 amino acids longer than that from Rv3645 and it was observed that CyaG AC chimeras were functional only when they had the CyaG linker. Obviously, there is a need

to understand the role of these linkers in signaling in more detail. This study shows that ACs can be manipulated successfully on connecting them with unrelated receptor protein.

6. Summary

Transmembrane signal regulation has been studied using chimeras of *E. coli* chemotaxis receptors and several bacterial ACs which share a common molecular domain organization. The chemotaxis receptors have a membrane anchored receptor with 2 TMs which is linked via a cytoplasmic HAMP domain to a C-terminal signal output domain. The investigated bacterial class III ACs have a hexahelical membrane anchor whose functional significance currently is unknown, and are linked via a cytoplasmic HAMP domain to the catalytic region. Both proteins need to dimerize for activity. To explore the possible role of the hexahelical anchor of bacterial ACs as sensory receptors, intramolecular networks of the chemotaxis receptors Tsr and Tar were joined with the mycobacterial AC Rv3645 and the cyanobacterial AC CyaG such that the sensory domain was from the chemoreceptor while the output domain from the bacterial ACs. The connecting cytoplasmic HAMP domain was either from the chemoreceptors or the ACs. Such hybrid chimeras were not only enzymatically active but could sense the ligands from the respective receptors because they regulated. Generation of functional chimeras demonstrated that the uniform construct design of the chimeras preserved register and packing of the signaling ensembles. Receptor mediated regulation was further confirmed by inserting single point mutations in the Tsr and Tar receptor segments which are known to abolish ligand binding. The individual mutants R69E and T156K in Tsr and R69E and T154I in Tar abolished serine and aspartate regulation respectively but on co-expression regulation was restored as expected from earlier chemotaxis experiments i.e. I generated a bacterial AC which could be regulated by chemotaxis receptors. The results suggest that the MCPs and bacterial ACs have a common mechanism of signal propagation. Successful swapping of HAMP domains between different chimeras demonstrated that the HAMP domains in one protein could communicate with heterologous input and output modules.

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8. APPENDIX

CyaG CDS (*Arthrospira platensis*)

ACC No. D49531.1
GI: 11990886
Protein ID: BAB19924.1

MRLFIIPPIPKLSLRLVLIVPFLLQITIAFGLTGWLSYRNRTRA**M**NDLATQLQVEVSNRIKQEMNTYLETP
HLINQINVDAINQGLISIDNIPALERYLWTQLQQFPTASYIGMGMENGDYIGAGRYDDGSIEFDIL**A**DST
NRQLEKWLTDDQGNRTELVSSRDNYDPR**V**RPWYLVAVEAGKPVWSEIYGYFGSQALVVSANRPIND**P**
EGNLLGVASSDLTLKRISEFLGTLEIGKTGQTFIVERNGYLVGTSTLEKTFITDEENRDTERILA**A**ESSDI
MTRETA**R**YLANKLA**Y**IS**Q**I**D**NHD**H**LSFYA**S**SRREFVQVTPMIDPRGI**H**WLIVVVVPEADFMEQIHENTR
ITILLFMAALVLAIILGILTARWISEPILRLSEASSAIASGARNATASAELNQ**K**VKVEKIRELGMLSESFNM
MIQNLRDSFIALE**N**TNRELEQRVLERTAAL**L**QEKERSEELLLNVLPKPIADQLKANKKAIASAIEEVTILF
ADIVGFTPLSARM**H**PI**D**LVSLLNEMFSIFD**H**LA**E**KHKLEKIKTIGDAYMVVGGL**S**LPQ**D**NHAEAIADMA
LEMQAAMKQFQGSYL**V**GSESFQIRIGINTG**S**VVAGVIGIKKFIYDLWGDAVNIASRMESSGTPGSIQVTE
ETY**N**RLK**K**NYIFK**E**RG**P**IPVKGKGQMTTYWLLGKKPVVDIS.

Residues marked in bold are those which were different from the original sequence obtained
from NCBI data base.

Rv3645 (*M. tuberculosis*)

Reference sequence, ACC. No. NC_000962
GI: 885620
Protein ID: NP_218162.1

Rv1264 (*M. tuberculosis*)

Reference sequence, ACC. No. NC_000962
ACC. No**.** Q11055.1
GI:1723089

Rv1625c (*M. tuberculosis*)

Reference sequence, ACC. No. NC_000962
GI: 57116894
Protein ID: NP_216141.2

CyaB1 (*Anabaena*)

ACC No. D89623
GI:15553050
Protein ID: BAA13998.2

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