Mycobacterial adenylyl cyclases Rv1625c and Rv0386: orthodox vs. unorthodox catalysis

Die mycobakteriellen Adenylatcyclasen Rv1625c und Rv0386: Orthodoxe gegenüber unorthodoxer Katalyse

DISSERTATION

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Lucila Isabel Castro Pastrana

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Dekan: Prof. Dr. H. Probst

Erster Berichterstatter: Prof. Dr. J. E. Schultz

Zweiter Berichterstatter: PD Dr. J. Linder

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List of abbreviations

AC (s)   Adenylyl cyclase (s)

BSA   Bovine serum albumine

CHAPS   3-(3-Cholamidopropyl)- dimethylammonio-1- propane sulfonate

cpm   Counts per minute

dNTPs   Desoxynucleoside triphosphates

DTT   Dithiotreitol

FPLC   Fast performance liquid chromatography

GC (s)   Guanylyl cyclase (s)

HEPES   N-2-Hydroxyethyl piperazine-N'-2-ethanesulphonic acid

IPTG   Isopropyl thiogalactoside

LB-broth   Luria-Bertani bacterial growth medium

MCS   Multiple cloning site

MWCO   Molecular weight cut-off

Ni-NTA   Nickel-nitrilotriacetic acid-agarose

O/N   Overnight

PEG   Polyethylen glycol

PMSF   Phenylmethansulfonylchlorid

PVDF   Polyvinylidene difluoride

RT   Room temperature

TEMED   N,N,N',N'-Tetramethylethylene diamine

TLCK N_α-Tosyl-L-lysin-chlormethylketon-hydrochlorid

TPCK   Tosyl-L-phenylalanin-chlormethylketon

X-Gal   5-bromo-4-chloro-3-indolyl-β-D-galactoside

For amino acid residues the one-letter code was used.

1 Introduction

1.1   Adenylyl cyclases: function, classification and evolution

Adenylyl cyclases are responsible for the synthesis of cAMP (adenosine 3´,5´-cyclic monophosphate) from adenosine triphosphate (ATP). Although they represent low abundance proteins in the cell, they produce a universal signalling molecule which mediates with high amplification diverse physiological processes in organisms as phylogenetically diverse as *Escherichia coli* and *Homo sapiens*. Transcription in bacteria and development in fungi and parasites are cAMP-regulated. In eukaryotes cellular functions like energy homeostasis, reproduction, brain function and cell differentiation are influenced by cAMP too. Cyclic AMP functions as a second messenger to relay extracellular signals to intracellular effectors such as protein kinase A. Regulation of intracellular concentrations of cyclic AMP are controlled primarily through modulation of AC activity and phosphodiesterases. The family of eukaryotic ACs consists of several membrane bound ACs and a single soluble AC with orthologs of the latter in rat, *Dictyostelium* and bacteria but absent from the genomes of *D. melanogaster*, *C. elegans*, *A. thaliana* and *S. cereviciae* (Roelofs and Van Haastert, 2002).

Cyclases (ACs and GCs) have been classified according to their amino acid sequence similarities rather than their substrate specificities. The class I type comprises ACs which produce cAMP as a consequence of its phosphorylation and are found in *E. coli*, *Salmonella*, *Pasteurella*, *Haemophilus* and *Vibrio* as single copy genes (Danchin, 1993). The second class of ACs comprises those which are calmodulin-activated, secreted toxins like the enzymes produced by the pathogenic organisms *Bordetella pertussis* (Ladant and Ullman, 1999) and *Bacillus anthracis* (Baillie and Read, 2001). [For class III ACs see below]

The fourth and fifth classes of ACs may be represented by cyclases with thermostable properties from *Aeromonas hydrophila* and from the ruminal anaerobe *Prevotella ruminicola*, respectively (Danchin, 1993).

The class III of nucleotide cyclases truly represents a conserved cyclase catalytic fold which are found in prokaryotes and in eukaryotes (Danchin, 1993). Recently, after analysis of amino acid sequence profiles, a division of the class III in to four subclasses,

class IIIa to IIId was suggested. This subclassification is partly based on length differences of an "arm" region. This region was thought to be essential for dimerization (Tesmer et al., 1997) and comprises the sequence between a conserved glycine and the downstream adenine-specifying residue (D1018 in AC type II from rat) (Linder and Schultz, 2003). The arm region contains 14 residues in subclass a (mammalian ACs, mycobacterial Rv1625c), 15 residues in subclass b in conjunction with a highly characteristic D to T/S switch in a substrate defining position (*Anabaena* cya B1, mammalian soluble AC), 7 to 11 amino acids in subclass c (mycobacterial Rv1264, Rv0386, *Streptomyces coelicolor* AC acc. num. P40135) and 13-14 residues in subclass d (*U. maydis* AC acc. num. A55481, *L. donovani* cyaA acc. num. Q27675) in which also a special motif surrounding the substrate-defining lysine is highly conserved (Linder and Schultz, 2003). From an analysis based on the available genome sequence from over 80 bacteria and other pathogenic organisms, it is becoming clear that the majority of cells have at least one cyclase belonging to class III cyclases and that the other classes of cyclases are more restricted in their occurrence. That demonstrates the versatility of the class III catalytic domain which apparently can be incorporated as a distinct functional domain into a larger polypeptide in conjunction with other protein domains with diverse physiological functions. Furthermore, it appears that the same class III fold can adapt to utilize ATP or GTP as a substrate, given the sequence similarity between mammalian ACs and GCs (Shenoy et al., 2002). From recent phylogenetic studies it has been concluded that within the family of ACs, soluble ACs were poorly conserved during evolution, whereas membrane-bound ACs have expanded to form the subgroups of prevailing ACs and GCs (Roelofs and Van Haastert, 2002).

Most class III cyclases, from bacteria to higher vertebrates, are constructed in a modular fashion as evident from their rather large size. The variability in the block-building of cyclases makes possible a fine regulation of their activity (Linder and Schultz, 2003) [see figure 1.1].

Adenylyl cyclases

Mammalian ACs (I-IX)

M. tuberculosis
Rv1625c

P. falciparum AC

M. tuberculosis
Rv1264

M. tuberculosis
Rv0386

Anabaena CyaB1

Guanylyl cyclases

Paramecium GC

soluble GC-$\alpha\beta$

Mammalian GCs (A-G)

Sea urchin GC

3
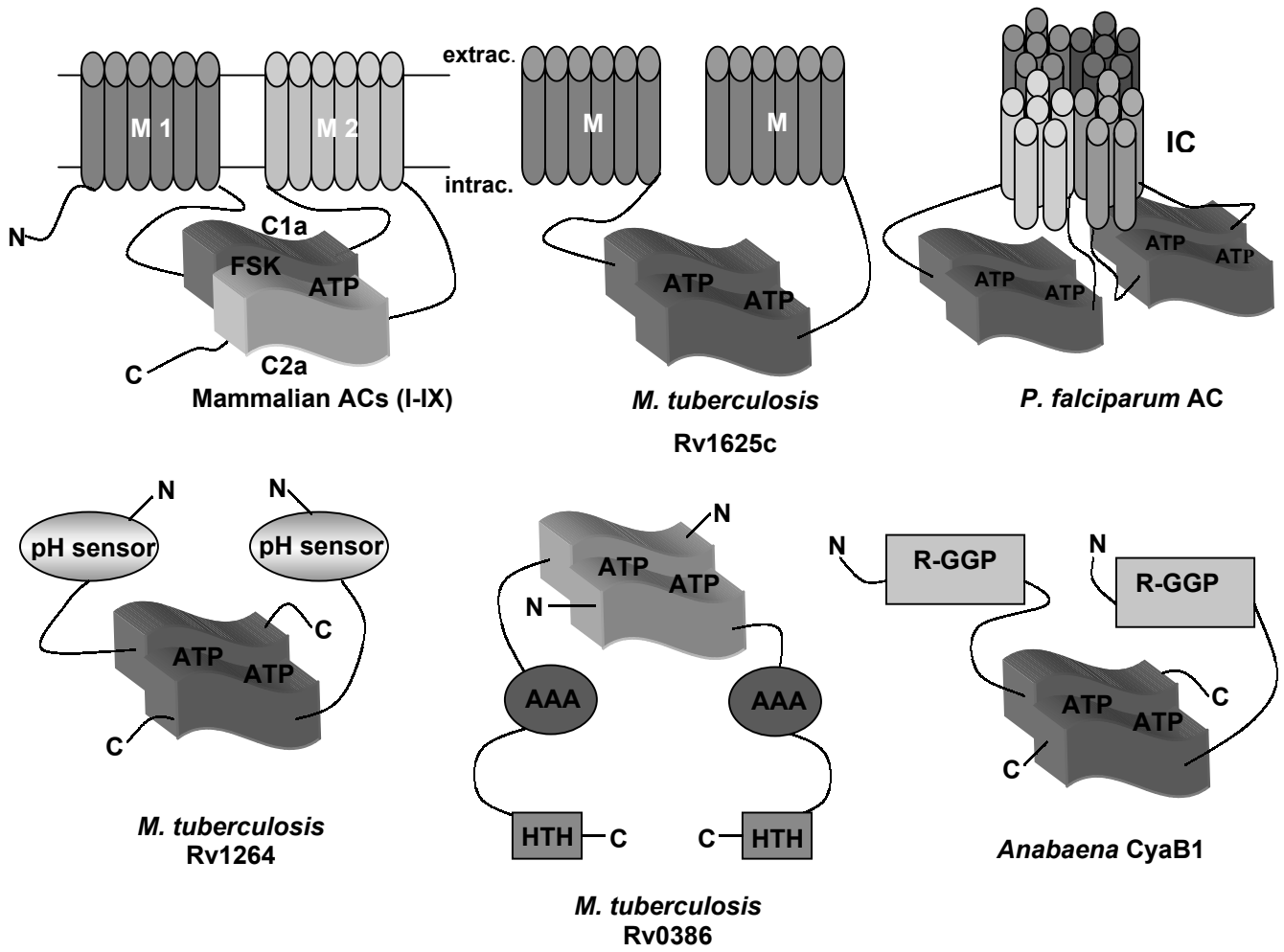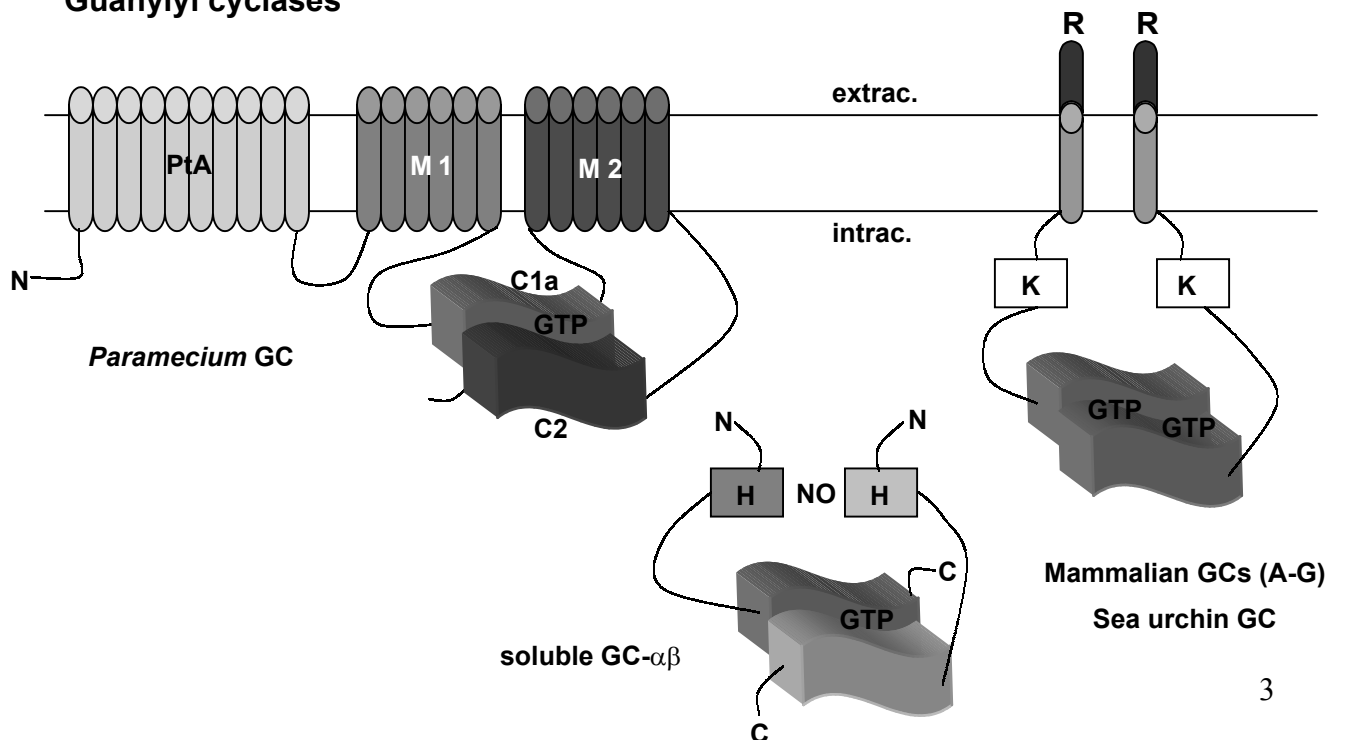
Fig. 1.1: Adenylyl and guanylyl cyclases (catalytic domain class III) are modular proteins. Drawn are the mammalian AC model (Tang and Hurley, 1998), the predicted topology for *M. tuberculosis* AC Rv1625c (Guo et al., 2001), Rv1264 (Linder et al., 2002), Rv0386, *Plasmodium falciparum* AC (Weber, 2003) and *Anabaena* CyaB1 (Kanacher, 2003). Predicted topology is also shown for mammalian GC's (isoforms A to G), soluble GC, sea urchin GC and for *Paramecium* GC (Linder et al., 2000). FSK=forskolin; IC=ion channel; AAA=AAA-ATPase; HTH=helix-turn-helix; R-GGP=regulator GAFA-GAFB-PAS; PtA=P-type ATPase; H=heme; NO=nitric oxide; R=receptor; K=kinase. The formation of homodimers is represented through identical colored catalytic domains, heterodimers show catalytic domains with different color intensity.

Evolutionarily, it has been shown that the 'palm' domain of the prokaryotic DNA polymerases I shares a common fold with the class III AC catalytic core, obviously both enzymes catalyse a phosphoryl transfer by a similar two-metal-ion mechanism suggesting a common ancestor. (Artymiuk et al., 1997 ; Tesmer et al., 1999). Interestingly, the catalytic domain of the γ-proteobacterial ACs (typified by *E. coli* CyaA) has similarity to the polymerase β-type nucleotidyl transferase superfamily, too. That emphasizes the general trend that cyclase signal transduction components evolved from enzymes involved in nucleotide processing (Aravind and Koonin, 1999).

In this work, an AC of *Mycobacterium tuberculosis* (Rv0386) with an N-terminal AC domain (class IIIc) and an attached C-terminal transcription factor domain is presented. Because here basically the AC function was studied, the meaning of combining a signal-transducing enzyme with a component of the basic nucleic acid processing is still an open question, the answer of which could be very useful for understanding evolution and maybe new regulatory mechanisms of ACs.

1.2   Mammalian adenylyl cyclases

Cyclic AMP is produced in mammals by nine isoforms of membrane-bound ACs and one soluble AC. The nine cloned ACs have a similar structure, with two hydrophobic domains comprising six predicted transmembrane helices each, and two cytoplasmic domains (Fig. 1.1). The cytosolic domains are similar to each other (roughly 30% identity), to the sequences within both bacterial and yeast ACs and even to those within GCs. Both together are responsible for catalytic activity (Taussig and Gilman, 1995; Sunahara et al., 1996). The nine isoforms share several regulatory features like activation by the α subunit of the heterotrimeric G protein Gs, activation by the diterpene

forskolin (with exception of type IX) and inhibition by a class of adenosine analogs known as P-site inhibitors. Individual forms can also be regulated by other G protein subunits, Ca^{2+}-calmodulin, Ca^{2+} or phosphorylation (Sunahara et al., 1996; Tesmer et al., 1997).

The cytosolic domains are called C1 and C2 and are subdivided into C1a, C1b, and C2a, C2b respectively, where C1a and C2a contain all of the catalytic apparatus. These C1a and C2a domains heterodimerize with each other in solution but can also form homodimers or even chimeric heterodimers if derived from different isoforms (Tang and Gilman, 1995; Yan et al., 1996; Whisnant et al., 1996). The structure of the type II AC C2 region revealed a wreathlike dimer arrangement which is likely to exist in other enzymes including many similar ACs and GCs. In this arrangement the interface of C1a and C2a domains accommodate the catalytic site and the forskolin regulatory site. Recombinant C1 and C2 domains are capable of carrying out catalysis when they are mixed and activated by forskolin (Zhang et al., 1997; Tang and Hurley, 1998). The two metal ions required for catalysis bind to specific residues found in the C1 region, the nucleotide binding pocket and other catalytic residues are contributed primarily by the C2 region. Similarly, forskolin binds to a site on the C1-C2 heterodimer through hydrophobic interactions with residues of both, C1 and C2 domains contributing (Dessauer et al., 1997; Tesmer et al., 1997; Zhang et al., 1997).

From crystallographic and mutational studies those amino acid residues were determined which participate in catalysis in mammalian ACs (Tesmer et al., 1997, 1999; Zhang et al., 1997). For definition of substrate specificity all important amino acids are contributed from the C2 region, they are D1018 (AC type II from rat) which recognizes the exocyclic amine and K938 which recognizes the unprotonated N_1 of adenine. Both residues are capable to discriminate against O^6 and N^2, and the protonated N_1 of guanine (Tesmer et al., 1997; Sunahara et al., 1998). The amino acids responsible for metal coordination are D396 and D440 (in canine AC type V). One Mg^{2+} ion binds together with ATP, while the second one acts kinetically as a free ion (Tesmer et al., 1997). Residues N1025 and R1029 (in rat AC type II) stabilize the transition state by stabilizing the negative charge on the pyrophosphate leaving group (Yan et al., 1997; Hurley, 1998). For binding forskolin, amino acids T410 and S942 (in rat AC type II) are implicated (Tesmer et al., 1997).

In the present work, the possibility of formation of active heterodimeric chimeras between mammalian C1 or C2 monomers and inactive mutated monomers from *M. tuberculosis* (Rv1625c, catalytic domain) was studied. The possibility of creating a non-existing forskolin-binding site by mutation of the mycobacterial catalytic domain was examined. Chimeras between mammalian and *Paramecium* GC monomers, as well as between mycobacterial AC and *Paramecium* GC monomers were also studied.

Table 1.1 shows the amino acid residues that are essential for catalysis classified according to the catalytic function they realize. The mammalian AC amino acid positions are compared with respect to their equivalent positions in Rv1625c and Rv0386 of *M. tuberculosis*, as well as in *Paramecium* GC.

	Purine binding		Metal binding		Transition-state stabilization		Forskolin binding	
	K938	**D1018**	**D396**	**D440**	**N1025**	**R1029**	**T410**	**S942**
Mammalian AC	ACII	ACII	ACV	ACV	ACII	ACII	ACII	ACII
	C2 rat	C2 rat	C1dog	C1dog	C2 rat	C2 rat	C1 rat	C2 rat
M. tuberculosis Rv1625c	**K296**	**D365**	**D256**	**D300**	**N372**	**R376**	*N372*	*D300*
M. tuberculosis Rv0386	**Q57**	**N106**	**D16**	**D61**	**N113**	**R117**	*N113*	*D61*
Paramecium GC	**E1681** (C1)	**S1748** (C1)	**D2233** (C2)	**D2277** (C2)	**N1755** (C1)	**R1759** (C1)	*L2356* (C2)	*K1685* (C1)

Table 1.1: Summary of the canonical amino acid residues responsible for catalysis in mammalian ACs (Tesmer et al., 1997, 1999; Zhang et al., 1997) in comparison with their equivalent residues in Rv1625c (Guo et al., 2001) and Rv0386 (present work) of *M. tuberculosis* and with *Paramecium* GC (Linder et al., 2000). Residues of the mycobacterial and *Paramecium* cyclases which are positional but not functional equivalent to those of mammalian ACs are in italics.

1.3   Adenylyl cyclases of *Mycobacterium tuberculosis*

The complete sequence of *Mycobacterium tuberculosis* H37Rv was published in 1998 with the annotation of only five genes (Rv1625c, Rv1318c, Rv1319c, Rv1320c and Rv1264) as putative ACs (Cole et al., 1998). Later on, using computational methods 15 putative ACs were detected (McCue et al., 2000). *M. tuberculosis* encodes several more

putative nucleotide cyclases than other prokaryotic species with the presence of predicted cytoplasmic (9), receptor-type membrane bound (1) and integral membrane (5) cyclases. Two of them (Rv1625c and Rv2435c) are classified as class a ACs together with the mammalian ACs (Cole et al., 1998; McCue et al., 2000).

To date, nine gene products of *M. tuberculosis*, Rv1625c and Rv1264 (Guo et al., 2001; Reddy et al., 2001; Linder et al., 2002), Rv0386 (present work), Rv1318c, Rv1319c, Rv1320c, Rv3645, Rv2212 and Rv1900c (Hammer, Zeibig, Motaal, Wetterer; unpublished) have been shown to possess AC activity.

The gene Rv1625c encodes a six-transmembrane-helices membrane-anchored AC with a single C-terminal catalytic domain, representing exactly one-half of a mammalian AC (Tang and Hurley, 1998). Certainly, by alignment of the mycobacterial catalytic domain with C1 from canine type V and C2 from rat type II ACs, considerable sequence identities were found. The most important is the conservation of each of the amino acids that have been identified crystallographically as participating in catalysis. A soluble enzymatically active catalytic domain from Rv1625c was constructed, expressed in *E. coli* and characterized. The dimerization was substantiated with construction of linked monomers. Mutations of the amino acid residues indispensable for catalysis by the mammalian heterodimers inactivated the mycobacterial cyclase. Finally, it was possible to reconstitute productive heterodimers from inactive mutant monomers. These molecular and biochemical properties of Rv1625c made it likely that this cyclase is an evolutionary precursor of the mammalian AC family (Guo et al., 2001).

The gene product Rv1264, predicted to be composed of a C-terminal AC catalytic domain and a novel N-terminal protein domain, could also be expressed in *E. coli* (Linder et al., 2002). Rv1264 has the same modular composition as ACs from *Streptomyces* and *Brevibacterium liquefaciens* (Peters et al., 1991; Danchin et al., 1993). The catalytic domain expressed alone displayed high AC activity, homodimerization was demonstrated and the catalysis depended on the same amino acids previously identified as critical in mammalian ACs. Because of the notably lower AC activity of the holoenzyme, an autoinhibitory function of the N-terminal domain was suggested (Linder et al., 2002).

Until today, several anti-mycobacterial agents interfering with the synthesis and assembly of components of the mycobacterial cell wall have controlled a lot of infection

cases. But the control of global tuberculosis has so far failed and the number of multidrug-resistant isolates is rising. Therefore, it is required to advance our understand of the complex biology of tuberculosis infection. The completed genome project is a major step toward this goal (Young, 2001).

From the genome sequence it is obvious that *M. tuberculosis* is equipped with a range of transcriptional regulators. These could confer a high capacity for adaptation as shown in several studies where a change in the pattern of gene expression occurred after transfer from laboratory conditions to the macrophage intracellular environment (Young, 2001). Also, the large number of putative cyclases in *M. tuberculosis* could point to the development of the ability to sense and respond to many intracellular and extracellular signals through this second messenger system (McCue et al., 2000). It is unknown if cyclase activity is necessary for pathogenesis, but it has been reported that macrophages with ingested mycobacteria increase cAMP levels which may inhibit the phagosome-lysosome fusion (Lowrie et al., 1975; McCue et al., 2000).

A recent computational study of genomic sequence comparison placed the gene Rv0386 between 19 genes in the genome of *M. tuberculosis* that may have been acquired by horizontal gene transfer from eukaryotes (Gamieldien et al., 2002), an event that could have conferred survival advantages to an ancient mycobacterium before becoming a pathogen of eukayotes (McCue et al., 2000). On the basis of studies of the mycobacterial AC Rv1625c, in this thesis the enzyme Rv0386 was basically studied as an AC without paying much attention to its putative feature as a transcription factor. Investigation of this enzyme was of particular interest because of its deviation from the canonical amino acid residues participating in catalysis, specifically those involved in the definition of substrate specificity. The possibility of an AC catalysis by a novel mechanism lead to attempts of crystallization.

2 Materials

2.1 Chemicals and materials

Amersham Pharmacia Biotech, Freiburg: ECL Plus Western Blot Detection System, Hyperfilm ECL, Thermo Sequenase Fluorescent Labeled Primer Cycle Sequencing Kit with 7-deaza-dGTP, Formamide, [2,8-^{3}H]-cAMP, [8-^{3}H]-cGMP

AppliChem, Darmstadt: HEPES, Acrylamide 4K-Solution 30%

Appligene, Heidelberg: Taq DNA-Polymerase with 10X reaction buffer

Bayer, Leverkusen: Aprotinin

BioGenes, Berlin: Specific antibodies anti-KD0386 and anti-DB0386

BIOLOG-Life Science Institute, Bremen: (Rp)-ATP-α-S

BIO-RAD, München: BIO-RAD Protein-Assay Dye Reagent Concentrate

Biozym Diagnostik, Hess. Oldendorf: Sequagel XR, Sequagel Complete Buffer Reagent, Chill-Out 14 Liquid Wax von MJ Research

Canberra PACKARD, Taunusstein: Ultima Gold XR LSC Cocktail

Dianova, Hamburg: Goat anti-mouse antibodies horseradish peroxidase conjugated, Goat anti-rabbit antibodies horseradish peroxidase conjugated

Fluka, Basel: Trypsin inhibitor from lima beans, TLCK, TPCK, SDS, PMSF, PEG 5000 monomethyl ether, PEG 6000, Nonidet P 40

Hampton Research, CA (USA): Reagent Kits Crystal Screen, Crystal Screen 2 and Crystal Screen Lite for the crystallization screen for proteins and other molecules, PEG 400, PEG 3350, PEG 8000

Hartmann Analytik, Braunschweig: [α-^{32}P]-ATP and [α-^{32}P]-GTP

ICN Biomedicals, Eschwege: [α-^{32}P]-ATP

Macherey-Nagel, Düren: Nucleotrap

Merck, Darmstadt: Imidazole, Glycin, sodium citrate, potassium-sodium tartrate, sodium acetate, sodium chloride, PEG 4000

Millipore, Bedford (USA): Centricon YM-10 Centrifugal Filter Devices (MWCO 10000)

MWG-Biotech, Ebersberg: Oligonucleotides

New England Biolabs, Schwalbach/Taunus: Restriction Endonucleases, BSA, T4-Polynucleotide Kinase, 10X Kinase buffer

Pall Corporation, Michigan (USA): Nanosep 10K OMEGA centrifugal devices

Peqlab, Erlangen: Agarose, peqGOLD Protein Marker

Promega, Madison (USA): Wizard MiniPreps Plasmid Purification Kit

Qiagen, Hilden: Ni^{2+}-NTA-Agarose, pQE-Expression vectors, *E. coli* M15 [pREP4], Purified mouse monoclonal RGS·His antibody (BSA-free)

Roche (Boehringer), Mannheim: Restriction endonucleases, Klenow-Polymerase, Alkaline Phosphatase, Rapid DNA Ligation Kit, dNTP's, ATP, GTP, CTP, Complete Protease Inhibitor Cocktail Tablets

Sartorius, Göttingen: Cellulose Acetate Filter with pore size 0.2 μm, Polycarbonate Filter Holder

SERVA Electrophoresis, Heidelberg: Coomasie-Brilliant-blue G250, VISKING Dialysis Tubing 8/32 and 27/32 with MWCO 12000-14000.

Sigma, Deissenhofen: Glycerol, MOPS, TRIS, EDTA, Xgal, IPTG, Ponceau S, Tween 20, TEMED, PMSF, BSA, ITP, UTP, $\alpha\beta$-CH_2-ADP, Cordycepin 5'-triphosphate, 2'3' GMP, 2' GMP, 8-Br-cGMP, monobutyryl cAMP, dibutyryl cAMP, 2'd3'-AMP

Spectrum Medical Instruments, Los Angeles (USA): Spectrapor Membrane Tubing MWCO 3500

Stratagene, Heidelberg: Plasmid pBluescript II SK (-), *E. coli* XL1-Blue MRF'

2.2 Equipment

Amersham Pharmacia Biotech, Freiburg: ÄKTA™ FPLC with Fraction Collector Frac-950, Pump P-920, Monitor UPC-900, Valve INV-907, Mixer M-925 and Software Unicorn Version 4.00, general accessories for FPLC and Anion Exchange Column MonoQ HR 5/5.

Biometra, Göttingen: TRIO-Thermoblock thermocycler

BIO-RAD, München: Trans-Blot SD Semi Dry Transfer Cell

Branson, Danbury, USA: Sonifier B-12, Ultrasound Bath Bransonic B12

Carl Zeiss, Göttingen: Microscope Axioskop 40/40 FL with fixed Polarisator with Lambda plate and Canon PowerShot G2 high quality digital camera with 4.0 M pixel CCD sensor and 3X optical zoom.

Eberhard-Karls-Universität, Tübingen: Gel electrophoresis chambers

Eppendorf, Hamburg: Thermostat 3401, Table Centrifuges 5410 & 5414, Cooling centrifuge 5402, BioPhotometer

Hampton Research, CA (USA): VDX 24 well polystyrene pre-greased plates, 22 mm siliconized glass cover slides (squares)

Heraeus, Osterode: Megafuge 1.0 R (BS 4402/A)

Idaho Technologies, Idaho Falls (USA): Air Thermo Cycler 1605

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

Macherey-Nagel, Düren: Porablot PDVF-Blotting membrane (0.25 x 3 m)

Millipore, Eschborn: Water purification system MilliQ UF Plus

MWG-Biotech, Ebersberg: LI-COR DNA sequencer model 4000

Sartorius, Göttingen: Balance BP 2100 S, Analytic balance handy

Savant, Farmingdale (USA): Vacuum centrifuge speed vac concentrator SVC100H

Schleicher & Schuell, Dassel: Whatmanpaper 3MM, Protran BA 83 Cellulosenitrate 0.2 µm (200 x 200 mm) Nucleic acid and protein transfer media

SLM Instruments, Urbana (USA): French Pressure Cell Press FA-078-E1, Manual Fill 20K Cell FA-073 (7.6 cm diam., 40 ml sample capacity)

Stratagene, Austin (USA): UV Stratalinker 2400

2.3 Buffers and solutions

2.3.1 Molecular biology

All solutions, buffers and culture media for molecular biology methods were sterilized 20 min at 120°C and 1 bar. pH was measured at room temperature.

Buffers for DNA

10X TAE			10X Klenow buffer	
400 mM	Tris/acetate pH 8.0		200 mM	Tris/HCl pH 7.5
10 mM	Na_2EDTA		60 mM	$MgCl_2$
			10 mM	DTT
			1 mg/ml	BSA

TE-buffer			10X TBE	
10 mM	Tris/HCl pH 7.5		1000 mM	Tris
1 mM	Na_2EDTA		890 mM	Boric acid
			25 mM	Na_2EDTA

4X Loading sample buffer (agarose gel)
0.05 %	Bromphenolblue
0.05 %	Xylenecyanol
50.0 %	Glycerol

Loading sample buffer (sequencing gel)
95 %	Formamide
20 mM	Na_2EDTA
0.05 %	Bromphenolblue
0.05 %	Xylenecyanol

10X CM buffer
100 mM	$CaCl_2$
100 mM	$MgCl_2$

10X Dephosphorylation buffer
500 mM	Tris/HCl pH 8.5
1 mM	Na_2EDTA

Bacterial culture media

LB-broth
1 %	Bacto Tryptone
0.5 %	Yeast extract
1 %	NaCl

LB-agar
1.5 %	Agar in LB-broth

LB-Amp-agar
100 µg	Ampicilin / ml LB-agar

2.3.2 Protein chemistry

Protein purification with Ni-NTA-agarose

Pellet wash buffer
50 mM	Tris/HCl pH 8.0
1 mM	EDTA

Cell Lysis buffer
50 mM	Tris/HCl pH 8.0
10 mM	β-mercaptoethanol
50 mM	NaCl

Wash buffer A
50 mM	Tris/HCl pH 8.0
10 mM	β-mercaptoethanol
2 mM	$MgCl_2$
400 mM	NaCl
5 mM	Imidazole

Wash buffer B
50 mM	Tris/HCl pH 8.0
10 mM	β-mercaptoethanol
2 mM	$MgCl_2$
400 mM	NaCl
15 mM	Imidazole

Wash buffer C
50 mM	Tris/HCl pH 8.0
10 mM	β-mercaptoethanol
2 mM	$MgCl_2$
10 mM	NaCl
15 mM	Imidazole

Elution buffer
50 mM	Tris/HCl pH 8.0
10 mM	β-mercaptoethanol
2 mM	$MgCl_2$
10 mM	NaCl
150 mM	Imidazole

Glycerol dialysis buffer
10 mM	NaCl
50 mM	Tris/HCl pH 7.5
2 mM	β-mercaptoethanol
20 %	Glycerol

Phosphate dialysis buffer
50 mM	Na_2HPO_4/NaH_2PO_4 buffer (pH 7.5)
10 %	Glycerol

Equilibration mixture for Ni-NTA-agarose
5 ml	Cell lysis buffer
225 mM	NaCl
13.5 mM	Imidazole pH 8.0
4.5 mM	$MgCl_2$

Membrane Suspension Buffer
50 mM	Tris/HCl pH 8.0
15 mM	β-Mercaptoethanol
250 mM	NaCl
20 %	Glycerol
2 %	CHAPS

High Saline Buffer
50 mM	Tris/HCl pH 8.0
10 mM	β-Mercaptoethanol
2 mM	$MgCl_2$
5 mM	Imidazole pH 8.0
250 mM	NaCl
20 %	Glycerol
2 %	CHAPS

Low Saline Buffer
50 mM	Tris/HCl pH 8.0
10 mM	β-Mercaptoethanol
2 mM	$MgCl_2$
15 mM	Imidazole pH 8.0
10 mM	NaCl
20 %	Glycerol
2 %	CHAPS

Elution Buffer with Detergent
50 mM	Tris/HCl pH 8.0
10 mM	β-Mercaptoethanol
2 mM	$MgCl_2$
300 mM	Imidazole pH 8.0
10 mM	NaCl
20 %	Glycerol
2 %	CHAPS

Tosyl-L-phenylalanin-chlormethylketon (TPCK)
2.2 mg/ml	in Ethanol (Stock)

N_α-Tosyl-L-lysin-chlormethylketon-hydrochlorid (TLCK)
2.2 mg/ml	in 1 mM HCl (Stock)

Phenylmethansulfonylchlorid (PMSF)   Lima bean Trypsin Inhibitor

  2.2 mg/ml in Isopropanol (Stock)   3.2 mg/ml in H_2O (Stock)

Aprotinin

  2.8 mg/ml in NaCl 0.9% (Stock)

SDS-Polyacrylamide gel electrophoresis

Resolving Gel buffer   Stacking Gel buffer
  1.5 M Tris/HCl pH 8.8   500 mM Tris/HCl pH 6.8
  0.4 % SDS   0.4 % SDS

10X Running buffer   Staining solution
  250 mM Tris   0.2 % Coomassie Brilliant
  1.92 M Glycin   Blue G-250
   1 % SDS   10 % Acetic acid
              40 % Methanol

4X Sample buffer
  130 mM Tris/HCl pH 6.8   Destaining solution
  10 % SDS   10 % Acetic acid
  10 % β-mercaptoethanol   30 % Ethanol
  20 % Glycerol
  0.06 % Bromphenolblue

Protein marker
  0.1 mg/ml BSA (66 kDa)
  0.1 mg/ml Ovoalbumine (45 kDa)
  0.1 mg/ml GAPDH (36 kDa)
  0.1 mg/ml Chymotrypsinogen A (25 kDa)
  0.1 mg/ml Trypsin inhibitor (20.1 kDa)
  0.1 mg/ml Cytochrom C (12.5 kDa)
   30 % 4X sample loading buffer

Western blot

TBS buffer (Tris buffer saline) pH 7.6   M-TBS
  20 mM Tris   5 % Milk powder non-fat
  150 mM NaCl   in TBS

Towbin-Blot-buffer

25 mM	Tris		TBS-T	
192 mM	Glycin		0.1 %	Tween 20 in TBS
20 %	Methanol			

Ponceau S Staining Solution
        0.1 % (w/v)   Ponceau S
                      in 5% (v/v) acetic acid

Cyclase enzyme test

Creatine kinase

    4 U /2.5 µl   Creatine kinase in
                      10 mM Tris/HCl pH 7.5

Creatine phosphate
        120 mM   in 10 mM Tris/HCl
                      pH 7.5

ATP or GTP Stock solution
        10 mM   adjusted to pH 7.2-
                      7.5 with NaOH

cAMP or cGMP Stock solutions
        40 mM   in H_2O (adjust pH to
                      7.5 with Tris buffer)

10X AC-Start Solution
  0.75 to 10 mM   ATP with
                   $2.5\text{-}4\times10^6$ Bq/ml
                   $[\alpha\text{-}^{32}P]$-ATP

10X GC-Start Solution
  0.75 to 10 mM   GTP with
                   $2.5\text{-}4\times10^6$ Bq/ml
                   $[\alpha\text{-}^{32}P]$-GTP

2X AC-Cocktail

43.5 %	Glycerol
100 mM	Tris-HCl pH 7.5 or MOPS pH 7.5
4 mM	cAMP with $2\text{-}4\times10^3$ Bq/ml $[2,8\text{-}^3H]$-cAMP
0.46 mg/2ml	Creatine kinase
6 mM	Creatine phosphate
2 to 5 mM	$MnCl_2$ or
5 to 10 mM	$MgCl_2$

2X GC-Cocktail

43.5 %	Glycerol
100 mM	MOPS pH 7.5
4 mM	cGMP with $2\text{-}4\times10^3$ Bq/ml $[8\text{-}^3H]$-cGMP
5 mM	$MnCl_2$ or
10 mM	$MgCl_2$

Adenylyl cyclase stop buffer (pH 7)

     3 mM   cAMP
     3 mM   ATP
     1.5 %   SDS

Guanylyl cyclase stop buffer

     1.5 %   SDS

Forskolin solution

     10 mM   Forskolin in DMSO

Crystallization buffers

Self-produced crystallization buffers were made using deionized purified water (MilliQ®) and were filtrated through 0.2 µm size pore filters applying vacuum to a filter holder device.

2.4 Oligonucleotides

Restriction sites are underlined, mutations are in bold and mutations together with restriction sites are bold and underlined.

Name	Sense	Sequence (5'→ 3')	Position	Comments
Sequencing primers for plasmids				
T7	s	GTA ATA CGA CTC ACT ATA GGG C	625-646	T7 pBluescript II SK(-)
T3	as	AAT TAA CCC TCA CTA AAG GG	772-791	T3 pBluescriptII SK(-)
U –pQE-IR	s	GAA TTC ATT AAA GAG GAG AAA	88-108	universal pQE30
R –pQE-IR	as	CAT TAC TGG ATC TAT CAA CAG G	212-233	reverse pQE30
Cloning primers of Rv1625c mutants				
myktub kat-s	s	AAA <u>GGA TCC</u> GAT ACT GCG CGT GCG GAG GCG	604-631	<u>BamHI</u> Primer from Dr. Guo.
myktub 372A-1	as	CAT CCG <u>GCT AGC</u> GAC **AGC** GAC CGC GTC GCC	1102-1131	<u>NheI</u>
myktub 372A-2	s	GTC AAT GTC **<u>GCT AGC</u>** CGG ATG GAA TCC	1111-1137	**<u>NheI</u>**

myktub 1-as	as	AAA <u>GAG CTC</u> TCA GAC CCC TGC CGT GCG	1314-1338	<u>SacI</u> Primer from Dr. Guo.
myktub 372T-1	as	CAT CCG <u>GCT AGC</u> GAC **AGT** GAC CGC GTC GCC	1102-1131	<u>NheI</u>
myktub 300S-1	as	AAC CAT GTA **A**<u>**CT CGA GCC**</u> GCT GAC CTT GAT	883-912	**<u>XhoI</u>**
myktub 300S-2	s	GTC AGC **<u>GGC TCG AGT</u>** TAC ATG GTT GTC	889-916	**<u>XhoI</u>**
Cloning primers of Rv0386 holoenzyme and AC catalytic domain				
myco0386/B-C-Hforw	s	AAA <u>GGA TCC</u> ATG AGC AAG TTG CTG CCA CGG GGC ACA GTG ACA TTG CTG TTG GCC GAC GTC GAG **GGT** TCC ACC	1-63	<u>BamHI</u>
myco0386/B-C-H-rev	as	AA<u>A AGC TT</u>**T CA**A TCG ATA CGC AAT TCG GGA TG	505-534	<u>HindIII</u>
myco0386/C-Cforw	s	CGT <u>ATC GAT</u> TTC CCG CCG CTG	517-537	<u>ClaI</u>
myco0386/C-Crev	as	GCC <u>ATC GAT</u> GAT CTC GTC AAG	1192-1212	<u>ClaI</u>
myco0386/C-Sforw	s	ACG CTT GAC GAG ATC <u>ATC GAT</u> GGC	1189-1212	<u>ClaI</u>
myco0386/C-Srev	as	CAG <u>GGC C</u>TT GTC <u>GGC CAA</u> TGC CCG	1855-1878	<u>SfiI</u>
myco0386/S-Sforw	s	CGG GCA TT<u>G GCC</u> GAC AA<u>G GCC</u> CTG	1855-1878	<u>SfiI</u>
myco0386/S-Srev	as	GGC <u>GGC C</u>AA CGC <u>GGC CGT</u> GGT CAA C	2460-2484	<u>SfiI</u>
myco0386/S-Hforw	s	TTG ACC AC<u>G GCC</u> GCG TT<u>G GCC</u> GCC	2461-2484	<u>SfiI</u>

myco0386/S-Hrev	as	AAA AGC TTT CAG GTA CGG CGG GCG GCC GC	3238-3258	HindIII
Cloning primers of Rv0386 mutants				
myco0386/ Q57Krev	as	ACC CTC ACC **CTT** CTC GAC AGG CCT TAC GCC	151-180	StuI
myco0386/ Q57Kforw	s	GGC GTA AGG CCT GTC GAG AAG GGT GAG GGT	151-180	StuI
myco0386/ Q57Arev	as	ACC CTC ACC **CGC** CTC GAC AGG CCT TAC GCC	151-180	StuI
myco0386/ Q57Aforw	s	GGC GTA AGG CCT GTC GAG **GCG** GGT GAG GGT	151-180	StuI
myco0386/ N106Drev	as	GTT GAT GGT GGG CCC GGC ATA **GTC** GCC TTC GTC	307-339	ApaI
myco0386/ N106Dforw	s	GAC GAA GGC **GAC** TAT GCC GGG CCC ACC ATC AAC	307-339	ApaI
myco0386/ N106Arev	as	GTT GAT GGT GGG CCC GGC ATA **GGC** GCC TTC GTC	307-339	ApaI
myco0386/ N106Aforw	s	GAC GAA GGC **GCC** TAT GCC GGG CCC ACC ATC AAC	307-339	ApaI
myco0386/ QKNDforw	s	AAG TGG CCG CCG CGT TAG ATC TGC AGC GAG CG	220-249	Bgl II
myco0386/ QKNDrev	as	AAC CGC GCT CGC TGC AGA TCT AAC GCG GCG GC	222-252	Bgl II
myco0386/ N106Sforw	s	GAC GAA GGC **TCA** TAT GCC GGT CCG ACC	307-333	NdeI

myco0386/ N106Srev	as	GGT CGG ACC GG<u>C ATA</u> <u>**TGA**</u> GCC TTC GTC	307-333	<u>Nde</u>I
Primers for transcription factor-, ATPase- and DNA-binding domains of Rv0386				
NHis/TF/ Rv0386forw	s	AAA <u>GGA TCC</u> TGT CAT CCC GAA TTG CGT ATC	502-522	<u>Bam</u>HI
NHis/DBDom/ Rv0386	s	AAA <u>GGA TCC</u> GCA TGG GCC GAA GGT GCC GCG	2980-3000	<u>Bam</u>HI
NHis/AADom/ Rv0386re	as	AAA <u>AAG CTT</u> GTG GCG CAT CGT CTC GCA	1501-1518	<u>Hind</u>III
Primer for N-terminally elongated adenylyl cyclase domain				
CDC1551ac- forw	s	AAA <u>GGA TCC</u> **ATG CGA CTG AGT GGA GCG GGG** ATG AGC AAG TTG CTG CCA CGG GGC ACA	1-27	<u>Bam</u>HI and **addition of MRLSGAG**
myco0386/cata/ C-His	as	AAA <u>AGA TCT</u> ATC GAT ACG CAA TTC GGG ATG	505-525	<u>Bgl</u> II
Primer for C-terminally His-tagged catalytic domain				
myco0386/cata/C-His (see above)				

2.5 Plasmids

Source: Stratagene

BamHI
SphI
SacI
KpnI
SmaI
XmaI
SalI
PstI
HindIII

*

PT5 — lac O — lac O — RBS — ATG — 6xHis — MCS — Stop Codons

* pQE-30   – –
pQE-31   AC
pQE-32   – G

Ampicillin

pQE-30, pQE-31,
pQE-32
3.4 kb

Col E1

Source: Qiagen

pQE-30

Eco RI/RBS   6xHis   Bam HI Sph I   Sac I   Kpn I Sma I   Sal I   Pst I   Hind III   t₀
                                                                           Xma I
ATG AGAGGATCG ▆ – – GGATCCGGATGCGAGCTCGGTACCCCGGGTCGACCTGCAGCCAAGCTT AATTAGCTGAG
RGS-His epitope

Nco I
Bam HI
Bgl II

PT5 — lac O — lac O — RBS — ATG — MCS — 6xHis — Stop Codons

Ampicillin

pQE-60
3.4 kb

Col E1

Source: Qiagen

pQE-60

Eco RI/RBS   Nco I   Bam HI Bgl II   6xHis Hind III   t₀
             CCATGG GAGGATCCAGATCT ▆ TAAGCTTAATTAGCTGAG

3 Methods

3.1 Polymerase chain reaction (PCR)

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

$$Ta = 2 \times (AT) + 4 \times (GC) - 4$$

(AT) and (GC) represent the number of A+T and G+C respectively in the primer sequence. If the annealing temperatures for each primer were different the lower one was employed.

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 max. 1 ng plasmid or 800 ng genomic DNA, 5-10% DMSO, 200 µM dNTPs, 1 pmol/µl primers, 1U Taq-DNA-Polymerase and 1X of the corresponding reaction buffer. The temperature program applied is shown in Table 3.1.

Denaturation		95°C	5 min
	Denaturation	95°C	1 min
20 - 40 cycles	Primer annealing	Ta	1 min
	Extension	72°C	1 min
Fill up		72°C	10 min

Table 3.1: Program used for polymerase chain reaction.

3.2 Isolation and purification of DNA

3.2.1 General

Plasmid DNA was isolated from bacterial cultures through a small-scale purification method, known as miniprep. The standard protocol of the Wizard Plus Minipreps DNA Purification System (PROMEGA) was carried out using 1-3 ml of O/N bacterial culture (12-16h by 37°C) and a vacuum manifold giving a yield of 1-10 ng DNA. DNA was

eluted with sterile water. The purified plasmid could be used directly for DNA sequencing and restriction digestion.

DNA fragments separated through electrophoresis were excised from the agarose gel with the help of a scalpel and extracted with the help of the Macherey-Nagel Nucleotrap-Gelextractions-Kit following the protocol of the manufacturers.

If a change in the buffer conditions was necessary in order to process the DNA samples, they were desalted following the corresponding protocol of the Nucleotrap-Gelextractions-Kit (Macherey-Nagel).

3.2.2 Agarose electrophoresis

To determine yield and purity of an isolated DNA or PCR reaction, to check digestion by a restriction enzyme and to determine the size of DNA molecules, agarose electrophoresis was carried out. 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 content was choosen according to the expected size (bp) of DNA-fragments:

$$\geq 2000 \text{ bp} \qquad 0.8 - 1 \text{ \%}$$
$$500 - 2000 \text{ bp} \qquad 1 - 1.8 \text{ \%}$$
$$\leq 500 \text{ bp} \qquad 2 \text{ \%}$$

TAE-buffer was used for electrophoresis, agarose gels were submerged in a horizontal electrophoresis apparatus. The DNA samples were mixed with loading sample buffer. Electrophoresis was performed at 80-100 V for 0.5-1 h at room temperature. The size markers EcoR I/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 submerged in a ethidium bromide bath (0.01 mg/ml) for 1-2 min. The gel was run 10 additional min and DNA fragments visualized on a UV light box (302 nm) and photographed.

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3.2.3 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 OD_{260}/OD_{280} was calculated to estimate purity. An OD ratio of >1.8 was desirable.

3.3 Enzymatic Methods

3.3.1 General molecular biology methods

Restriction enzyme digestions were performed by incubating 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 more compatible buffer or the One-Phor-All-buffer was used. If no possible compatibility was found, digestions were performed sequentially with a desalting step in-between.

3.3.2 Generation of blunt ends

After PCR or a restriction digest, 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 (1 U/µl) in a 10 µl reaction volume were mixed. The reaction mixture was incubated for 10 min at 37°C. Then 1 µl of dNTPs (25 mM) was added and the incubation continued for 30 min. The sample was heated to 70°C for 10 min for inactivation.

3.3.3 5'-Phosphorylation of PCR products

This was necessary for blunt end ligation. The reaction was composed of 1-2 µg DNA or of the Klenow-treated DNA solution, 1 mM ATP, 10 U T4-Polynucleotide Kinase and 1X T4-PNK-buffer in 15 µl (37 °C, 1 h).

3.3.4 5'-Dephosphorylation of plasmid vectors

To suppress circularization of plasmid DNA, the 5'-phosphates from both ends were removed with calf alkaline phosphatase. 500 ng DNA, 1 U/pmol of enzyme and 1X dephosphorylation buffer were mixed in 10 µl and incubated at 37°C for 1 h.

3.3.5 Ligation of DNA fragments

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

3.4 Transformation of recombinant DNA

3.4.1 Competent cells of *E. coli*

From a new plate of XL1-blue MRF⁻, BL21 (DE3) [pREP4], BL21 STAR (DE3) [pREP4] or BL21 Rosetta (DE3) [pREP4] cells, a single colony was picked and grown up in 5 ml LB broth (37°C, O/N). 1 ml was transferred into 50 ml LB and grown at 37°C to 0.3-0.4 OD_{600} (approx. 2-3 h). The culture was cooled on ice (10 min) and transferred to Falcon tubes. Cells were recovered at 2500 x g (15-20 minutes, 4°C). Pellets were suspended in 10 ml of ice cold $CaCl_2$ (0.1 M) and centrifuged (2500 x g, 15 min, 4°C). This step was repeated with addition of 20 % glycerol. The suspended pellets were standing on ice for 2 hours, aliquoted (100 µl) and stored at –80°C.

3.4.2 Rapid transformation

One aliquot of competent cells was thawed on ice. 100 ng plasmid DNA (max.) was added and mixed. After 5-15 min on ice the mixture was spread on LB agar plates containing the appropriate antibiotic and incubated at 37 °C O/N.

3.4.3 Standard transformation

The entire DNA ligation reaction (21 µl from a Rapid Ligation Kit protocol) was diluted in 1X CM buffer to 50 µl. This was added to competent cells (100 µl), mixed gently and incubated on ice (30-45 min). Cells were then heat-shocked (1 to 2 min, 42°C) and incubated on ice (10 to 20 min). 400 µl of LB-broth were added and cells were incubated

at 37 °C for 45-60 min with agitation (210-230 rpm). 50 to 200 µl of the mixture were spread on LB agar plates with appropriate antibiotics. Plates were incubated in an inverted position for 12-16 h at 37 °C. For transformation with pBluescript II SK (-) vector it was possible to make a blue-white screen by spreading 40 µl of IPTG 0.1 M and 40 µl of X-Gal (2 %) over the LB agar plate prior to plating. Bacteria carrying recombinant DNA formed white colonies, those carrying plasmids without insert formed blue colonies.

3.4.4 Glycerol stock cultures

1-3 ml of a O/N bacterial culture was centrifuged (2 min, 10,000 x g). The pellet was suspended in 750 µl of a mixture of LB-broth/Glycerol 4:1, storage at –80 °C.

3.5   DNA Sequencing

Miniprep DNA was sequenced using sequenase and 7-deaza-dGTP (the latter to prevent band compression caused by DNA secondary structure). The Thermo Sequenase Primer Cycle Sequencing Kit was used. The DNA/Primer-Mix varied in its composition and volume depending on the type of vector used. DMSO was added to avoid primer-dimer formation. Primers used were fluorescent-labeled: for pBluescript T_3 and T_7 primers (Ta= 56°C), and for pQE30 U-pQE-IR800 and R-pQE-IR800 primers (Ta= 54 °C) were used.

DNA fragment cloned in pBluescript		DNA fragment cloned in pQE30	
DNA	1-2 pmol (~130 ng/kb) (8 µl)	DNA	1-2 pmol (~130 ng/kb) (10 µl)
Primer [2 pmol/µl]	1.5 µl	Primer [4 pmol/µl]	2 µl
DMSO	0.7 µl	DMSO	0.7 µl
Final volume (with H_2O)	21 µl	Final volume	18 µl

Table 3.2: Components of the DNA/Primer-Mix used for DNA sequencing depended on the vector used for cloning.

4 - 5 µl of DNA/Primer-Mix were added to 2 µl of G, A, T or C-reagent mix of the corresponding kit following the instructions of the supplier. Each reaction was overlayed with one drop chill-out-wax and run in a Thermocycler using the following program:

Step	Temperature	Time	Cycles
First denaturation	95 °C	2 min	1
Denaturation	95 °C	20 s	
Annealing	Ta of the primers used	20 s	25-30
Extension	70 °C	20 s	
Termination	4 °C	∞	1

Table 3.3: Program used for DNA sequencing.

At the end of the reaction 6 µl of Formamide loading dye of the kit were added. 1 µl of each sample was loaded onto a 6% polyacrylamide gel and electrophoresed with the help of a LI-COR Sequencing apparatus at 50 W (max. 1500 V or 37 mA, 50°C). Running buffer was TBE-buffer, DNA fragments were detected through Laser fluorescence at 800 nm. Data obtained was analyzed with the BaseImageIR V. 4.0 Software.

3.6 Cloning

All constructs were cloned in the MCS of pBluescriptII (SK-) as a first cloning step and for sequencing. Finally, they were cloned for expression into pQE30 or pQE60. As a result, all proteins had either an N-terminal MRGSH$_6$GS or a C-terminal RSH$_6$ extension.

3.6.1 Mammalian and protozoan cyclases

Clones of the mammalian and the *Paramecium* cyclases cytosolic regions were from Hoffmann (1999).

3.6.2 *M. tuberculosis* Rv1625c

3.6.2.1   Site-directed mutagenesis

A DNA construct of Rv1625c (mycoAC$_{204-443}$) with 5'-BamHI and 3'-SacI sites in pQE30 was used as a template (Guo et al., 2001). Single amino acid mutations were introduced by site-directed mutagenesis using respective PCR.

3.6.2.1.1 Mutants N372A and N372T

Table 3.4 and Figure 3.1 outline the cloning steps used for mycoAC$_{204-443}$ N372A and N372T, respectively.

Mutant	Fragment	Primers	Length of the PCR product (bp)
N372A	1	myktub kat-s myktub 372A-1	534
	2	myktub 372A-2 myktub 1-as	237
N372T	1	myktub kat-s myktub 372T-1	534
	2	myktub 372A-2 myktub 1-as	237

Table 3.4: Primers used for cloning the mutants N372A and N372T. See sequence of the primers in chapter 2.

Amplification conditions were: Ta= 52°C, 5 % DMSO, 20 cycles. Products were digested with BamHI/NheI to obtain fragment 1 and NheI/SacI for fragment 2 of both N372A and

N372T. The fragments were 5' phosphorylated, purified by agarose gel electrophoresis and cloned into pQE30 plasmid digested with BamHI/SacI and dephosphorylated. Recombinants were isolated from *E. coli* XL-1 blue, and introduced into *E. coli* BL21(DE3)[pREP4] for protein expression with prior confirmation of the mutations by double-stranded sequencing.
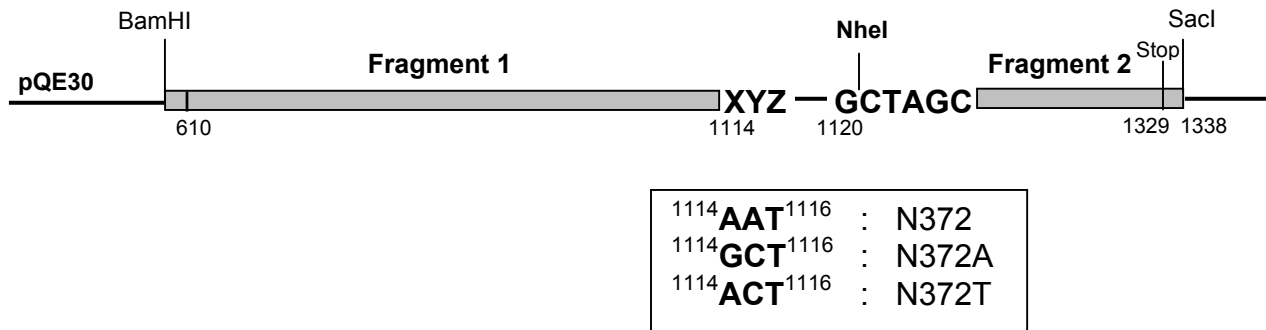
Figure 3.1: Restriction sites and sequence changes used for the mutants N372A and N372T. Numbering of the bases corresponds to positions in the holoenzyme. The silent insertion of an NheI restriction site and the codon changes corresponding to the respective mutations are in bold.

3.6.2.1.2 Mutant D300S

For cloning the mutant D300S two PCR products were amplified. For fragment 1 (312 bp) primers myktub kat-s and myktub 300S-1 with the insertion of a 5'-BamHI and a 3'-XhoI restriction sites were used. Fragment 2 (456 bp) was obtained with myktub 300S-2 and myktub 1-as with the insertion of a 5'-XhoI and a 3'-SacI site (figure 3.2). PCR conditions were: Ta= 52°C, 5 % DMSO, 20 cycles. PCR products were isolated, purified, digested with the respective enzymes and ligated to a dephoshorylated BamHI/SacI digested pQE30 vector. Recombinants were isolated from XL-1blue cells and introduced into BL21 cells for protein expression after sequencing.
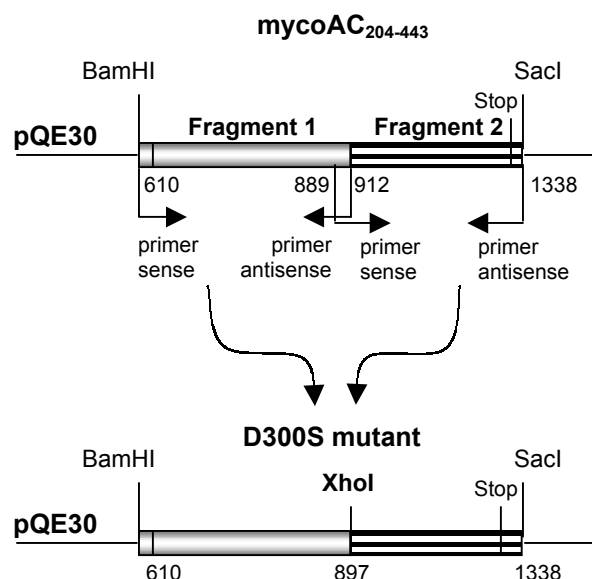
Figure 3.2 : Cloning of D300S in pQE30. Within the XhoI site the codon changes for the respective mutation were inserted by PCR (^{898}TCG^{900} to ^{898}GAC^{900}). Numbering is according to the holoenzyme Rv1625c.

3.6.3 *Mycobacterium tuberculosis* Rv0386

3.6.3.1 Holoenzyme

The *M. tuberculosis* genomic DNA was provided by Dr. Boettger (University of Zürich Medical School). The gene Rv0386 was obtained in 5 PCR reactions, i.e. in five different fragments which covered the entire ORF (3255 bp). For PCR conditions see Table 3.5. See appendix for Rv0386 DNA sequence (restriction sites used for cloning are outlined).

PCR fragment	Primers	Length (bp)	$T_{annealing}$ (°C)	Cycles	% DMSO
1	myco0386/B-C-Hforward myco0386/B-C-H-reverse	545	58	40	5
2	myco0386/C-Cforward myco0386/C-Creverse	696	60	40	5
3	myco0386/C-Sforward myco0386/C-Sreverse	690	62	40	5
4	myco0386/S-Sforward myco0386/S-Sreverse	630	62	40	5
5	myco0386/S-Hforward myco0386/S-Hreverse	806	70	40	5

Table 3.5: Primers used and PCR conditions for cloning Rv0386.

The primer myco0386/B-C-Hforward inserted a silent mutation at position 55 to eliminate a BamHI site. After PCR, the DNA products were purified by agarose gel electrophoresis, blunted, phosphorylated and ligated into different vectors (Table 3.6).

PCR fragment	Vector	Expected direction
1	pBSK(-), ClaI digested, dephosphorylated and blunted	antisense
2,3 and 4	pBSK(-), EcoRV digested, dephosphorylated and blunted	sense
5	pBSK(-), EcoRV digested, dephosphorylated and blunted	antisense

Table 3.6: Vectors used for cloning the DNA fragments obtained by PCR (see Figures 3.3 and 3.4).

The ligation products were transformed into XL-1 blue cells. The recombinant DNA was analyzed by restriction. The direction of the five PCR products within vectors was controlled through digestion with BamHI and HindIII. To search for clones with fragment I in antisense direction, an additional control digestion with only BamHI was carried out. Similarly, for fragment V a digest with only HindIII identified the clones with antisense direction. All clones were verified through sequencing (Figures 3.3 and 3.4).
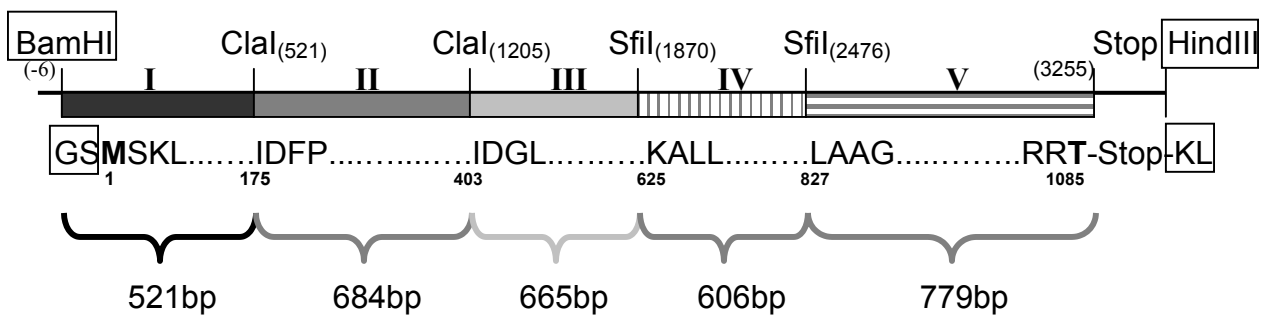
Figure 3.3: Overview of the sequence of Rv0386 to be expressed. Note the five fragments. The restriction sites BamHI and HindIII were introduced through primers. ClaI and SfiI sites were from the original sequence.

Figure 3.4: Steps used for cloning Rv0386. Levels above correspond to vector positions and levels below to Rv0386 positions.

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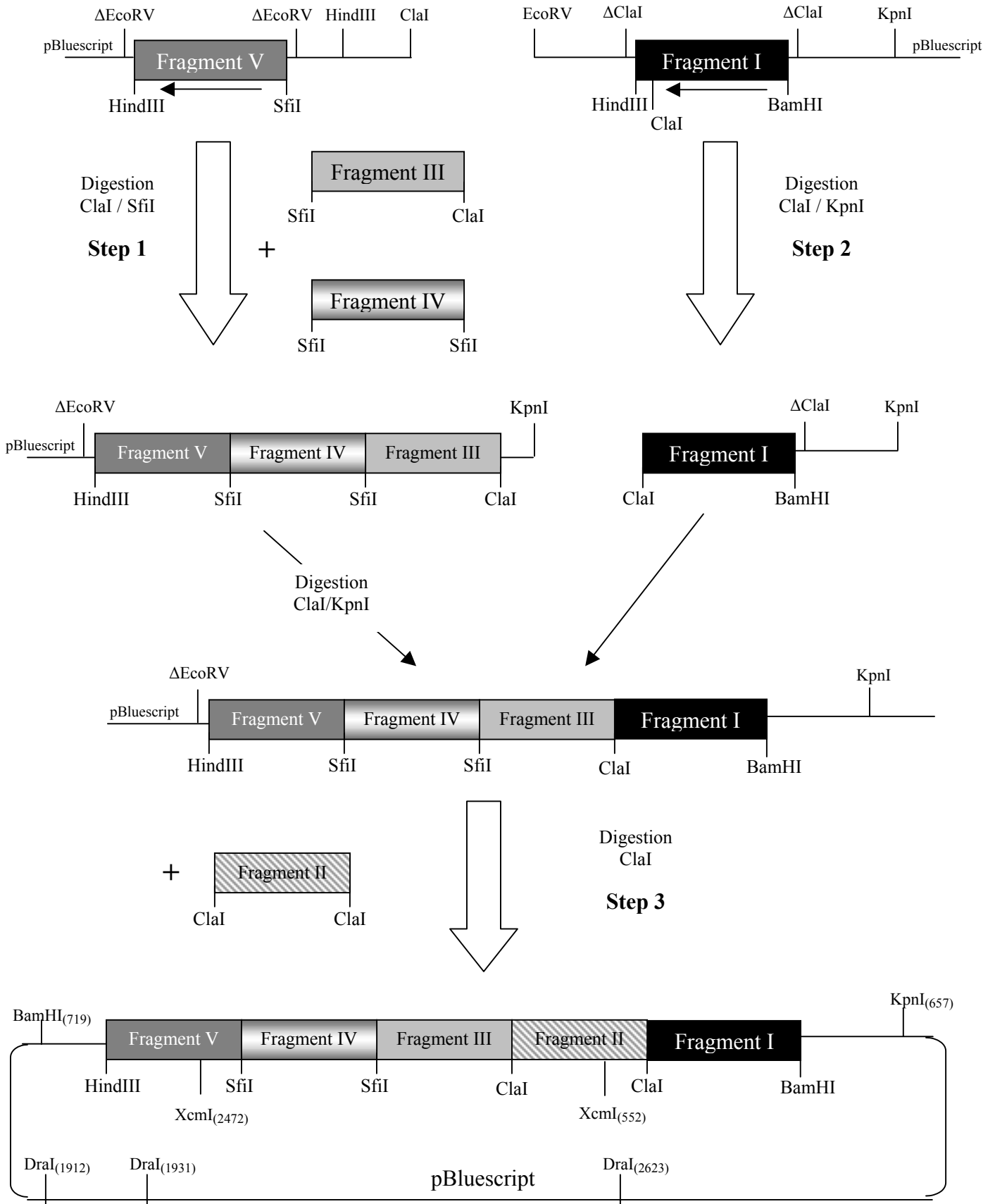
Step 1: DNA fragment V in pBluescript (in antisense direction) was digested with SfiI (2h, 50°C) and subsequently with ClaI (O/N, 37°C). The product was dephosphorylated and used as a vector. Simultaneously, fragments IV and III in pBluescript were digested with SfiI and fragment 3 additionally with ClaI. Both products were phosphorylated to function as inserts. Vector and the two inserts were triple ligated and transformed in XL-1 blue cells. Clones were first controlled by digestion with HindIII and ClaI. A second control digest was carried out with PstI (Figure 3.5). One of the correct clones was chosen.
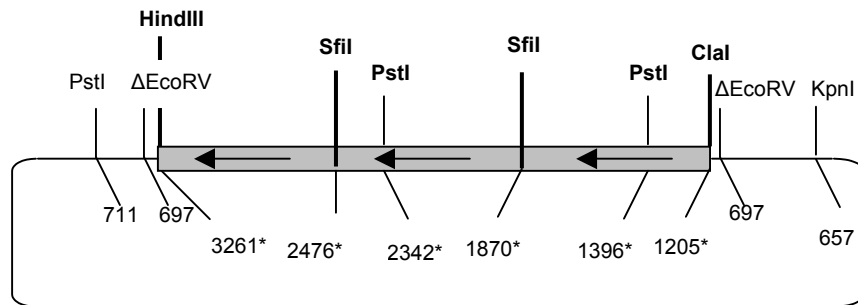
Fig. 3.5: Restriction sites used for controlling the insertion of fragment IV (SfiI/SfiI) in the correct direction between fragments III (ClaI/SfiI) and V (SfiI/HindIII). Names of the enzymes in bold correspond to restriction sites present in the holoenzyme sequence. *Numbering corresponds to positions in Rv0386. The other restriction sites and numberings correspond to pBluescript.

Step 2: Fragment I cloned in pBluescript in antisense direction was digested with ClaI and KpnI (1h, 37°C). The product was the insert. Construct containing fragments III, IV and V cloned together in pBluescript (step 1) was digested with ClaI and KpnI and dephosphorylated to act as the vector (KpnI comes from pBluescript). Vector and insert were ligated and transformed in XL-1 blue cells. The obtained clones were controlled by digestion with BamHI and HindIII.

Step 3: Fragments I/III/IV/V (in pBluescript) were digested with ClaI and then dephosphorylated to be the vector. Fragment II in pBluescript was digested with ClaI to serve as a insert. Vector and insert were ligated and transformed in XL-1 blue cells. Clones were analyzed by digestion with BamHI, XcmI and HindIII to verify the direction of fragment II.

Step 4: The whole construct in pBluescript (in antisense direction) was digested with BamHI, HindIII and DraI to be the insert for the vector pQE30. DraI digests pBluescript into 3 fragments but does not cut the insert. This permitted the easy isolation of the insert from pBluescript because of the size difference. pQE30 was likewise digested with BamHI and HindIII and dephosphorylated. Insert and vector were ligated, transformed in XL-1 blue cells and clones were controlled by digestion with BamHI and HindIII. One of the correct clones was chosen for transformation into BL21 cells for expression and a glycerol stock culture was prepared.

3.6.3.2   Adenylyl cyclase domain

Fragment I used for cloning the holoenzyme (figure 3.4) was digested with BamHI and HindIII from pBluescript and ligated into pQE30 digested correspondingly. Finally, it was transformed in BL21 cells for expression.

3.6.3.3   Putative AAA-ATPase domain

Using the pQE30 cloned holoenzyme as a template, a PCR (Ta= 48°C, 10 % DMSO, 30 cycles) with primers NHis/TF/Rv0386forw and NHis/AAADom/Rv0386rev was carried out. These primers inserted 5'-BamHI and 3'-HindIII sites. The DNA fragment between positions 502 and 1518 was amplified (Figure 3.6). The products were digested with BamHI and HindIII and phosphorylated. DNA of pQE30 was digested similarly and dephosphorylated. Insert and vector were ligated and transformed in XL-1 blue cells. Recombinant DNA from minipreps was sequenced to verify sequence fidelity. A correct clone was then transformed in BL21 cells and in BL21-STAR cells.
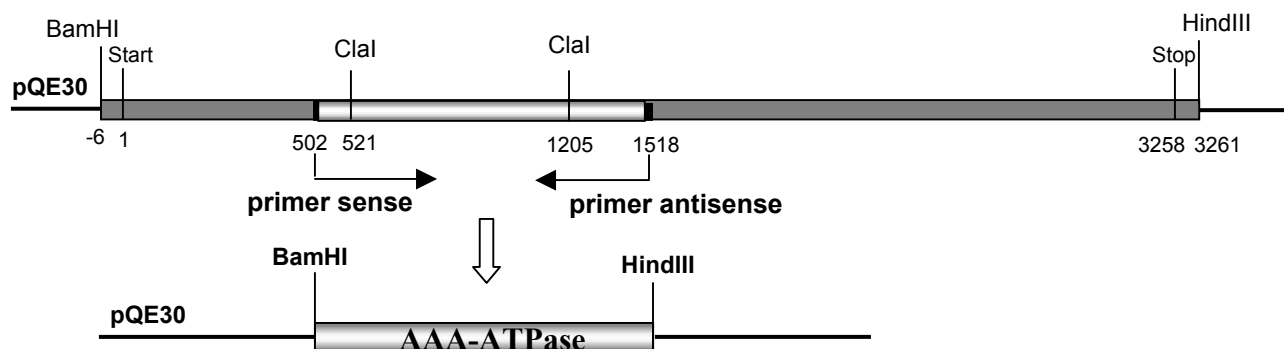
Fig. 3.6: Cloning steps of the AAA-ATPase domain of Rv0386 in expression vector pQE30. DNA from position 502 to 1518 was amplified. Numbering corresponds to positions in the holoenzyme sequence.

3.6.3.4   Putative Transcription factor domain

DNA between positions 502 and 1121 of Rv0386 was amplified by PCR (Ta= 54°C, 5 % DMSO, 30 cycles) with primers NHis/TF/Rv0386forw and myco0386/C-Crev (Figure 3.7). The holoenzyme in pQE30 was used as a template. The primers introduced 5'-BamHI and 3'-BglII restriction sites. PCR products were purified from agarose gel, blunted and phosphorylated. DNA of pBluescript was blunt digested with EcoRV and dephosphorylated. Insert and vector were ligated and transformed in XL-1 blue cells. Recombinant DNA of a correct clone was digested with BamHI and Bgl II and a 631 bp fragment was purified. The holoenzyme construct in pQE30 was digested with BamHI and BglII, dephosphorylated and used as a vector. With this step the fragment between positions –6 and 1121 of the holoenzyme sequence was removed and replaced with the 631 bp fragment. The DNA of the obtained clones was analyzed by digestion with BamHI, Bgl II and HindIII (631, 2142 and 3460 bp fragments were expected). A correct clone was chosen for transformation into BL21 and BL21-STAR cells.

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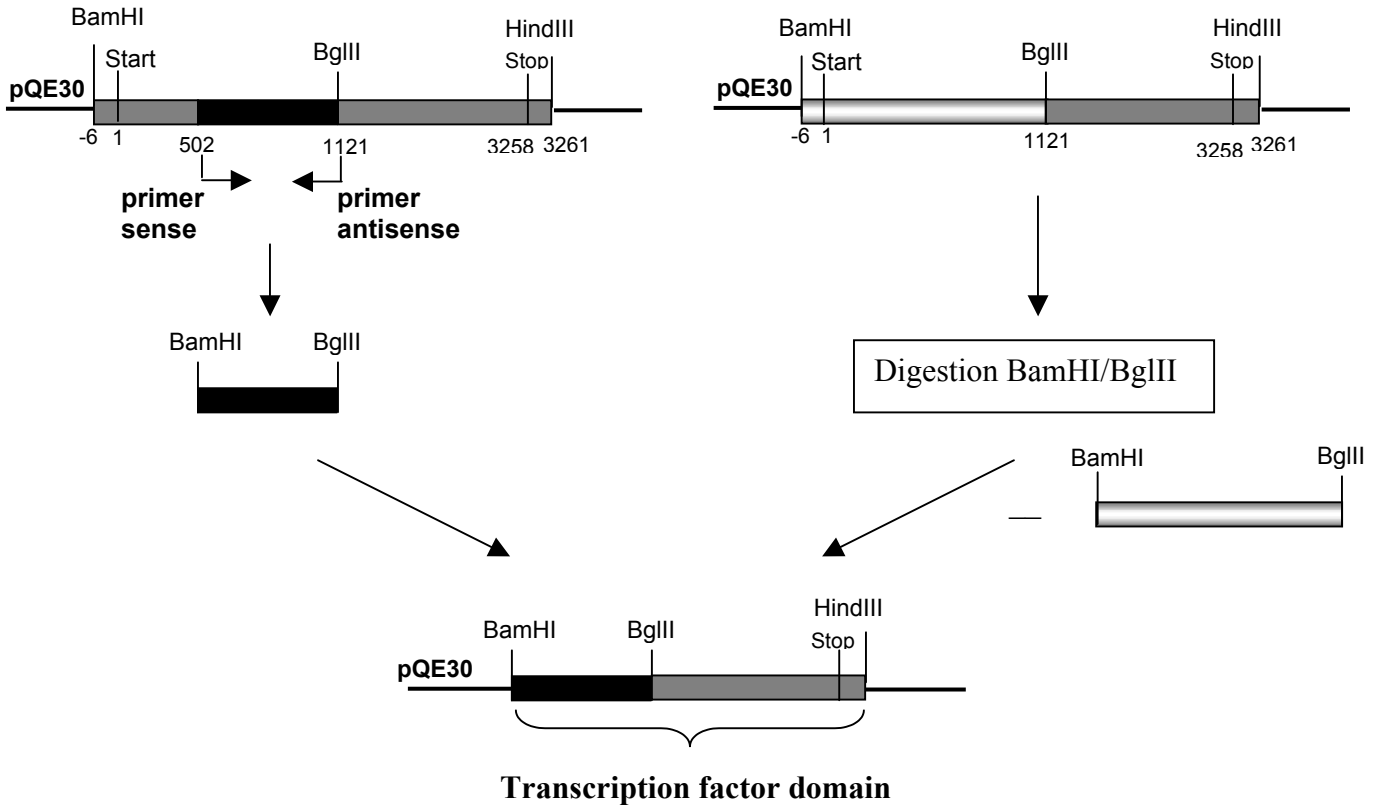
Fig. 3.7: Cloning steps for the transcription factor domain of Rv0386 into vector pQE30. Numbering is according to the holoenzyme.

3.6.3.5 Putative DNA-binding domain

Amplification of fragment 2980 to 3261 was performed with primers NHis/DBDom/Rv0386 and myco0386/S-Hrev (Ta= 64°C, 5 % DMSO, 30 cycles). 5'-BamHI and 3'-HindIII sites were introduced (Figure 3.8). Fragment V used for cloning the holoenzyme (SfiI$_{2476}$ to HindIII$_{3261}$) was used as a template. The product was isolated, purified and digested with BamHI and HindIII. pQE30 was cut similarly and dephosphorylated. Insert and vector were ligated and transformed in XL-1 blue cells. Recombinant DNA of the clones was sequenced and a correct one was used for transformation into BL21 cells.
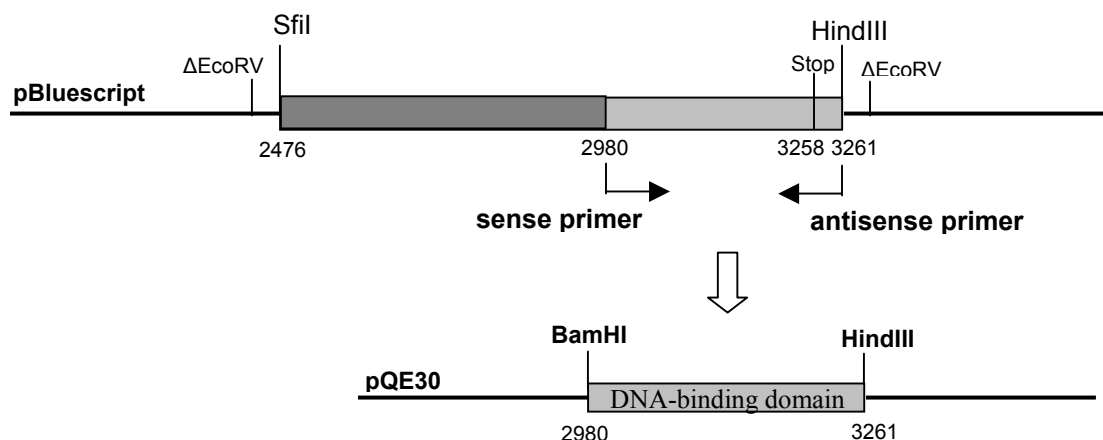
Fig. 3.8: Cloning steps of the DNA-binding domain of Rv0386 in pQE30. Numbering corresponds to the nucleotide positions in the holoenzyme.

3.6.3.6   Site-directed mutagenesis

Mutation primers were used to introduce the desired changes. See appendix for DNA sequence with outlined restriction and mutation sites.

3.6.3.6.1 Q57K and Q57A mutants

In table 3.7 and figure 3.9 the primers and the strategy are delineated. PCR conditions: Ta= 60°C, 5 % DMSO, 40 cycles. The template was the construct of the AC domain in pQE30 vector.

Mutant	Fragment	Primers	Length of the PCR product (bp)
Q57K	1	myco0386/B-C-Hforw myco0386/Q57Krev	180
	2	myco0386/Q57Kforw myco0386/B-C-Hrev	390
Q57A	1	myco0386/B-C-Hforw myco0386/Q57Arev	180
	2	myco0386/Q57Aforw myco0386/B-C-Hrev	390

Table 3.7: Primers used for the cloning of Q57K and Q57A .

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Fragments 1 and 2 were purified, blunted, ligated with dephosphorylated pBluescript vector ΔEcoRV and transformed into XL-1 blue cells. Clones were verified through sequencing. Fragment 1 in pBluescript was digested with StuI and HindIII and dephosphorylated to serve as a vector. Fragment 2 was obtained by a StuI/HindIII digest. Vector and insert were ligated and transformed in XL-1 blue cells. Clones were controlled by a BamHI/HindIII digestion. Then the mutated catalytic domain was cut out of pBluescript, inserted in a dephosphorylated pQE30 vector Δ(BamHI/HindIII) and transformed into BL21 cells.
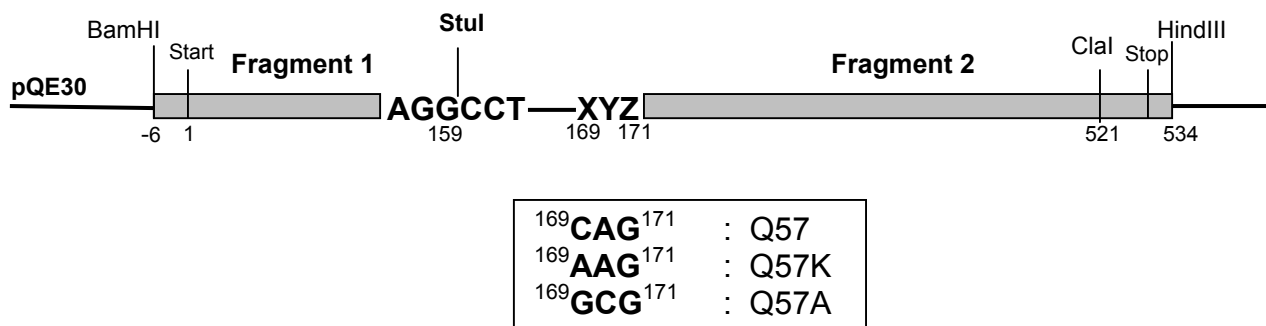
Fig. 3.9: Site-directed mutagenesis for cloning Q57K and Q57A in pQE30. A silent restriction site for StuI and the corresponding nucleotide changes for each mutation were inserted by PCR. Numbering is according to the holoenzyme.

3.6.3.6.2 N106D and N106A mutants

In table 3.8 and figure 3.10 the primers and the strategy are delineated. PCR conditions: Ta= 60°C, 5 % DMSO, 40 cycles. Template was the AC domain in pQE30.

Mutant	Fragment	Primers	Length of the PCR product (BP)
N106D	1	myco0386/B-C-Hforw myco0386/N106Drev	339
	2	myco0386/N106Dforw myco0386/B-C-Hrev	234
N106A	1	myco0386/B-C-Hforw myco0386/N106Arev	339
	2	myco0386/N106Aforw myco0386/B-C-Hrev	234

Table 3.8: Primers used for cloning of N106D and N106A.

Each fragment was purified, blunted, phosphorylated, ligated with a blunt dephosphorylated pBluescript ΔApaI and transformed in XL-1 blue cells. Clones were verified through sequencing. Correct clones were digested with BamHI/ApaI and ApaI/HindIII to cut out fragments 1 and 2, respectively. Both fragments were ligated with dephosphorylated pQE30 Δ(BamHI/HindIII) and transformed in XL-1 cells. Clones were controlled by digestion with BamHI and HindIII and a correct one was transformed in BL21 cells.
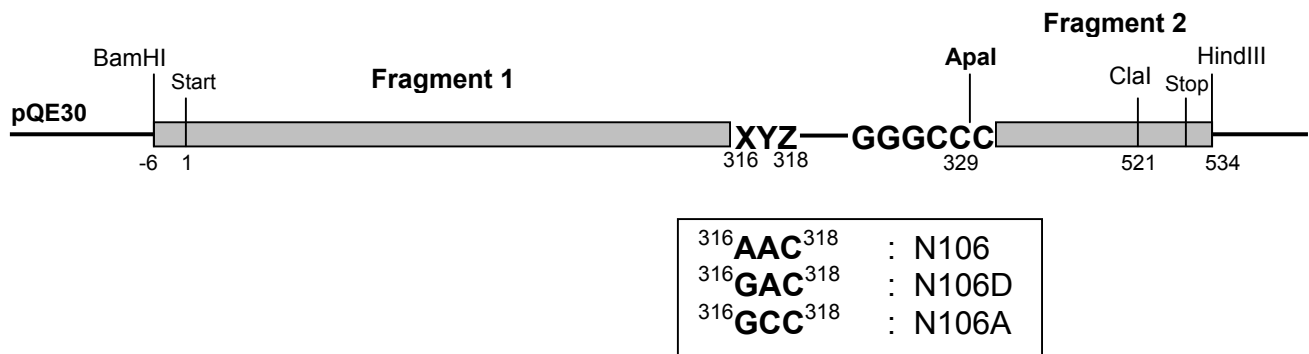
Fig. 3.10: Cloning of N106A and N106D in pQE30. Silent restriction site for ApaI and nucleotide changes for each mutant were inserted by PCR. Numbering is according to the holoenzyme.

3.6.3.6.3 Q57K/N106D mutant

Two PCR products were amplified with primers myco0386/B-C-Hforw and myco0386/QKNDrev for fragment 1, and myco0386/QKNDforw and myco0386/B-C-Hrev for fragment 2. PCR conditions: Ta= 56°C, 5 % DMSO and 20 cycles. Template for fragment 1 (258 bp) was the mutant Q57K in pQE30 vector and for fragment 2 (312 bp) mutant N106D in pQE30. 5'-BamHI and 3'-BglII sites for fragment 1 and 5'-BglII and 3'-HindIII sites for fragment 2 were inserted. PCR products were purified, blunted, phosphorylated and ligated to a blunt dephosphorylated pBluescript SK(-) vector ΔEcoRV. DNA of the clones was sequenced. Fragment 1 was cut from pBluescript with BamHI/BglII and fragment 2 with BglII/HindIII. Both fragments were simultaneously ligated into a dephosphorylated pQE30 vector Δ(BamHI/HindIII). The final construct was controlled by BamHI/Bgl II/HindIII digestion and transformed into BL21 cells (figure 3.11).
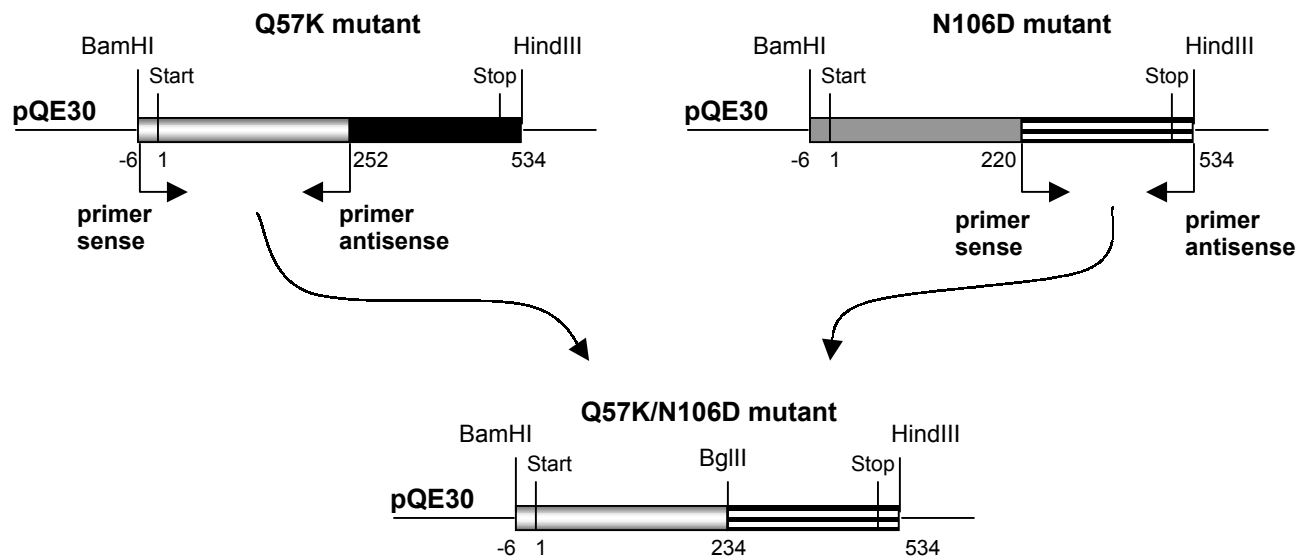
Fig. 3.11: Cloning of Q57K/N106D in pQE30. Silent restriction site for BglII was inserted by PCR. Numbering is according to the holoenzyme.

3.6.3.6.4 N106S mutant

Mutant	Fragment	Primers	Length of the PCR product (bp)
N106S	1	myco0386/B-C-Hforw myco0386/N106Srev	342
	2	myco0386/N106Sforw myco0386/B-C-Hrev	228

Table 3.9: Primers used for cloning of mutant N106S.

PCR conditions: Ta= 58°C for fragment 1 and 54°C for fragment 2, 5 % DMSO and 30 cycles. The AC domain in pQE30 was the template. Cloning strategy was similar as for mutant D300S of Rv1625c (see above). Products were purified, digested with BamHI/NdeI (fragment 1) or NdeI/HindIII (fragment 2), desalted and ligated into dephosphorylated pQE30 Δ(BamHI/HindIII) in a triple ligation. Recombinants were isolated from XL-1 blue cells and introduced into BL21 for expression with prior sequence confirmation.

3.6.3.7 N-terminally elongated AC domain (with N-terminal His-tag)

By comparison of Rv0386 with its homologue MT0399 from *Mycobacterium tuberculosis* CDC1551 (Fleischmann et al., 2002), an almost total identity was observed with the exception of 7 extra N-terminal amino acids in MT0399 (MRLSGAG). These amino acids are just ahead of the START codon of Rv0386. To determine the potential influence of this addition they were inserted through PCR. The original 'gtg' START of MT0399 was changed to 'atg'.

Primers CDC1551ac-forw and myco0386/B-C-Hrev were used. PCR conditions: Ta= 58°C, 5% DMSO and 30 cycles. The AC domain in pQE30 was the template. The 564 bp PCR product obtained was purified from agarose gel, digested with BamHI and HindIII and directly ligated with suitably prepared pQE30 vector. After transformation into XL-1 blue cells, it was controlled by sequencing. Finally, it was transformed into BL21 cells for expression.

3.6.3.8 N-terminally elongated AC domain (with C-terminal His-tag)

The PCR product was amplified with primers CDC1551ac-forw and myco0386/cata/C-His. PCR conditions: Ta= 58°C, 5 % DMSO, 30 cycles and AC domain in pQE30 as template. The product (564 bp) was purified, digested with BamHI and BglII and ligated into a dephosphorylated pQE60 vector Δ(BamHI/BglII). After transformation into XL-1 blue cells clones were sequenced. Finally, it was transformed into BL21 cells for expression.

3.6.3.9 C-terminally His-tagged AC catalytic domain of Rv0386

With primers myco0386/B-C-Hforw and myco0386/cata/C-His the AC catalytic domain was amplified. PCR conditions: Ta= 58°C, 5% DMSO and 30 cycles. The AC domain in pQE30 was the template. The 543 bp PCR product was purified from agarose gel, digested with BamHI and BglII and directly ligated into suitably prepared pQE60. After transformation into XL-1 blue cells, it was controlled by sequencing. Finally, it was transformed into BL21 cells for expression.

3.6.3.10 AC domain mutant R7G

This mutant was unintentionally constructed due to a wrong nucleotide in the primer myco0386/B-C-Hforw. This changed nucleotide introduced a mutation by changing a C

in the codon **C**GG (codes for arginine) into a G forming the wrong codon **G**GG (codes for glycine). This mutant was cloned presuming that it was the catalytic domain wild type (see above).

3.7 Protein chemistry methods

3.7.1 Expression of proteins in *E. coli*

Most proteins were expressed in *E. coli* BL21 (DE3) [pREP4]. Rv0386 holoenzyme and its transcription factor as well as its ATPase domain were also expressed in *E. coli* BL21 STAR (DE3) [pREP4]. In *E. coli* Rosetta [pREP4] cells only the holoenzyme Rv0386 was expressed.

3.7.1.1   Pre-cultures

For expression in BL21 [pREP4], BL21 STAR [pREP4] and Rosetta [pREP4] cells, 5 ml of LB-broth with 50 µg/ml kanamycin and 100 µg/ml ampicillin were inoculated. With a sterile tip a small amount of the frozen glycerol stock culture was inoculated and incubated O/N at 37 °C (210-230 rpm).

3.7.1.2   Expression

All mammalian, protozoan and mycobacterial constructs were expressed in *E. coli* BL21 [pREP4] from frozen stocks. Mycobacterial Rv0386 constructs were also expressed in *E. coli* BL21 STAR and ROSETTA. 5 ml pre-culture were inoculated and grown in 200 ml LB broth containing 100 µg/ml ampicillin and 50 µg/ml kanamycin (30°C, 220 rpm) to A_{600} 0.4-0.6 (approx. after 1.5-3 h). Expression of the mammalian, protozoan and Rv1625c mycobacterial proteins was induced with 30 µM IPTG and Rv0386 proteins with 60 µM IPTG (22-25°C for BL21 or 12-16°C for STAR and ROSETTA, 220 rpm). Cells were harvested after 3-5 h for constructs in BL21 cells or after 16-18 h for constructs in BL21 STAR and ROSETTA cells (10 min, 5000 x g, 4°C). Supernatant was discarded and the pellet washed with 30 ml Pellet Wash Buffer and centrifuged again (6000 x g, 15-20 min, 4 °C). Cell pellets were then stored at –80°C.

3.7.2 Purification of soluble proteins from *E. coli*

Frozen -80°C pellets were thawed on ice (10 min), suspended in 25 ml of Cell Lysis buffer and sonificated for lysis (microtip setting 4, on ice, 3 x 10 s). These suspensions were called cell homogenate. For mammalian ACs lysis buffer contained protease inhibitors (76 nM aprotinin, 62.5 µM TPCK, 59.6 µM TLCK, 126.3 µM PMSF and 0.16 µM lima bean trypsin inhibitor).The cell homogenate was centrifuged (16,000 x g, 30 min, 4°C). Occasionally, lysozyme (0.2 mg/ml, on ice, 30 min) and DNAse (0.02 mg/ml with 5 mM $MgCl_2$, on ice, 30 min) were added prior to centrifugation. The supernatant and the pellet were separately tested for AC activity.

Supernatants were mixed with 250 mM NaCl and 15 mM imidazole. Then 250 µl of pre-equilibrated Ni^{2+}-NTA-Agarose were added (rocking for at least 1 h on ice). Samples were centrifuged (5 min, 2500 x g). Supernatant was transferred into a 10 ml syringe with a Wizard mini-column attached. The solution was let through and the resin was then washed with 3 ml of each of Washing buffers A, B and C. Elution was performed with 400 µl Elution Buffer.

3.7.3 Purification of insoluble proteins from *E. coli*

Frozen -80°C pellets were thawed on ice (10 min), suspended in 25 ml cell lysis buffer containing Complete® protease inhibitors (1/2 tablet) and passed twice through the french press for cell lysis (20,000 psi). The homogenate was centrifuged (4°C, 3000 x g, 30 min). The pellet from this step was suspended in 500 µl lysis buffer to be analysed later. The supernatant was centrifuged (4°C, 100,000 x g, 1 h). Supernatant was discarded and the pellet was suspended in 25 ml of membrane suspension buffer containing 2 % CHAPS (or other detergent) and incubated on ice for 1 h with gentle agitation. The membrane suspension was centrifuged (4°C, 100,000 x g, 1 h). The pellet was suspended in 500 µl of membrane suspension buffer. Supernatant was mixed with 15 mM imidazole pH 8.0 and incubated O/N with gentle agitation with 200 µl Ni-NTA-agarose (pre-equilibrated with 2 % CHAPS). Ni-NTA bound protein was washed and eluted. Two washing steps were made, the first with 6 ml of High Saline Buffer and the second one with 6 ml of Low Saline Buffer. Finally, the protein was eluted with 400 µl of Elution Buffer. Each washing fraction was stored and analysed later.

3.7.4   Protein concentration

3.7.4.1   Bio-Rad protein determination

1 mg/ml of BSA was used as a standard. 1-10 µg of protein were pipetted to 800 µl distilled water and mixed. 200 µl of Dye Reagent Concentrate (5X) were added and mixed. Samples were measured at OD_{595}. The presence of $\leq$ 2% of the detergent CHAPS did not interfere.

3.7.4.2   Dialysis

To eliminate imidazole from the eluate and change buffer samples were dialyzed (VISKING Dialysis Tubing, MWCO12000-14000). The DNA-binding domain of Rv0386 (~12000 Da) was dialyzed with a SPECTRAPOR membrane (MWCO 3500). Samples were dialyzed at least for 3 hours at 4°C.

3.7.4.3   Sample Concentration

To produce antibodies against the Rv0386 DNA-binding domain (made by BioGenes) and to carry out the crystallization experiments with the Rv0386 AC domain, concentration of proteins was required. Samples were centrifuged until the desirable protein concentration was achieved (4 °C, 2500-3500 x g, 2-4 h, CENTRICON Centrifugal Filter Devices MWCO 10000). For crystallization, samples were centrifuged with the Nanosep 10K Omega centrifugal devices MWCO 10000 (14,000 x g, 4°C, 10-30 min).

3.7.5 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

An electrophoresis apparatus from HOEFER was used. In Tables 3.10 a and b the recipe for polyacrylamide gels and the effective separation range of proteins are shown:

a

Resolving Gel (pH 8.8)	7.5 %	10 %	12.5 %	15 %
Resolving Gel buffer	3 ml	3 ml	3 ml	3 ml
H_2O	6 ml	5 ml	4 ml	3 ml
Acrylamide/Bisacrylamide 37.5 : 1	3 ml	4 ml	5 ml	6 ml
TEMED	10 µl	10 µl	10 µl	10 µl
APS 10 %	80 µl	80 µl	80 µl	80 µl
Effective Separation Range (kDa)	**45 - 200**	**20 - 200**	**14 - 70**	**5 - 70**

b

Stacking Gel (pH 6.8)	4 %
Stacking Gel buffer	1 ml
H_2O	2.4 ml
Acrylamide / Bisacrylamide 37.5 : 1	0.6 ml
TEMED	10 µl
APS 10 %	40 µl

Table 3.10: (**a**) Components required for different concentrations (%) of resolving gels for SDS-PAGE; (**b**) components of the stacking gel.

Soluble protein samples were mixed with 1X sample buffer and pellets and cell homogenates with 4X sample buffer. Usually, they were heated at 95 °C for 5 minutes before loading. Gels were run at a constant current: 20 mA, 200 V for 50-65 minutes. Gels were stained with Coomassie Blue for 30 min with gentle agitation. After destaining 0.1 to 0.5 µg protein/band was detectable.

Usually, SDS-PAGE was performed simultaneously with pellets and supernatants of *E. coli* containing an empty vector (pQE30) as a control. Protein Marker components (10 µl contained 1 µg of each protein) are listed below.

Protein Marker	MW (kDa)	peqGold Marker	MW (kDa)
Cytochrom C	12.5	Lysozyme	14.4
Trypsin Inhibitor	20.1	β-Lactoglobulin	18.4
Chymotrypsinogen A	25	RE Bsp981	25
GAPDH	36	Lactate dehydrogenase	35
Ovalbumin	45	Ovalbumin	45
BSA	66	Bovine Serum Albumin	66
		β-Galactosidase	116

3.7.6 Anion exchange chromatography

The holoenzyme Rv0386 and its catalytic domain were additionally purified with anion exchange chromatography with an ÄKTA FPLC at 4 °C. All buffers were made with MilliQ water, filtrated and degassed. Protein was measured at 280 nm. All fractions were tested for AC activity. About 1.4 mg (800 µl) catalytic domain protein or 0.3 mg (800 µl) holoenzyme diluted to 10 ml with respective "A" buffers (see Table 3.11), were applied through a 10 ml loop to a pre-equilibrated column (Mono Q).

Protein	pI	Buffer A	Buffer B
Catalytic domain Rv0386	6.6	50 mM Tris pH 8.0 2 mM β-mercapto ethanol 10 % glycerol	Buffer A + 1 M NaCl
Holoenzyme Rv0386	5.8	50 mM Tris pH 7.0 10 mM β-mercapto ethanol 2 mM MgCl$_2$ 20 % Glycerol	1 M NaCl

Table 3.11: Compositions of buffer A and buffer B used for anion exchange chromatography of the catalytic domain and the holoenzyme Rv0386. pI= isoelectrical point.

Columns were developed with a stepwise NaCl gradient. Flow rate was 1 ml/min. Elution began at 0% NaCl (only buffer A) for 3 fractions of 5 ml. After that fractions were 1 ml. After 10 fractions the NaCl concentration was increased (100, 200, 300, 400, 500 mM and 1 M NaCl steps). 70 fractions were collected.

3.7.7 Cyclase enzyme tests

They were carried out according to Salomon et al., 1974, and Steinlen, 1988.

3.7.7.1   Adenylyl cyclase test

A standard test contained in 100 µl 75 µM [α-^{32}P]-ATP (25 kBq, 30 °C, 10 min). 40 µl of protein sample including reagents like forskolin, inhibitors or detergents was mixed with 50 µl of AC Test Cocktail. The assay was started with 10 µl of AC-Start solution. Final concentrations were: 50 mM buffer (Tris-HCl pH 7.5 or MOPS pH 7.5), 22 % glycerol, 2-5 mM MnCl$_2$ or 5-10 mM MgCl$_2$, 2 mM [2,8-^{3}H]-cAMP (100 Bq), 3 mM creatine phosphate and 1 U creatine kinase (the latter as an ATP-regenerating system when testing impure protein samples). [2,8-^{3}H]-cAMP was used as an internal standard.

The reaction was stopped by addition of 150 µl stop buffer. Water was added to 1 ml and the mixture was applied to a Dowex column (9 x 1 cm glass column with 1.2 g Dowex 50). After washing with 3 ml water the samples were eluted with 5 ml water on Al$_2$O$_3$ columns (10 x 0.5 cm plastic column with 1 g active, neutral Al$_2$O$_3$ 90). Samples were immediately eluted with 4.5 mL 0.1 M Tris-HCl pH 7.5, mixed with 4 ml Ultima Gold XR Scintillator Solution and counted. Dowex columns were regenerated with 1 x 5 ml HCl (2N), 1 x 10 ml and 1 x 5 ml water, and Al$_2$O$_3$ columns with 2 x 5 ml Tris-HCl pH 7.5 (0.1 M). Specific activity (A = pmol·mg^{-1}·min^{-1}) was calculated with the following formula:

$$A = \frac{\text{Substrate (µM) x 100 µl}}{\text{Time (min)}} \times \frac{1000}{\text{Protein (µg)}}$$

$$\times \frac{\text{cpm [}^3\text{H] }_{total}}{\text{cpm [}^3\text{H] }_{sample} - 3\% \text{ [}^{32}\text{P] }_{sample}} \times \frac{\text{cpm [}^{32}\text{P] }_{sample} - \text{cpm [}^{32}\text{P] }_{blanc}}{\text{cpm [}^{32}\text{P] }_{total}}$$

The subtraction of 3 % of the phosphorous counts from the tritium value for each sample was made because of spillover of ^{32}P into the tritium channel. For AC reconstitution assays where proteins were mixed at different concentrations the lowest one was used for calculations. Activities lower than double background (in cpm) were considered zero.

3.7.7.2   Guanylyl cyclase test

Assays were carried out simultaneously with an AC test for comparison. The same protocol used for AC tests was used with [8-^{3}H]-cGMP (100 Bq) contained in the GC Test Cocktail and 75 µM [α-^{32}P]-GTP (25 kBq) as a substrate. The Dowex columns for GC test contained 4 g of this material, and the Al$_2$O$_3$ columns 0.8 g. The elution of the Dowex columns was with 2 ml water (after a washing step with 3 ml water) and of the Al$_2$O$_3$ columns with 4 ml 0.1 M Tris-HCl pH 7.5. Regeneration of the Dowex columns was with 1 x 5 ml 2 N HCl, 1 x 10 ml water and 1 x 5 ml with water, the Al$_2$O$_3$ columns were washed with 2 x 4 ml 0.1 M Tris-HCl pH 7.5. The calculation of the enzymatic activity was carried out as above.

3.7.8 Production of specific antibodies

Production of antibodies against the AC domain (KD0386) and the DNA-binding domain (DB0386) of Rv0386 was done by BioGenes (Berlin) using purified proteins dialyzed against 50 mM phosphate buffer (10 % glycerol, filtered). Specific IgG anti-KD0386 were delivered in Tris-Glycin-Buffer pH 7.5 containing 0.02% Thimerosal. Specific IgG anti DB0386 were delivered in Tris-Glycin-Buffer pH 7.5, 250 mM NaCl and 0.02% Thimerosal. The sensitivity and specificity of the antibodies were tested through Dot-Blot and Western-Blot, respectively.

3.7.8.1   Dot Blot

This was used to determine the optimal concentrations for subsequent binding assays. A set of serial dilutions of the purified protein (concentrations of 0.1 ng to 1 µg pro spot) were applied to a Protran nitrocellulose membrane. The membrane was then incubated with different dilutions in M-TBS of the antibodies and developed as described for Western blotting.

3.7.8.2   Western Blot

Samples were separated by SDS-PAGE. The Semi-Dry-Electrotransfer method of Towbin et al. (1979) was used. At the end of the electrophoresis the gel was removed and attached to a PVDF membrane, which were soaked for 10 min in methanol, 10 min in water and 10 min in Towbin buffer. Gel and membrane were sandwiched between

pieces of Whatman 3 MM paper soaked in Towbin buffer. The sandwich was then placed between graphite plate electrodes with the transfer membrane on the anodic side. A current of 2-3 mA/cm^2 of gel (20 V) was applied for 2-3 hours. The gel was stained with Coomassie Brilliant Blue. The transfer membrane was stained with Ponceau S for no longer than 5 minutes. When protein was visible the membrane was destained by washing with deionized water and the positions of proteins were marked with a pencil. The membrane was then incubated for 1 hour at RT in M-TBS blocking solution. The membrane was rinsed twice for 5 min with TBS-T buffer and then incubated for 1 h with the primary antibody solution (mouse monoclonal RGS·His antibodies, antibodies anti-KD0386 or antibodies anti-DB0386, in M-TBS diluted). The primary antibody was then discarded and the membrane rinsed once for 15 minutes and twice for 5 minutes with TBS-T. The membrane was incubated immediately with the secondary antibody solution (goat anti-mouse or goat anti-rabbit horseradish peroxidase conjugated antibodies, in M-TBS diluted). After 1 hour the secondary antibody was discarded and the membrane washed with TBS-T (see above). For chemiluminiscence a mixture of 2 ml solution A and 50 µl solution B of the ECL-Plus Western Blotting Detection Kit was prepared. The mixture was uniformly distributed over the whole surface of the membrane. After a 5 min reaction a film exposure with Hyperfilm-ECL was taken. Exposure periods varied from 10 seconds to 10 minutes.

3.7.9 Crystallization

Within the scope of this study conditions for crystallization and first attempts to optimize the obtained crystals of the Rv0386 catalytic domain were made. The hanging drop vapor diffusion technique (Figure 3.12) and the 24 well plates of Hampton Research were used.
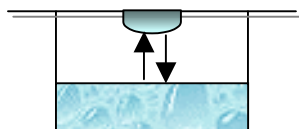
Fig. 3.12: Scheme of the hanging drop method where drops are prepared on a siliconized microscope glass cover slip by mixing the protein sample and the precipitant solution. The cover slip is placed over a small well (reservoir) containing the precipitating solution. The drop and the liquid reservoir of reagent are placed in vapor equilibration.

The reagent kits Crystal Screen, Crystal Screen 2 and Crystal Screen Lite of Hampton Research were used. For crystallization of Rv0386$_{(1-175)}$ with N-terminal His-tag the set of Crystal Screen buffers 4-6, 8-20, 22, 25, 27, 29-41, 43, 45, and 47-50 were used. The buffers 1-3, 6-11, 14, 16, 19, 22, 23, 28-30 and 39-46 of the set Crystal Screen 2 were also used. Finally the buffers 4-10, 13, 14, 16, 17, 19, 22, 23, 25-29, 31, 36, 41, 43, and 47-50 of the set Crystal Screen Lite were utilized. In the case of Rv0386$_{(1-175)}$ with C-terminal His-tag, all buffers of the three screening sets were examined with the protein dialyzed after Ni-NTA purification in glycerol dialysis buffer. All further optimization steps were performed only with the protein Rv0386$_{(1-175)}$ with N-terminal His-tag.

The crystallization variables that were examined for optimization were: glycerol, buffer system in the sample solution and its pH, protein concentration, composition of the precipitant solution, temperature of incubation, proportions of the components in the drop mixture, protein sample purification steps and addition of substrates, inhibitors, cofactors and other substances.

Protein sample solutions were dialyzed against glycerol dialysis buffer (10 and 20% glycerol) containing different buffer systems like MOPS, Tris-HCl and HEPES with pH's 7.5 and 8.5 and then stored at –20°C, 15°C, 4°C and RT. All these conditions were examined for stability tests by testing the AC activity. Conditions where the protein showed no aggregation and less lost of AC activity were examined in the crystallization screens. Protein samples were centrifuged by 14,000 rpm at 4°C for 30 min before setting the crystallization experiments to remove aggregates and amorphous debris. For the first screenings 1 μl of protein sample (protein concentration between 5 and 11 μg/μl) was mixed with 1 μl of each screening buffer (drop mixture 1:1). In the reagent reservoir 0.5 ml of the precipitant solution were mixed with glycerol until obtaining a

concentration of about 2-5% more than the glycerol present in the drop mixture. The 24 well plates were incubated at 12 and 16°C and examined under a polarization microscope.

After obtaining the first crystals the components of the successful precipitant solutions were varied to optimize form, amount and size of the crystals. The protein concentration of the sample was also varied (increased upto 30 µg/µl) and the composition of the drop mixture (proportions 2:1, 1:2, 3:1, 1:3 of protein:precipitant were examined). Ni-NTA purified protein further purified with anion exchange chromatography was also examined for crystal formation. Additions of Mn^{2+} or Mg^{2+}, nucleotides like ATP (substrate), GTP (substrate) or CTP, P-site inhibitors, cGMP and cAMP analogs were examined for optimization.

4 Results

4.1 Chimeras of *M. tuberculosis* Rv1625c mutants with mammalian adenylyl and *Paramecium* guanylyl cyclases

4.1.1 Introductory remarks

Biochemical and mutational studies showed that the mycobacterial AC Rv1625c forms a homodimer where two identical and equivalent catalytic sites are generated (Guo et al., 2001). The possibility to make reconstitutions between mutated mycobacterial and mammalian cytosolic monomers as well as with *Paramecium* GC (ParaGC) monomers was examined (Fig. 4.1 and 4.2). From *M. tuberculosis* the mutants $Rv1625c_{204-443}R376A$ and D300A from Dr. Guo and $Rv1625c_{204-443}N372A$, N372T and D300S (generated here) were used. The mammalian cytosolic domains, the C2 region of ParaGC and its triple mutant ParaGC-C2 KWQ were from Hoffmann, 1999 (Table 4.1).

Name	Domain (function)	Amino acid position	Origin
AC I	C1 (C1)	304-519	Bovine
AC II	C2 (C2)	848-1114	Rat
AC V	C1 (C1)	403-647	Rabbit
AC VII	C1 (C1)	220-486	Bovine
AC IX	C1 (C1)	323-570	Mouse
	C2 (C2)	1012-1355	
Rv1625c wild type	C (C1+C2)		
D300A*	C (C2)		
D300S*	C (C2)	204-443	*M. tuberculosis*
R376A*	C (C1)		
N372A*	C (C1)		
N372T*	C (C1)		
ParaGC-C2	C2 (C1)	2178-2415	*Paramecium*
ParaGC-C2-KWQ**	C2 (C1)		
*Point mutants of *M. tuberculosis* Rv1625c AC domain.			
* *Triple mutant $_{2345}LVR_{2347}$ to $_{2345}KWQ_{2347}$ of *Paramecium tetraurelia* guanylyl cyclase.			

Table 4.1: Cytoplasmic domains used in this work (Hoffmann T.R., 1999; Guo et al., 2001).

a

```
                            Me
                            ↓
Rv1625c     231 :IAERLKEPE----------RNIIADKYDEASVLFADIVGFTERASSTAPA
IC1_BOV     304 :------------------------------VSILFADIVGFTGLASQCTAQ
VC1_RABBIT  451 :INAKQEDM--------MFHKIYIQKHDNVSILFADIEGFTSLASQCTAQ
VIIC1_BOV   249 :IIERLKERGDRRYLPDNNFHNLYVKRHQNVSILYADIVGFTRLASDCSPK
IXC1_MOU    368 :--NRKKKSSIQK--APIAFRPFKMQQIEEVSILFADIVGFTKMSANKSAH
ParaGC-C2  2207 :---------LEEFFRPNEEKRVLREQADEVTLLFADIAGFTEYSSKVQPE


                                    Pu        Me
                                    ↓         ↓
Rv1625c     271 :DLVRFLDRLYSAFDELVDQHGLEKIKVSGDSYMVVS------GVPRPRPD
IC1_BOV     325 :ELVKLLNELFGKFDELATENHCRRIKILGDCYYCVS------GLTQPKTD
VC1_RABBIT  492 :ELVMTLNELFARFDKLAAENHCLRIKILGDCYYCVS------GLPEARAD
VIIC1_BOV   299 :ELVVVLNELFGKFDQIAKANECMRIKILGDCYYCVS------GLPVSLPN
IXC1_MOU    414 :ALVGLLNDLFGRFDRLCEQTKCEKISTLGDCYYCVA------GCPEPRAD
ParaGC-C2  2248 :QVVNMLRNLFTEFDKNSLLHNVFKLYTIGDCYVVMGMVDYGKGIQRNPSQ


                                                      _____
Rv1625c     315 :HTQALADFALDMTNVAAQLKDPRGNP-VPLRVGLATGPVVAGVVGSRRFF
IC1_BOV     369 :HAHCCVEMGLDMIDTITSVAEATEVD-LNMRVGLHTGRVLCGVLGLRKWQ
VC1_RABBIT  536 :HAHCCVEMGMDMIEAISLVREVTGVN-VNMRVGIHSGRVHCGVLGLRKWQ
VIIC1_BOV   343 :HARNCVKMGLDMCEAIKQVREATGVD-ISMRVGIHSGNVLCGVIGLRKWQ
IXC1_MOU    458 :HAYCCIEMGLGMIKAIEQFCQEKKEM-VNMRVGVHTGTVLCGILGMRRFK
ParaGC-C2  2298 :EAVNVVRMGFAMIDAIRRVRAHINHPTLDMRIGVHTGSIIGGVLGTELVR


                   Pu       Ts    Ts
                   ↓        ↓     ↓
Rv1625c     364 :YDVWGDAVNVASRMESTDSVGQIQVPDEVYERLKDDFVLRERGHINVK-G
IC1_BOV     418 :YDVWSNDVTLANVMEAAGLPGKVHITKTTLACLNGDYEV-EPGHGHER--
VC1_RABBIT  585 :FDVWSNDVTLANHMEAGGKAGRIHITKATLNYLNGDYEV-EPGCGGER--
VIIC1_BOV   392 :YDVWSHDVSLANRMEAAGVPGRVHITEATLKHLDKAYEV-EDGHGQQR--
IXC1_MOU    507 :FDVWSNDVNLANLMEQLGVAGKVHISEATAKYLDDRYEM-EDGRVIERLG
ParaGC-C2  2348 :YDIYGPDVLIANKMESKGAKGFVQVSQETKDIIEREF-------------
                        △
                        Fsk
```

b

```
Rv1625c     202 :-------LRDTA--RAEAVMEAEHDRSEALLANMLPASIAERLKEPE---
IIC2_RAT    825 :YYCRLDFLWKNKFKKEREEIETMENLNRVLLENVLPAHVAEHFLARSLKN
IXC2_MOU   1012 :VEADLH----------RTKIQSMRDQADWLLRNIIPYHVAEQ-----LKV
ParaGC-C1  1589 :LEV---FLGRINMKENQIMSD-------ILSILLPQFIRDRINKAG---
```

                                    Me
                                     ⇩

```
Rv1625c     240 :RNIIADKYDEASVLFADIVGF----TERASSTAPADLVRFLDRLYSAFDE
IIC2_RAT    875 :EELYHQSYDCVCVMFASIPDFKEFYTESDVNKEGLECLRLLNEIIADFDD
IXC2_MOU   1047 :SQTYSKNHDSGGVIFASIVNFSEFYEEN--YEGGKECYRVLNELIGDFDE
ParaGC-C1  1626 :QYDIQEDQGMVAVLFCDIIDFDQL-----IKNEQSNVVDILDKLFRRFD-
```

                          Pu   **Me**
                           ⇩   ⇩

```
Rv1625c     286 :LVDQ---HGLEKIKVSGDSYMVVSGVPRPRPD-----------HTQALAD
IIC2_RAT    925 :LLSKPKFSGVEKIKTIGSTYMAATGLSAIPSQEHAQEPERQYMHIGTMVE
IXC2_MOU   1095 :LLSKPDYNSIEKIKTIGATYMAASGLNTAQCQEGGHPQE----HLRILFE
ParaGC-C1  1670 :LLCQQ--HEVQKIETVGKTYMAAAGLKIHVSQKSNP--------VNKVIS
```

                                     △
                                   Fsk   **Pu**
                                                                  ⇩

```
Rv1625c     222 :FALDMTNVAAQLKDPRGN-PVPLRVGLATGPVVAGVVGSRRFFYDVWGDA
IIC2_RAT    975 :FAYALVGKLDAINKHSFN-DFKLRVGINHGPVIAGVIGAQKPQYDIWGNT
IXC2_MOU   1141 :FAKEMMRVVDDFNNMLWFNFKLRVGFNHGPLTAGVIGTTKLLYDIWGDT
ParaGC-C1  1710 :LALDMKRYVMSNE------TFQIKIGIHYGNVIAGVIGHHKPQFSLIGDT
```

      Ts **Ts**
       ⬇   ⬇

```
Rv1625c     371 :VNVASRMESTDSVGQIQVPDEVYERLKD-DFVLRERGHINVKGKGVM---
IIC2_RAT   1024 :VNVASRMDSTGVLDKIQVTEETSLILQTLGYTCTCRGIINVKGKGDLKTY
IXC2_MOU   1191 :VNIASRMDTTGVECRIQVSEESYRVLSKMGYDFDYRGTVNVKGKGQMKTY
ParaGC-C1  1754 :INTASRICSTAESWDVAISEQAYRQTNKYELVYVQRDVVA-KGKGKLITY
```

Fig 4.1: a) Alignment of the catalytic domain of Rv1625c with the cytosolic C1 loop from bovine AC type I (IC1), rabbit AC type V (VC1), bovine type VII (VIIC1), mouse type IX (IXC1) and with the cytosolic C2 loop from the *Paramecium* GC. **b)** Alignment of the catalytic domain of Rv1625c with the cytosolic C2 loops from rat AC type II (IIC2), mouse type IX (IXC2) and the C1 domain from the *Paramecium* GC. Me= metal-cofactor binding; Pu= purine-specifying; Ts= transition state stabilizing; Fsk= forskolin-binding. Conservation scale: inversed (80-100%), dark gray (60-80%), gray (40-60%). The top bar specifies the "arm region" (sequence between a conserved G and the downstream adenine-specifying residue in ACs). Boxed residues are the AC-specific dimer-formation stabilizing motif (KWQ) and its corresponding motif in *Paramecium* and *Mycobacterium*.
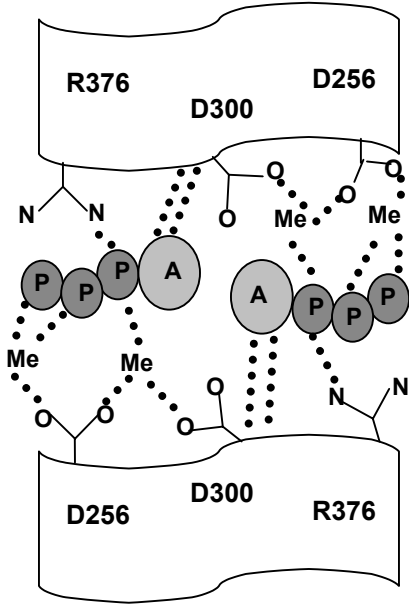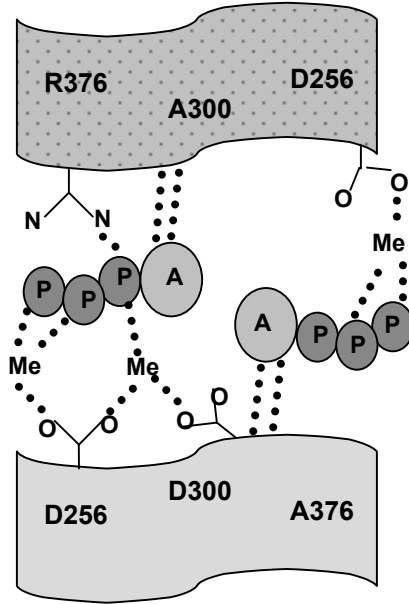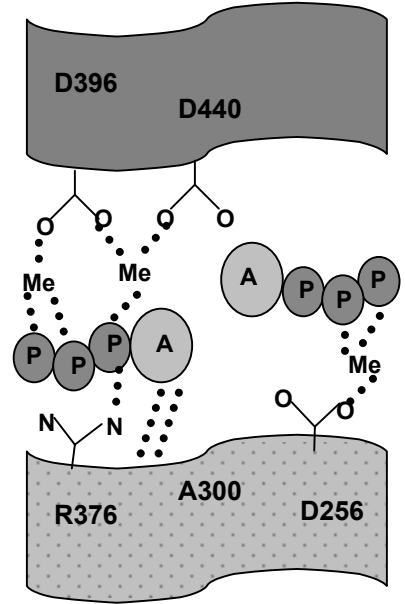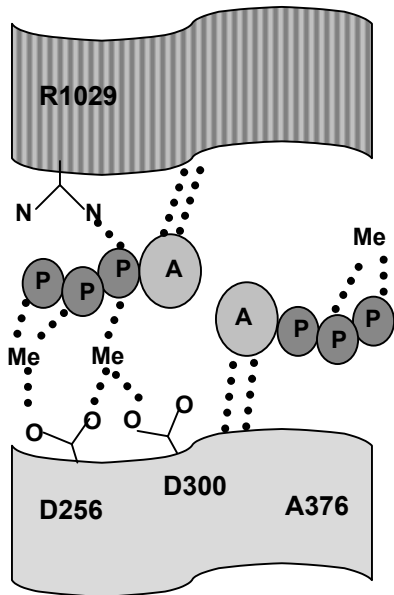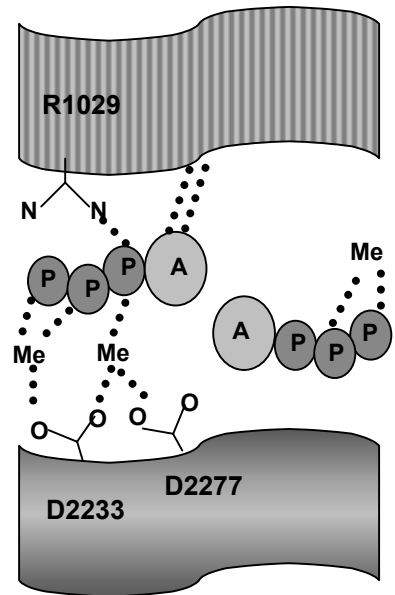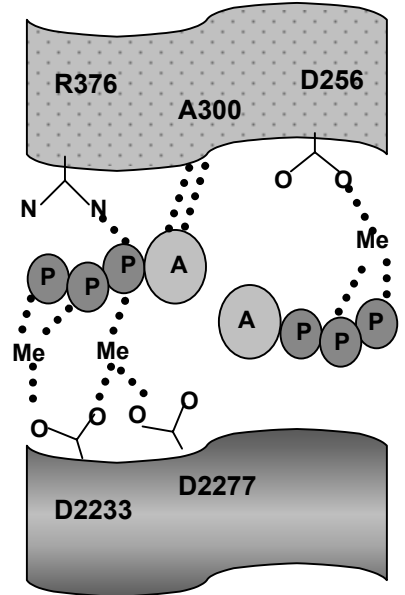
Fig. 4.2: (**A**) Graphic representation of the homodimeric catalytic center of the mycobacterial Rv1625c AC forming two catalytic sites (adapted from Guo et al., 2001). Residues D256, D300 and R376 are outlined; binding of the adenine ring A is indicated by dotted lines. (**B**) Heterodimer with only one catalytic site from the reconstitution of Rv1625c mutant monomers D300A with R376A. (**C to F**) Symbolized heterodimers between mycobacterial D300A and the C1 domain from canine type V; mycobacterial R376A reconstituted with C2 from rat type II; *Paramecium* GC C2 domain reconstituted with mycobaterial D300A and with the C2 domain from rat typ II. P= phosphate; Me= divalent metal cation.

## 4.1.2 Expression and purification of mammalian, mycobacterial and *Paramecium* ACs

The cytosolic domain constructs of the mammalian AC's IC1, VC1, VIIC1, IXC1, IIC2 and *Paramecium* GC (Hoffmann, 1999) as well as the mycobacterial Rv1625c cytosolic domain mutants D300A and R376A (Guo et al., 2001) were available as glycerol stock in BL21 cells. Total amount of protein obtained after purification based on 600 ml culture were: 200 µg of IC1, VC1 and IXC1; 300 µg of VIIC1, IIC2 and ParaGC-C2; 500 µg of ParaGC-C2 KWQ mutant; and 3 mg of D300A and R376A (Fig. 4.3).

The mammalian monomers considerably lost activity after 3 days of storage with 50% glycerol. An example is the chimera VIIC1/IIC2 which had only a residual specific activity of 0.14 $nmol \cdot mg^{-1} \cdot min^{-1}$ (about 6%) [Table 4.2]. Therefore assays were carried out within hours after elution from the affinity resin. Mycobacterial mutants D300A and R376A were stable at –20°C with 50% glycerol. *Paramecium* ParaGC-C2 and ParaGC-C2-KWQ mutant also lost most of their activity after only 4 days of storage with 50% glycerol. The chimera ParaGC-C2/D300A retained only 11% from its activity after 4 days (6.5 $nmol \cdot mg^{-1} \cdot min^{-1}$; Table 4.3).

The three point mutants Rv1625c$_{204-443}$N372T, N372A and D300S were expressed in BL21 cells. After Ni^{2+}-NTA purification (addition of 20 µg/ml DnaseI after sonification was omitted) and dialysis with glycerol dialysis buffer (to remove excess imidazole) pure proteins were obtained. All mutants were expressed in good quantities, after purification about 1 mg protein was purified from 600 ml culture (Fig. 4.4).

They were stable at -20°C with 20% glycerol.
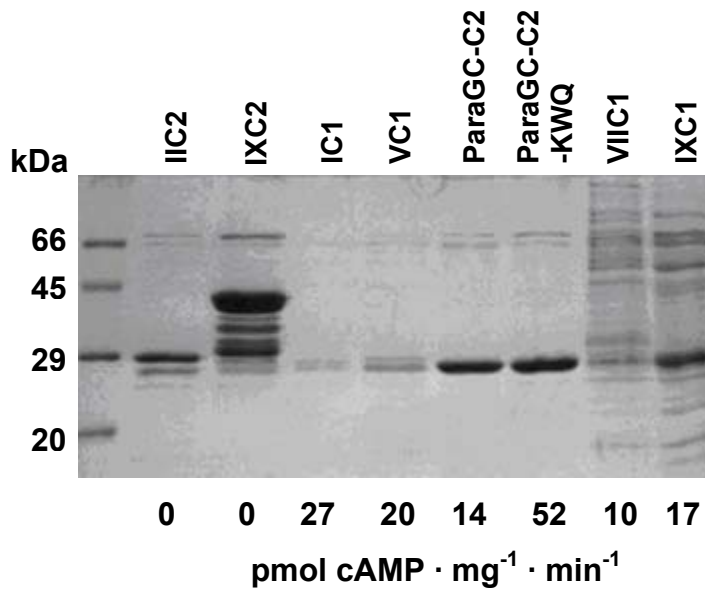
Fig. 4.3: 15% SDS-PAGE analysis of the purified mammalian and *Paramecium* soluble cyclases (Coomassie blue staining). Applied were: about 7-9 µg of IIC2 (29kDa), ParaGC-C2 (29kDa) and ParaGC-C2-KWQ (29kDa); 12-16 µg of IXC2 (36kDa), IC1 (27kDa), VC1 (27kDa), VIIC1 (29kDa) and IXC1 (29kDa). Molecular mass standards are on the left. The specific activities indicated below each lane were determined with 75 µM ATP at 4 µg IC1 (1.5 µM), 8 µg VC1, ParaGC-C2 and C2-KWQ (3 µM), 7 µg IIC2 and IXC2 (2.5 and 2 µM), 11 µg IXC1 (4 µM) and 15 µg VIIC1 (5 µM) protein/assay.
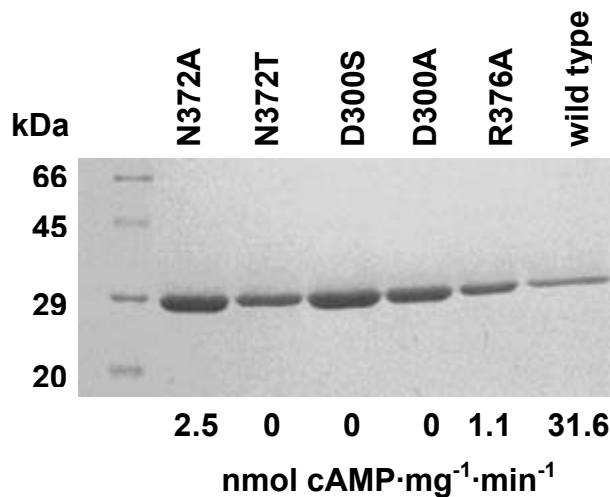
Fig. 4.4 : Purification and activity of all mutants of *Mycobacterium* Rv1625c$_{204-443}$ used in this work (SDS-PAGE analysis, Coomassie blue staining). The catalytic domain wild type is also present on the gel. All proteins were Ni^{2+}-NTA purified and dialyzed. About 1-2 µg of each protein were applied. Molecular weight marker is shown on the left. All mutants have a molecular weight of about 27 kDa like the wild type. The specific activities indicated below each lane were determined with 75 µM ATP (30°C, 10 min) at a protein concentration of 0.1 µg/assay (40 nM).

4.1.3 Mammalian/*Mycobacterium* chimeras

The reconstitution of mammalian/mammalian monomers and of R376A with D300A (Guo et al., 2001) were used as a control. In most cases it was not possible to reconstitute robustly active heterodimeric catalytic complexes from mammalian monomers with the mycobacterial mutants. The low activities obtained were <<1% in comparison to that of the mycobacterial R376A/D300A chimera. Mutants D300S and N372T (generated to investigate the lack of forskolin stimulation of Rv1625c) in conjunction with mammalian monomers could not be stimulated by forskolin. Forskolin stimulation and cofactor dependence of chimera R376A/IIC2 were further investigated (not shown). Activity of this chimera was not detectable with Mg^{2+} as a cofactor and an insignificant stimulation with forskolin was observed.

Chimeras	µM in the assay (µg)		specific activity nmol · mg^{-1} · min^{-1} (cpm)		specific activity with forskolin nmol · mg^{-1} · min^{-1} (cpm)	
IC1 / IIC2[a]	1.5 / 2.5	(4 / 7)	0.1	(629)	0.5	(2051)
VC1 / IIC2[c]	3 / 2.5	(8 / 7)	0.1	(813)	0.5	(3500)
VIIC1 / IIC2[c]	5 / 2.5	(15 / 7)	2.5	(12672)	2.7	(26731)
IXC1 / IIC2[a]	3.7 / 3.7	(11 / 11)	0.04	(563)	0.03	(366)
IC1 / D300A(C2)[b]	5 / 1.6	(13 / 4)	0.2	(1116)	not done	
VC1 / D300A(C2)[b]	5 / 1.6	(14 / 4)	0.1	(526)	not done	
VIIC1 / D300A(C2)[b]	5 / 1.6	(15 / 4)	0.1	(596)	not done	
IXC1 / D300A(C2)[b]	3.8 / 1.6	(11 / 4)	0.8	(643)	not done	
IC1 / D300S (C2)[b]	3 / 1.6	(9 / 4)	not detectable		not detectable	
VC1 / D300S (C2)[b]	3 / 1.6	(9 / 4)	not detectable		not detectable	
R376A(C1) / IIC2[a]	0.4 / 5	(1 / 14)	9.4	(14432)	not done	
N372A (C1) / IIC2[b]	0.16 / 2.5	(0.4 / 7)	1.6	(711)	1.3	(538)
N372A (C1) / IXC2[b]	0.16 / 1.9	(0.4 / 7)	2	(713)	1.6	(604)
N372T (C1) / IIC2[b]	0.16 / 2.5	(0.4 / 7)	0.6	(311)	0.9	(423)
N372T (C1) / IXC2[b]	0.16 / 1.9	(0.4 / 7)	0.5	(237)	1.2	(390)
R376A / D300A[a] (C1) / (C2)	0.4 / 1.6	(1 / 4)	351	(498939)	not done	

Basal activities of R376A and N376A were 6.3 and 0.4 nmol·mg^{-1}·min^{-1}, respectively. Basal activities for IC1, VC1, IXC1, VIIC1 were 27, 20, 17 and 10 pmol/mg/min, respectively, and for the other monomers zero. DMSO (used for forskolin dilution) controls were not made.

Table 4.2: Reconstitution of mammalian/mammalian and mammalian/mycobacterial heterodimers. The functional analogies of the mutant mycobacterial catalytic loops to the C_1 and C_2 catalytic domains of mammalian AC's are in parentheses. The reconstituted activity of the mycobacterial chimera R376A/D300A was used as control for the mycobacterial monomers. Assay conditions: 75 µM ATP, 30°C, 10 min, 2 mM MnCl$_2$, forskolin 120 µM. Data are from single representative experiments (n=2[a] reproducible; n=1[b] and n=2[c] bad reproducible, experiments). The detection limit of the assays (double background) was 10 pmol·mg^{-1}·min^{-1}.

4.1.4 *Paramecium*/*Mycobacterium* chimeras

Reconstitution of AC activity by chimeras between *Paramecium* GC C1 monomer and mammalian C1- and C2-monomers (IC1, IIC2, VC1, VC2, VIIC1, VIIC2, IXC2) failed, all chimeras were inactive (Hoffmann, 1999). Therefore, in this work only ParaGC-C2 and C2-KWQ were used for reconstitution with IIC2 and this was used as a control for the reconstitution with D300A, N372T, N372A and D300S. The reconstitution of IIC2 with the mutant ParaGC-C2-KWQ was in this study 3-fold more active in comparison with ParaGC-C2/IIC2 (Table 4.3), as reported by Hoffmann, 1999. The chimeras ParaGC-C2/D300A and ParaGC-C2-KWQ/D300A had comparable activity as ParaGC-C2/IIC2. Since in the mixtures of *Paramecium* monomers with N372A and N372T only C1 domains were mixed, no significant reconstitution was observed, as expected.

Chimera	µM in the assay (µg)		specific activity nmol·mg^{-1}·min^{-1} (cpm)	
ParaGC-C2 / IIC2[b]	5.1 / 3.7	(15 / 11)	50.3	(77741)
ParaGC-C2KWQ / IIC2[b]	9.2 / 3.7	(27 / 11)	148.3	(221496)
C1 / C2				
ParaGC-C2 / D300A[a]	5.1 / 1.6	(15 / 4.4)	57.5	(40617)
ParaGC-C2KWQ / D300A[a]	9.2 / 1.6	(27 / 4.4)	54.9	(34137)
C1 / C2				
ParaGC-C2 / D300S[b]	3.4 /1.6	(10 / 4)	2.2	(7739)
ParaGC-C2KWQ / D300S[b]	3.4 /1.6	(10 / 4)	2.6	(9777)
C1 / C2				
ParaGC-C2 / N372T[b]	3.4 / 0.16	(10 / 0.4)	0.4	(210)
ParaGC-C2KWQ / N372T[b]	3.4 / 0.16	(10 / 0.4)	0.6	(290)
C1 / C1				
ParaGC-C2 / N372A[b]	3.4 / 0.16	(10 / 0.4)	0.6	(274)
ParaGC-C2KWQ / N372A[b]	3.4 / 0.16	(10 / 0.4)	0.7	(306)
C1 / C1				
Basal activities of ParaGC-C2, ParaGC-C2-KWQ and N372A were 0.02, 0.03 and 0.4 nmol/mg/min, respectively. Basal activities of the other monomers were zero.				

Table 4.3: Reconstitution of C1-C2-like catalytic sites between IIC2, D300A and *Paramecium* GC-C2 and C2-KWQ. Assay conditions: 75 µM ATP, 30°C, 10 min, 2 mM MnCl$_2$. Data from a representative experiment are shown (n=2[a]; n=1[b]). Detection limit (double background) was 16 pmol·mg^{-1}·min^{-1}.

4.1.4.1 Characterization of the chimera ParaGC-C2/D300A

This chimera was 200-fold more active with Mn^{2+} compared to Mg^{2+} (Table 4.4).

Protein(s)	µM in the assay (µg)		specific activity with 2 mM Mn^{2+} nmol·mg^{-1}·min^{-1} (cpm)		specific activity with 5 mM Mg^{2+} nmol·mg^{-1}·min^{-1} (cpm)	
ParaGC-C2	3	(8.5)	0.01	(200)	0	(51)
D300A	3	(8.5)	0.01	(170)	0	(52)
ParaGC-C2 / D300A	3/3	(8.5/8.5)	6.77	(70000)	0.03	(279)

Table 4.4: Cofactor dependence of the AC activity of the chimera ParaGC-C2/D300A. Test conditions: 75 µM ATP, 30°C, 10 min, Tris-HCl pH 7.5, 8.5 µg protein. Detection limit: 12 pmol·mg^{-1}·min^{-1}. Data from n=1 experiment are shown.

ParaGC-C2 was titrated with increasing amounts of D300A to determine the K_d value (Fig. 4.5). Within the experimental conditions no saturation was observed, i.e. the K_d probably is rather high (>100 µM).
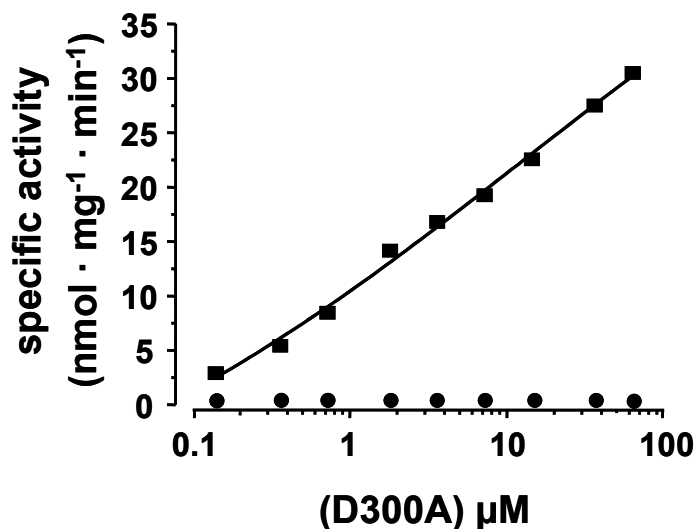
Fig. 4.5: Titration of 155 nM ParaGC-C2 with D300A (0.144-63 µM). Assay was with 75 µM ATP, 10 min, 30°C, 2 mM MnCl$_2$. Circles show D300A alone.

The kinetic characterization was carried out with 40 nM ParaGC-C2 and 9 µM D300A. Basal activities were zero for both monomers. By increasing ATP (10 µM to 1 mM) the

chimera showed a substrate dependence according to the Michaelis-Menten model. Lineweaver-Burk treatment yielded a K_M of 201 µM and a V_{max} of 29.5 nmol·mg^{-1}·min^{-1} (Fig. 4.6).
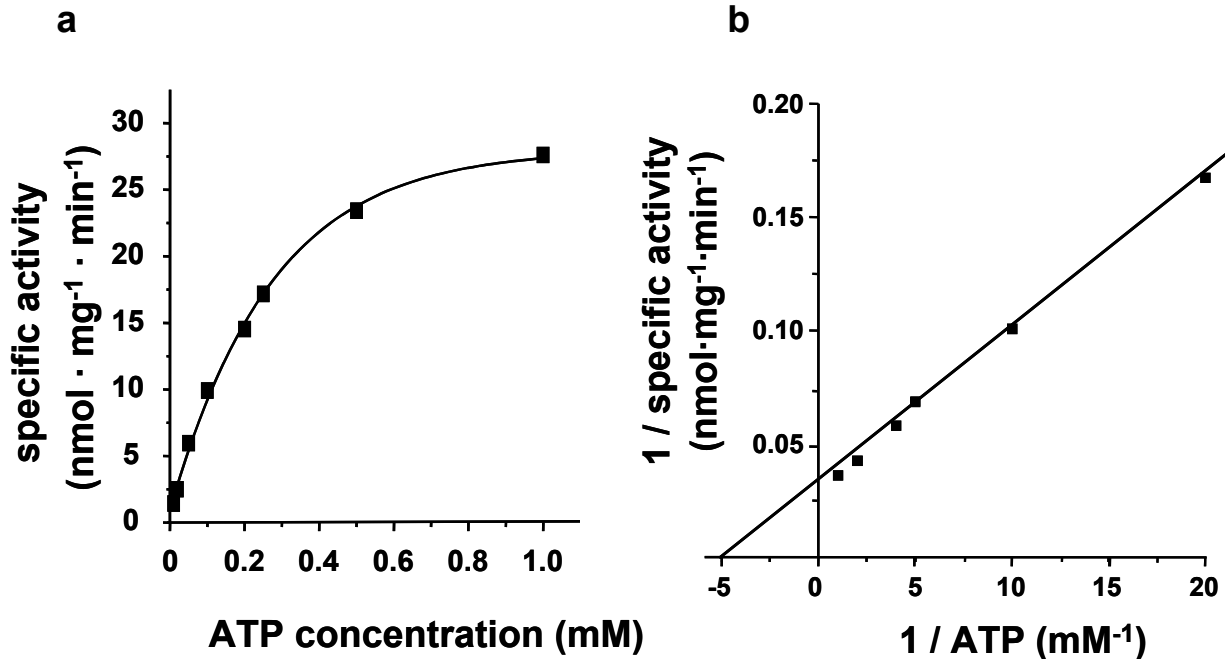
Fig. 4.6: Substrate dependence of the chimera ParaGC-C2/D300A (30°C, 10 min, Tris-HCl pH 7.5, 2 mM MnCl$_2$). (**a**) Michaelis-Menten graphic; (**b**) Lineweaver-Burk graph (values of the two smallest ATP concentrations were not drawn for graph clarity but used for calculation of K_M and V_{max}).

The effects of mammalian AC activators/inhibitors on the activity of ParaGC-C2/D300A were similar to those observed for *Paramecium* GC and Rv1625c separately (Table 4.5; Linder et al., 2000; Guo et al., 2001).

Substance	specific activity (nmol·mg^{-1}·min^{-1})	residual activity (%)
Chimera alone	**10.08**	**100%**
Forskolin (0.1 mM)	9.41	93.3
2'd, 3'-AMP (1 mM)	8.28	82.1
2' d, 3'-GMP (1mM)	7.96	78.9
GTPγs (1mM)	3.02	30

Table 4.5: Influence of forskolin and P-site inhibitors on the chimera 860 nM ParaGC-C2/ 9 µM D300A. Assay conditions were 75 µM ATP, 30°C, 10 min, Tris-HCl pH 7.5.

4.1.5 Mycobacterium/Mycobacterium chimeras

Essentially inactive C1 and C2-like mutant monomers could reconstitute each other with Mn^{2+} as a cofactor (Table 4.6). Mg^{2+}-mediated catalysis either was rather poor (0.3 nmol cAMP·mg^{-1}·min^{-1} for N372T[0.2µM]/D300S[4µM] and 2.5 nmol·mg^{-1}·min^{-1} for R376A[0.2µM]/D300S[4µM]) or was not detectable for other chimeras.

Protein concentration		Specific activity
40 nM	40 nM	(nmol cAMP · mg^{-1} · min^{-1})
R376A (C1)		1.1
	+ D300A (C2)	24.8
	+ D300S (C2)	31.7
N372A (C1)		2.5
	+ D300A (C2)	14.9
	+ D300S (C2)	4.2
N372T (C1)		0
	+ D300A (C2)	5.3
	+ D300S (C2)	1.6

Table 4.6: AC reconstitution in pairs from point-mutated Rv1625c monomers. The functional analogies to the C1 and C2 domains are in parentheses. Assay conditions: 75 µM ATP, 30°C, 10 min, Tris-HCl pH 7.5, 2 mM MnCl$_2$. Basal activities of monomers D300A and D300S were zero.

Forskolin stimulation assays with the catalytic domain wild type and the chimera N372T/D300S were carried out. Since forskolin was diluted in DMSO, controls with addition of DMSO alone and DMSO/Forskolin were tested (Table 4.7). It was concluded that the stimulation observed with DMSO/Forskolin was due to DMSO.

Protein (s)	µM in the assay (µg protein)	Specific activity (nmol·mg^{-1}·min^{-1})		
		alone	With 1 µl DMSO	With 1 µl 50 µM Forskolin (0.5 µM in the assay)
Rv1625c$_{204-443}$	0.144 (0.4)	16.6	22.4	22.9
N372T/D300S	1.6/1.6 (4.4/4.4)	3.7	4.1	4.0

Table 4.7: DMSO stimulation in forskolin assays of Rv1625c$_{204-443}$ and chimera N372T/D300S. Assay conditions: 75 µM ATP, 10 min, 30°C, 2 mM MnCl$_2$, Tris-HCl pH 7.5.

4.1.5.1 Titration of N372A, N372T and R376A with D300A or D300S

For each chimera curves with 85 and 850 µM ATP were established to determine if the association of the monomers might be substrate-dependent. Mutants N372A, N372T and R376A (an inactive protein concentration was used) were titrated with increasing amounts of D300A and D300S (Fig. 4.7 to 4.12). The apparent Kd values were derived graphically. The high specific activities obtained were due to an unusual high radioactive substrate used (from ICN Biochemicals).

Fig. 4.7: Reconstitution of AC activity of 45 nM **N372A** with increasing amounts of **D300A**. Assay conditions were 30 °C, Tris-HCl pH 7.5, 2 mM $MnCl_2$ and (**a**) 85 µM ATP for 10 min; or (**b**) 850 µM ATP for 4 min. (**a**) Kd= 7 µM; (**b**) Kd= 6 µM.
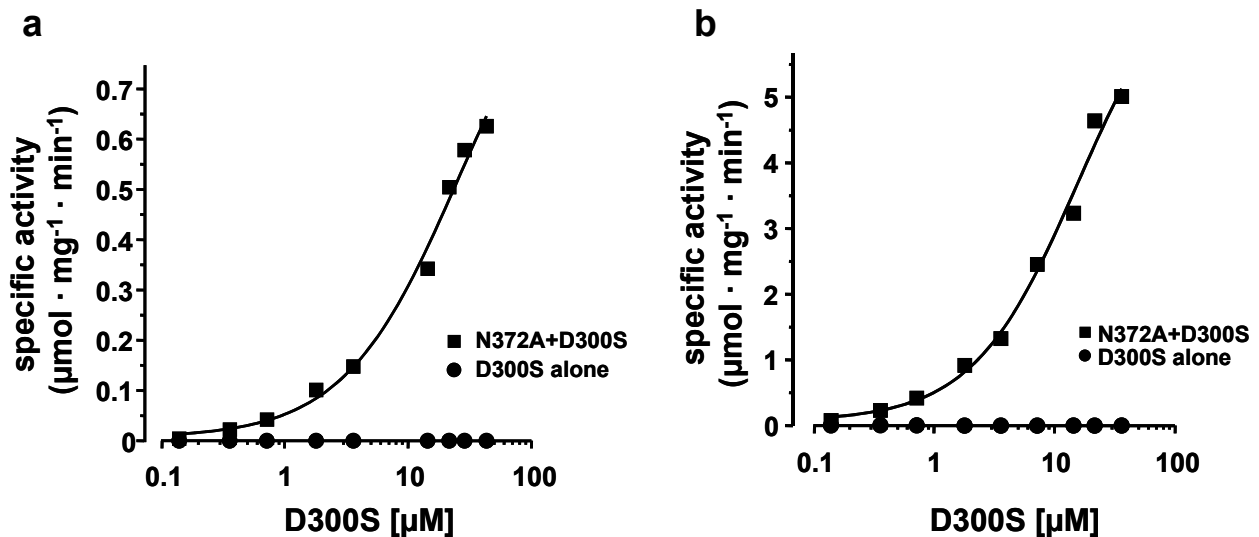
Fig. 4.8: Reconstitution of AC activity of 45 nM **N372A** with increasing amounts of **D300S**. Assay conditions were 30 °C, Tris-HCl pH 7.5, 2 mM $MnCl_2$ and (**a**) 85 µM ATP for 10 min; or (**b**) 850 µM ATP for 4 min. (**a**) Kd= 14 µM; (**b**) Kd= 8 µM.
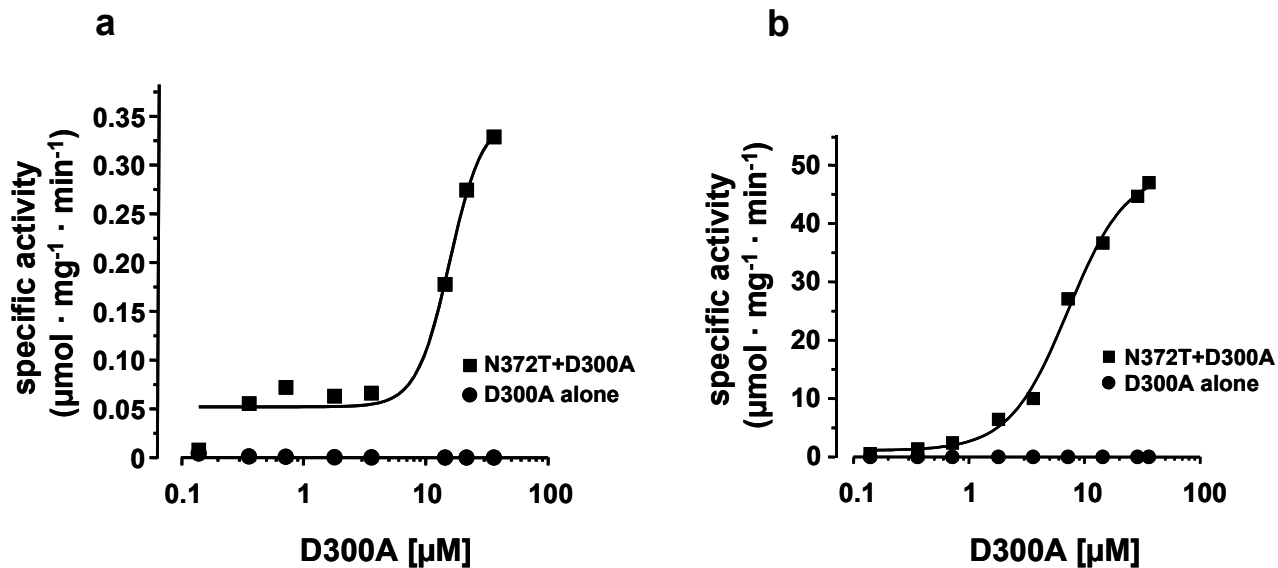
Fig. 4.9: Reconstitution of AC activity of 45 nM **N372T** with increasing amounts of **D300A**. Assay conditions were 30 °C, Tris-HCl pH 7.5, 2 mM $MnCl_2$ and (**a**) 85 µM ATP for 10 min; or (**b**) 850 µM ATP for 4 min. (**a**) Kd= 12-14 µM; (**b**) Kd= 6-7 µM.
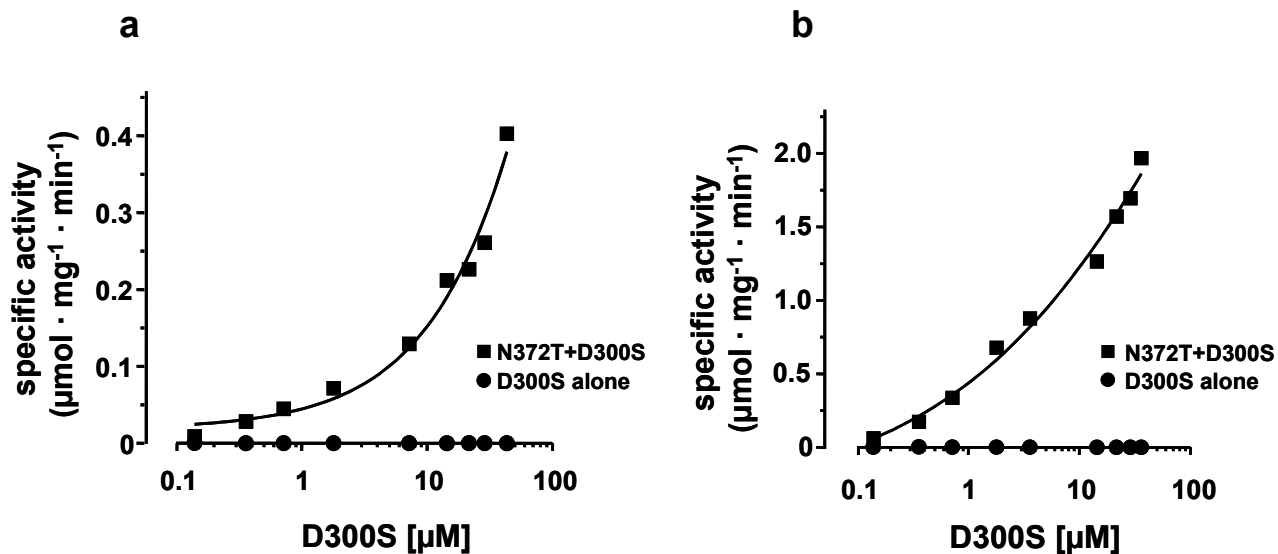
Fig. 4.10: Reconstitution of AC activity of 45 nM **N372T** with increasing amounts of **D300S**. Assay conditions were 30 °C, Tris-HCl pH 7.5, 2 mM $MnCl_2$ and (**a**) 85 µM ATP for 10 min; or (**b**) 850 µM ATP for 4 min. (**a**) and (**b**) Kd= not determinable.

a

b
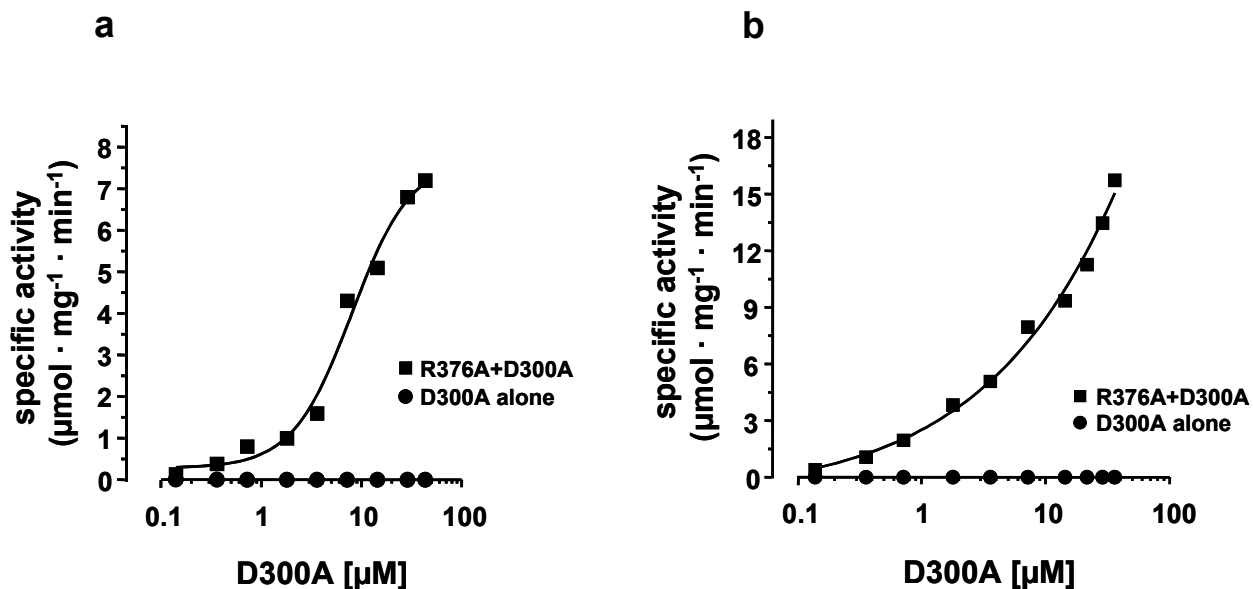
Fig. 4.11: Reconstitution of AC activity of 45 nM **R376A** with increasing amounts of **D300A**. Assay conditions were 30 °C, Tris-HCl pH 7.5, 2 mM $MnCl_2$ and (**a**) 85 μM ATP for 10 min; or (**b**) 850 μM ATP for 4 min. (**a**) Kd= 6-8 μM; (**b**) Kd not determinable.
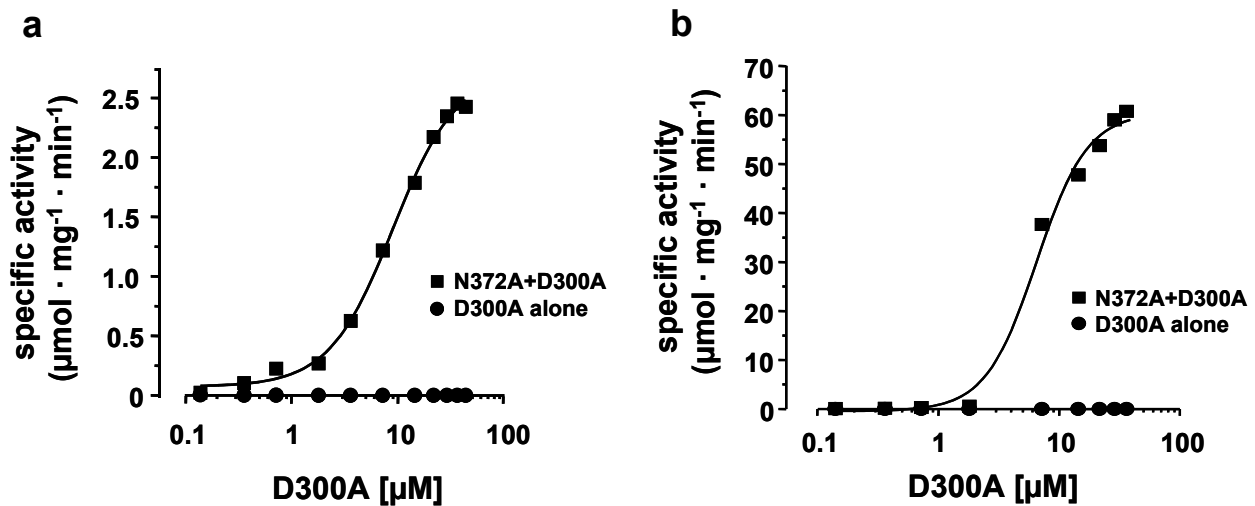
a

b
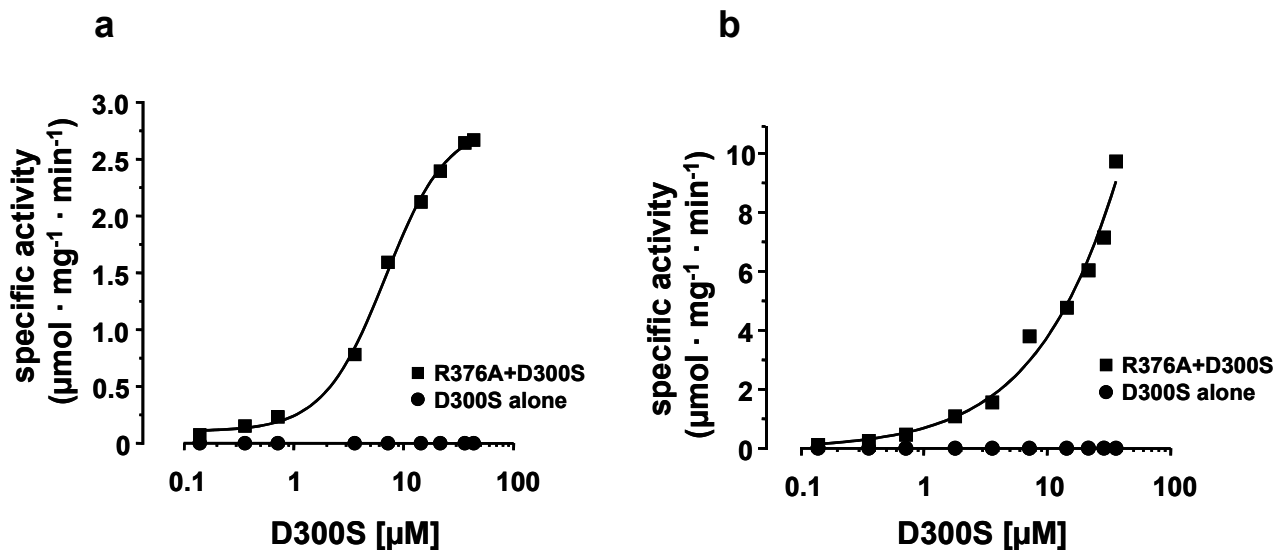
Fig. 4.12: Reconstitution of AC activity of 45 nM **R376A** with increasing amounts of **D300S**. Assay conditions were 30 °C, Tris-HCl pH 7.5, 2 mM $MnCl_2$ and (**a**) 85 μM ATP for 10 min; or (**b**) 850 μM ATP for 4 min. (**a**) Kd= 6 μM; (**b**) Kd not determinable.

The high activities reached for all chimeras could be explained by the formation of large amounts of productive heterodimers by the excess of D300A or D300S over N372A, N372T and R376A. Apparently, the association of the monomers of chimeras N372A/D300A, N372T/D300A and N372A/D300S was improved when using high ATP

concentration (850 µM) since their respective apparent K_d values decrease with respect of titration using 85 µM ATP. On the contrary, a K_d for chimeras R376A/D300A and R376A/D300S could not be determined by testing with 850 µM but with 85 µM ATP (possibly because of presence of limiting substrate concentrations). Affinity of the monomers of chimera N372T/D300S showed to be very low since no K_d value was determinable. These reconstitution assays with the new mutants are further evidence for the catalysis of the mycobacterial Rv1625c adenylyl cyclase through dimerization. The K_d values obtained for all chimeras is higher than the K_d of the wild type monomers (Guo et al., 2001), presumably indicating conformational changes due to mutations that had affected the dimerization process.

Expression and characterization of the adenylyl cyclase Rv0386 of *Mycobacterium tuberculosis*

Sequence features of Rv0386

At the Sanger Institute Website, the *M. tuberculosis* gene product Rv0386 is annotated as a probable transcriptional regulator (LuxR/UhpA family) with some similarity to AFSR_STRCO P25941 regulatory protein afsr and many putative *M. tuberculosis* regulatory proteins. It has been reported to contain an ATP/GTP-binding motif A (P-loop) and a probable helix-turn-helix motif. AC Rv0386 was analyzed by several programs to collect information about sequence features and similarities to other proteins of *M. tuberculosis* and other organisms. Rv0386 was analyzed as entity as well as in separated domains (AC, ATPase-, DNA-binding and transcription factor) with the program Protein-Protein-BLAST (NCBI; Nov-26-2003; see 8.2 in appendix). Most similarities were found with *M. tuberculosis* hypothetical proteins Rv2488c and Rv0890c and with transcriptional regulators of Bradyrhizobium, Mesorhizobium and Streptomyces sp.. Analysis with the program DNA-STAR did not detect neither significant hydrophobic nor transmembrane segments in the sequence of Rv0386. Also with this program protein sequence alignments of Rv0386 with other ACs (e.g. Rv1625c, AC type II from rat), GCs (e.g. *Paramecium* GC), proteins containing the ATPase P-loop motif (e.g. AfsR and L6tr) and DNA-binding proteins (e.g. *B. pertussis* BVGA, *S. coelicolor* SCO3008 and *M. tuberculosis* Rv0890c) were made (see 8.3 in appendix). Additionally, an analysis

with the program PESTfind (Rechsteiner and Rogers, 1996) did not detect potential proteolytic signals in Rv0386.

4.2.2 Expression and characterization of the Rv0386 adenylyl cyclase catalytic domain

4.2.2.1 Expression and purification

The protein was expressed for 4 h with 60 µM IPTG with a protein yield of approximately 2.7 mg/600 ml culture after Ni^{2+}-NTA-agarose purification. The protein was dialyzed with glycerol dialysis buffer (20%) and stored at –20°C. Pellet, supernatant and purified AC domain were analyzed by SDS-PAGE (20 kDa including N-terminal His-tag) and tested (Fig. 4.13).
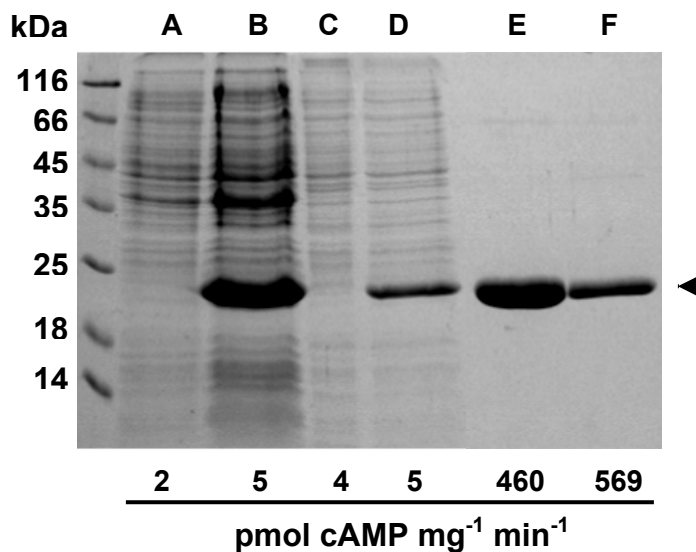
Fig. 4.13: 15% SDS-PAGE. Lanes A (control) and B (construct), pellets of pQE30 in BL21 cells. Lanes C and D, supernatants from A and B. Lane E, AC domain after Ni-NTA purification. Lane F, AC domain after dialysis. The specific activities indicated below each lane were determined for lanes A to E with 75 µM ATP (30°C, 2 mM $MnCl_2$, buffer Tris-HCl pH 7.5, 10 min assay) and 7, 26, 62, 5 and 9 µg protein, respectively. Specific activity in lane F was determined with 75 µM ATP (30°C, 5 mM $MnCl_2$, MOPS pH 7,5, 10 min assay) and 4 µg protein (1.9 µM). Same protein amounts tested were correspondingly applied at each lane.

4.2.2.2 Characterization of the AC activity

4.2.2.2.1 Optimal temperature

The temperature optimum was around 30-37°C (Fig. 4.14a). The activation energy of 76.1 kJ/mol was derived by an Arrhenius plot (Fig. 4.14b). This value is within the range reported for other mycobacterial ACs (Guo et al., 2001; Linder et al., 2002).

a

Temp (°C)	specific activity (nmol/mg/min)
0	0.030
22	0.451
25	0.547
30	0.721
37	0.720
45	0.498
56	0.122

b

E_A = -Rm
R   = 8.3145 J · K^{-1} · mol^{-1}
m = -9149.16 K
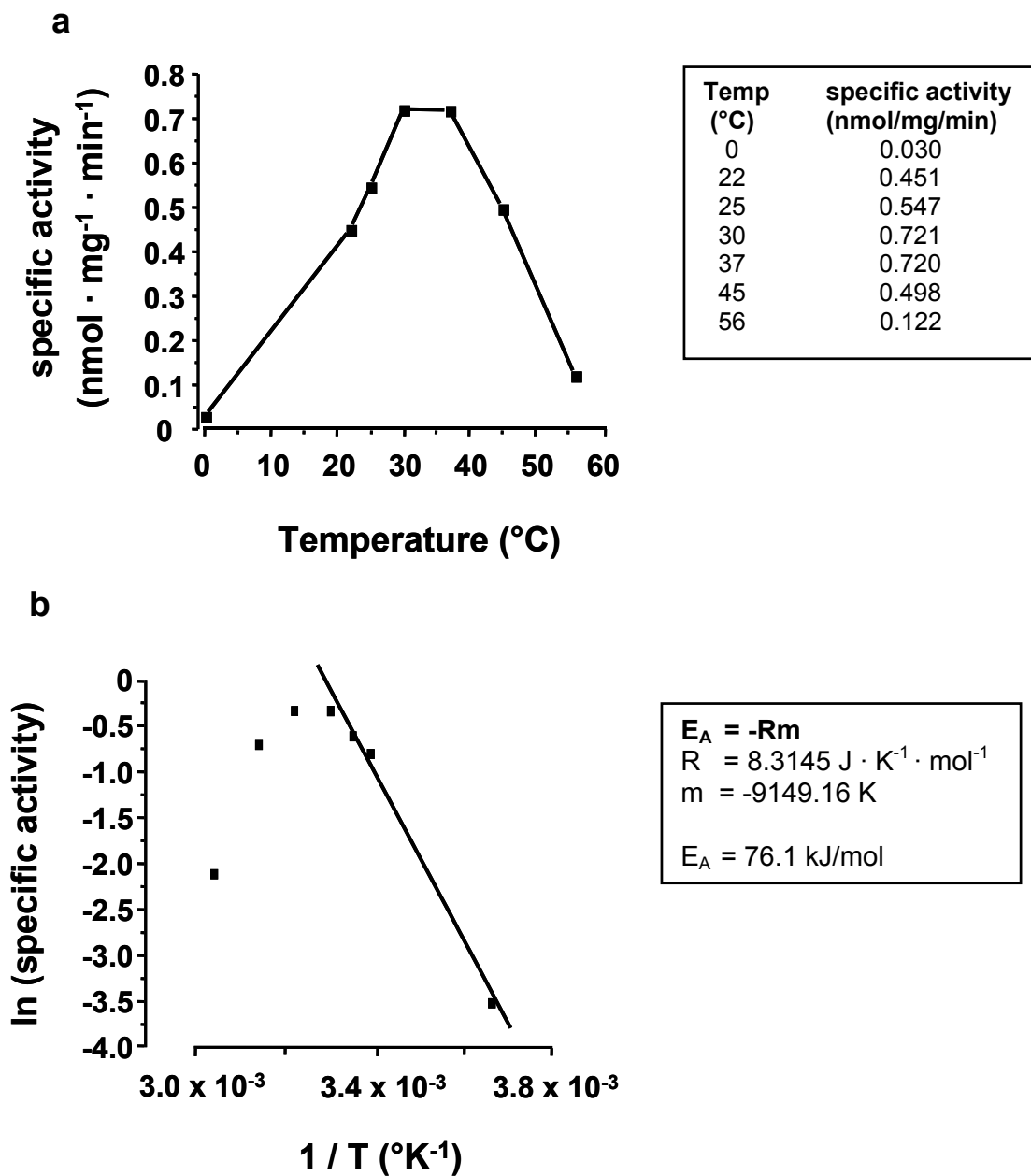
E_A = 76.1 kJ/mol

Fig. 4.14: a) Temperature dependence. Assay conditions: 75 µM ATP, 10 min, 11.6 µg protein (5.6 µM), MOPS pH 7.5. **b**) Arrhenius plot. An activation energy of 76.1 kJ/mol is

necessary for Rv0386 catalytic domain in order to convert ATP into cAMP. y = - 9149.16 x + 30.03

4.2.2.2.2 Mn^{2+}/Mg^{2+} dependence

ACs use predominantly Mn^{2+} as divalent cation although Mg^{2+} probably will be the physiologically more important divalent cation. In most class III ACs, Mg^{2+}-supported activity is rather low (Kasahara et al., 1997; Hurley, 1999; Guo et al., 2001; Linder et al., 2002). The *in vitro* activity of the Rv0386 catalytic domain is absolutely Mn^{2+} dependent (Fig. 4.15).
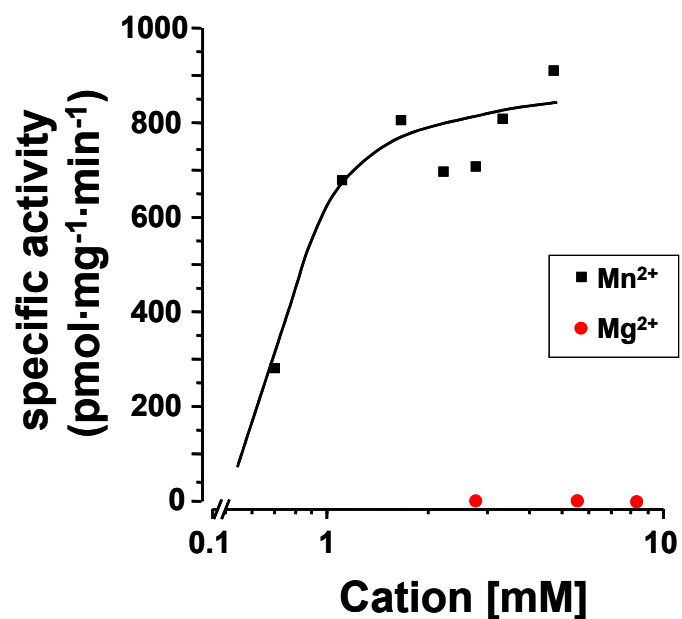
Fig. 4.15: Cofactor dependence of the AC activity. Mn^{2+} was tested from 0.7 mM to 4.7 mM and Mg^{2+} from 2.7 mM to 8.3 mM. Assay conditions: 75 µM ATP, 10 min, 2.3 µM protein.

4.2.2.2.3 pH dependence

The activity of the catalytic domain was strongly affected by the pH and dependent on the buffer system. A pH range from 3.1 to 9.9 was tested using 10 different buffer systems (Fig. 4.16) pH 8.3 was the best for the activity corresponding to Tris/HCl pH 8.5. Because at this value Mn^{2+} is oxidized, pH 7.4 corresponding to MOPS pH 7.5 was used in assays.
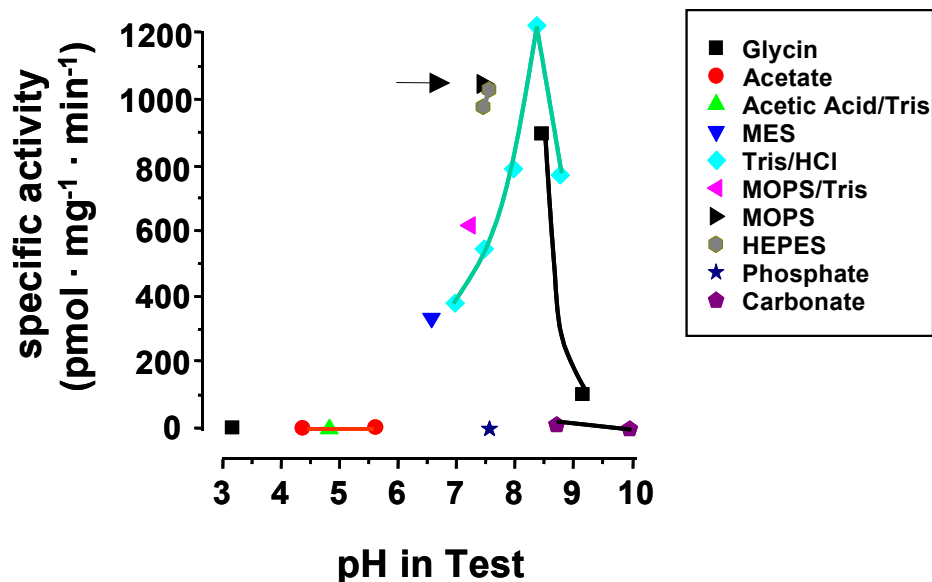
Fig. 4.16: pH dependence. Assay conditions: 75 µM ATP, 4.38 µM protein, 10 min.

4.2.2.2.4 Effect of imidazole

Elution buffer of Ni-NTA-agarose purification contains 150 mM imidazole. That corresponds to a maximal final concentration in the usual AC assay of 60 mM. On the other hand, imidazole was shown to inhibit Rv1625c (personal communication Dr. Guo). Therefore its effect on Rv0386 was examined (Fig. 4.17). Surprisingly, it was observed that at 60 mM AC activity is inhibited >>90%. Therefore the protein was always dialyzed after purification (glycerol dialysis buffer) to remove rest of the imidazole.
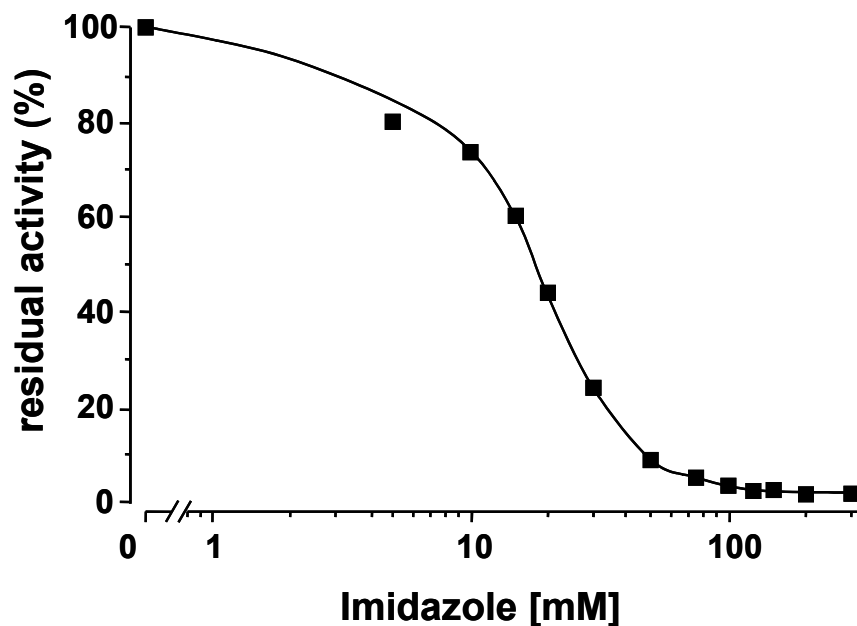
Fig. 4.17: Inhibition of the AC activity through imidazole (5-300 mM). Assay conditions were 75 µM ATP, 10 min, 30°C and 2.2 µM protein concentration.

4.2.2.2.5 Effect of detergents

A set of detergents used for purification of insoluble proteins was tested. A slight activation with Triton X-100, Polidocanol (dodecyl polyethylene glycolether) and Nonidet P-40 was observed. The addition of SDS (anionic detergent) ablated the activity completely. The detergent CHAPS (zwitterionic steroid detergent) reduced but did not completely abolished it (Table 4.8).

Detergent	% in test	AC-activity (%)
Triton X-100	1	124
Polidocanol	1	130
Nonidet P-40	1	144
CHAPS	1	33
	2	9
	3	6
	4	9
SDS	1	1

Table 4.8: Influence of detergents on the AC activity. Assay conditions: 2.2 µM (4.5 µg) protein, 75 µM ATP, 3 mM Mn^{2+}, 30°C, MOPS pH 7.5, 10 min.

4.2.2.2.6 Stability tests

The best storage temperature was –20°C (with 20% glycerol). The best buffer system that maintained protein stability was Tris/HCl pH 7.5, which retained 90% of activity after 1 month and 50% after 2.5 months. In Tris/HCl pH 8.5, the protein was fully active after 2 weeks. At 4°C and 25°C a residual activity of 85% and 51% was present after 2 weeks, respectively (data not shown). For crystallization purposes the stability of the protein with 10 % glycerol stored at –20°C was also tested. After one day full activity was retained in Tris/HCl pH 7.5, and after 2 weeks residual activity was 81%.

4.2.2.2.7 Protein dependence

From 46.5 to 465 nM protein specific activity increased 4.5-folds but further increments resulted in a slight decrement of activity (Fig. 4.18). These results suggested rapid formation of a homodimer with further formation of less active oligomers upon increasing protein concentrations. Half maximal activity was attained at 140 nM protein, indicating a high affinity of the catalytic domains for each other.

µM	nmol·mg^{-1}·min^{-1}
0.046	1.9
0.093	3.5
0.116	2.5
0.233	4.8
0.465	8.7
1.86	5.8
4.64	6.6
6.96	5.2
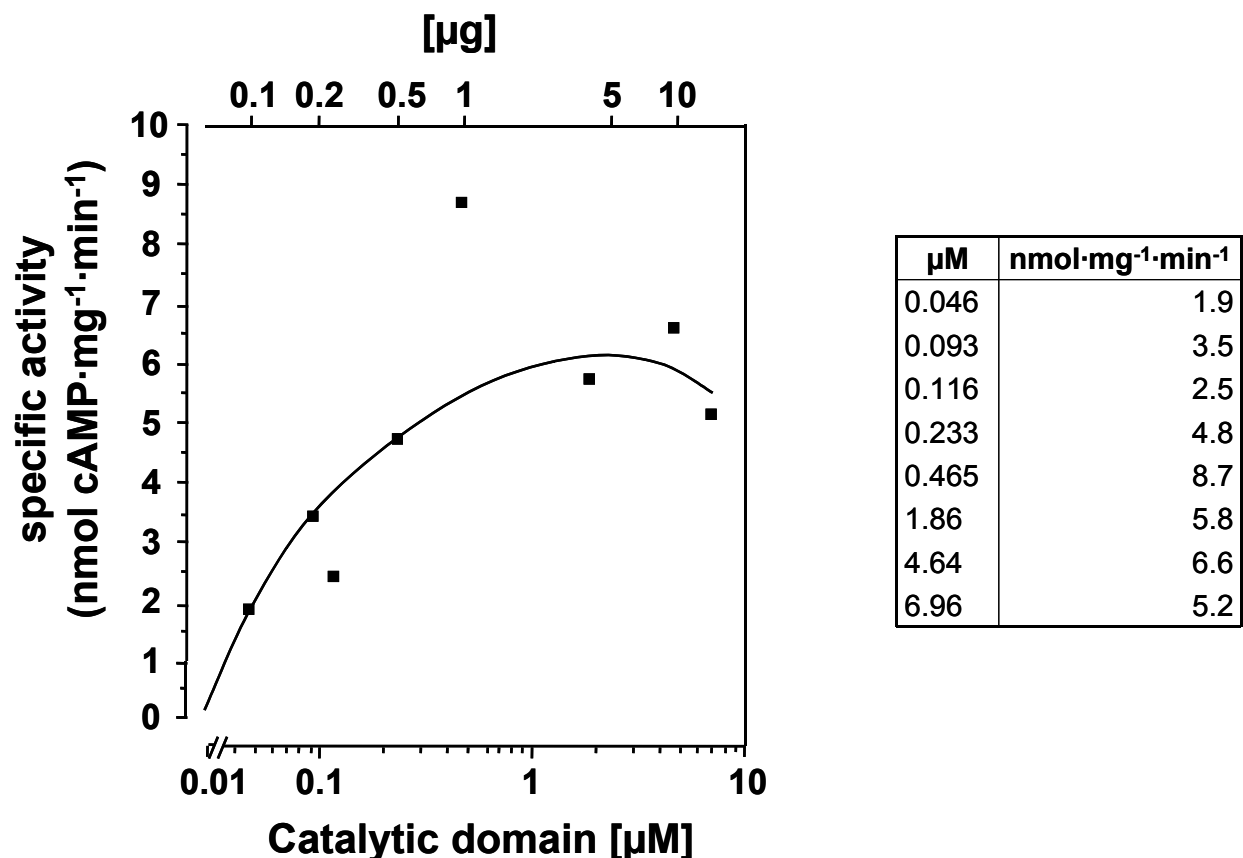
Fig. 4.18: Protein dependence of Rv0386 catalytic domain. Assay conditions were 850 µM ATP, 5 mM Mn^{2+}, MOPS buffer pH 7.5, 30 °C and 20 min. Protein concentrations tested were from 46.5 nM to 6.9 µM (96 ng to 14 µg).

4.2.2.2.8 Enzyme kinetics

The kinetic properties were analyzed using 5 mM Mn^{2+} and substrate concentrations from 10 µM to 2.3 mM. The K_M for Mn-ATP was 417 µM, the V_{max} was 14.4 pmol/min (4.8 nmol/mg/min). The K_M value obtained is within the range of 30-400 µM observed for purified membrane-bound and soluble ACs (Tang and Hurley, 1998). V_{max} is rather low compared with that of the catalytic domain of other ACs, e.g. Rv1625c (2.1 µmol·mg^{-1}·min^{-1}; Guo et al., 2001), Rv1264 (1.25 µmol·mg^{-1}·min^{-1}; Linder et al., 2002) and cyaB1 (309 nmol·mg^{-1}·min^{-1}; Kanacher et al., 2002). The Hill coefficient was 1.0 which indicated no cooperativity (Fig. 4.19a,b,c).

a
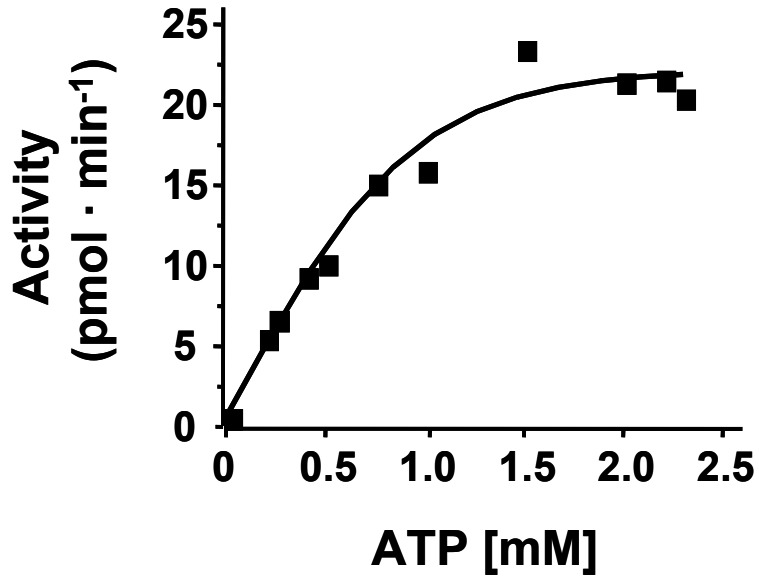
mM	pmol·min^{-1}
0.01	0.4
0.02	0.4
0.2	5.4
0.25	6.5
0.4	9.2
0.5	10
0.75	15
1	15.8
1.5	23.3
2	21.3
2.2	21.5
2.3	20.3

b
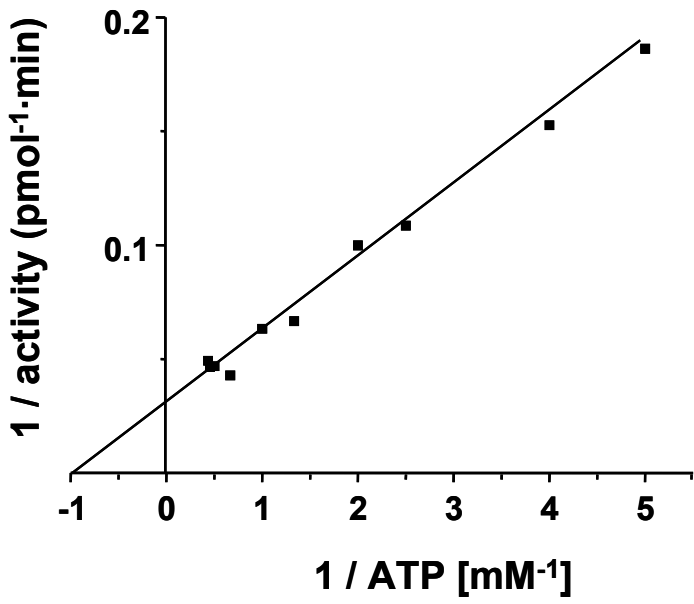
1/activity	1/ATP
100	2.63
50	2.22
5	0.19
4	0.15
2.5	0.11
2	0.1
1.3	0.06
1	0.06
0.66	0.04
0.5	0.05
0.45	0.05
0.43	0.05

c
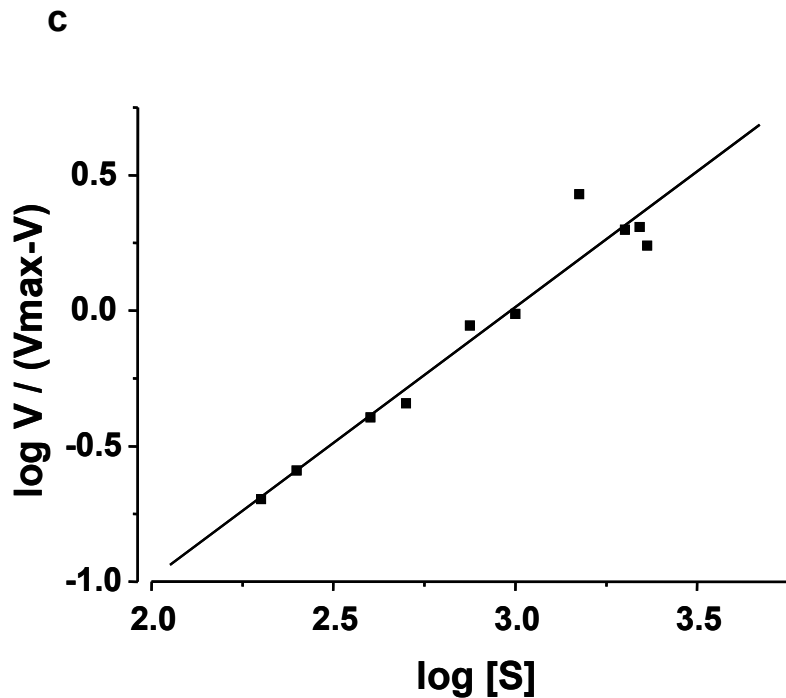
Fig. 4.19: **a)** Michaelis-Menten kinetic of the catalytic domain. Assays were conducted at 1.45 µM (3 µg) protein, MOPS pH 7.5, 30°C and 10 min; **b)** corresponding Lineweaver-Burk linear fit; **c)** corresponding Hill-Plot (R^2= 0.9835; y= 1.0148x - 3.0329). **b** and **c)** the two smallest values were not drawn for graph clarity but taken for K_M and Vmax calculation. V= activity (pmol·min^{-1}); [S]= ATP.

4.2.2.2.9 Influence of inhibitors, substrate analogs and other substances

Activity was tested in presence of forskolin, 2'-deoxy-3'-adenosine monophosphate (2'd3'-AMP) [P-site inhibitor], cyclic AMP (cAMP), guanosine 2'&3'-monophosphoric acid (2'3' GMP; mixed isomers), guanosine 2'-monophosphoric acid (2'-GMP); 8-bromoguanosine 3',5'-cyclic monophosphate (8-Br-cGMP); O^2-monobutyryl adenosine-3',5'-cyclic monophosphoric acid (monobutyryl cAMP); $N^6,O^{2'}$- dibutyryl adenosine 3',5'-cyclic monophosphoric acid (dibutyryl cAMP); 3'-deoxyadenosine 5'-triphosphate (Cordycepin 5'-triphosphate; ATP-analog); α,β-methylen adenosine diphosphate ($\alpha\beta$-CH_2-ADP; ADP-analog) and adenosine-5'-O-(1-thiotriphosphate), Rp-diastereomer (Rp-ATP-α-S, ATP-analog).

Substance tested [mM]	% activity of corresponding basal	Specific activity nmol·mg^{-1}·min^{-1} (control with no addition)
Forskolin [0.1]	114	6.3 (5.5)
2'd3' AMP [1]	102	5.6 (5.5)
2'3' GMP [1]	119	6.6 (5.5)
2' GMP [1]	129	7.1 (5.5)
8-Br-cGMP [1]	131	7.2 (5.5)
cAMP [1]	103	6.4 (6.2)
(Rp) ATP αS [1]	119	7.4 (6.2)
Monobutyryl cAMP [1]	29	1.8 (6.2)
Dibutyryl cAMP [1]	58	3.2 (5.5)
αβ CH$_2$ ADP [1]	13	0.8 (6.2)
Cordycepin 5' triphosphate [1]	23	1.4 (6.2)

Table 4.9: Effect of inhibitors, substrate analogs and other substances on AC activity. Assay conditions: 850 µM ATP, 30°C, 10 min, MOPS pH 7.5. Substances were tested in two separate assays.

Only αβ-CH$_2$-ADP, cordycepin and monobutyryl cAMP showed a significant inhibitory effect on Rv0386. Dibutyryl cAMP could be also considered as an inhibitor. Neither forskolin nor the P-site inhibitor did show considerable activation or inhibition, respectively.

4.2.2.2.10 Influence of purine and pyrimidine nucleotides

ITP and CTP had no significant effect (Fig. 4.20). The activation showed by addition of GTP and UTP may be explained as an allosteric effect. It is possible that GTP or UTP bound to one of the catalytic sites and activated the enzyme allosterically.
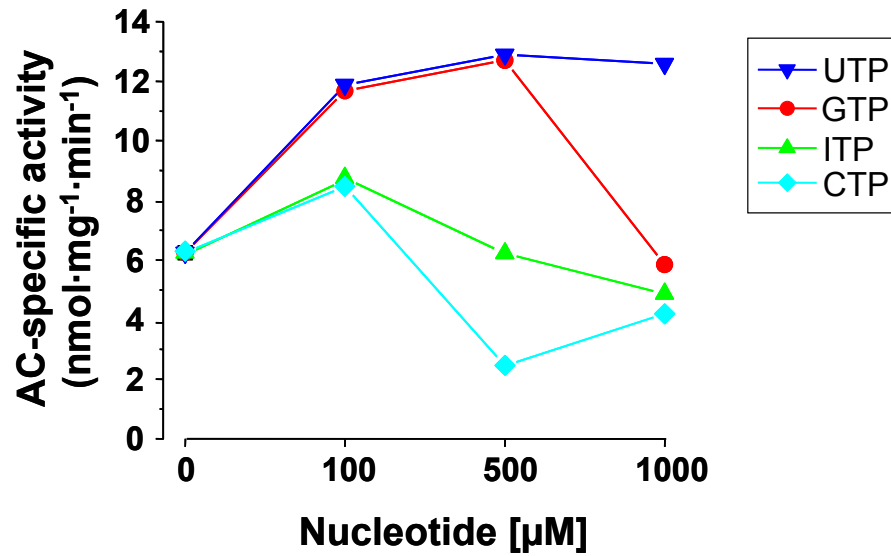
Fig. 4.20: Influence of nucleotides on the AC activity. Assay conditions were: 850 µM ATP, 30 °C, 5 mM MnCl$_2$, MOPS pH 7.5, 10 min. Protein concentration tested was 465 nM (0.96 µg) showing a specific activity of 6.2 nmol cAMP mg^{-1} min^{-1}.

4.2.2.3   Characterization of the GC activity

The catalytic domain was tested for GC activity at a GTP concentration of 75 µM in MOPS buffer pH 7.5 at 30°C, 5 mM Mn^{2+}. Simultaneously an AC assay was carried out for comparison. The catalytic domain has a GC activity of 30% of the AC activity. That resulted surprising since it is not reported about other ACs with any GC side activity. No GC activity was detectable with Mg^{2+} (Table 4.10).

Protein (µM)	AC activity pmol · mg^{-1} · min^{-1}		GC activity pmol · mg^{-1} · min^{-1}		% GC to AC activity
	5 mM Mn^{2+}	10 mM Mg^{2+}	5 mM Mn^{2+}	10 mM Mg^{2+}	
1.94	226	0	68	0	30
2.15	308	0	85	0	27.6
4.84	435	0	142	0	32.6

Table 4.10: Determination of the guanylyl cyclase side activity of Rv0386.

The process of dimerization ocurred slower than with ATP as a substrate (see Fig. 4.19 and 4.21 for comparison). The specific activity increased only 3-folds from 665 nM to 14.5 µM protein. An EC_{50} value of 0.88 µM was derived graphically.

µM	nmol·mg⁻¹·min⁻¹
0.66	0.3
1.33	0.5
2.37	0.6
3.83	0.7
7. 67	0.8
9.7	0.7
12.1	0.8
14.5	0.8
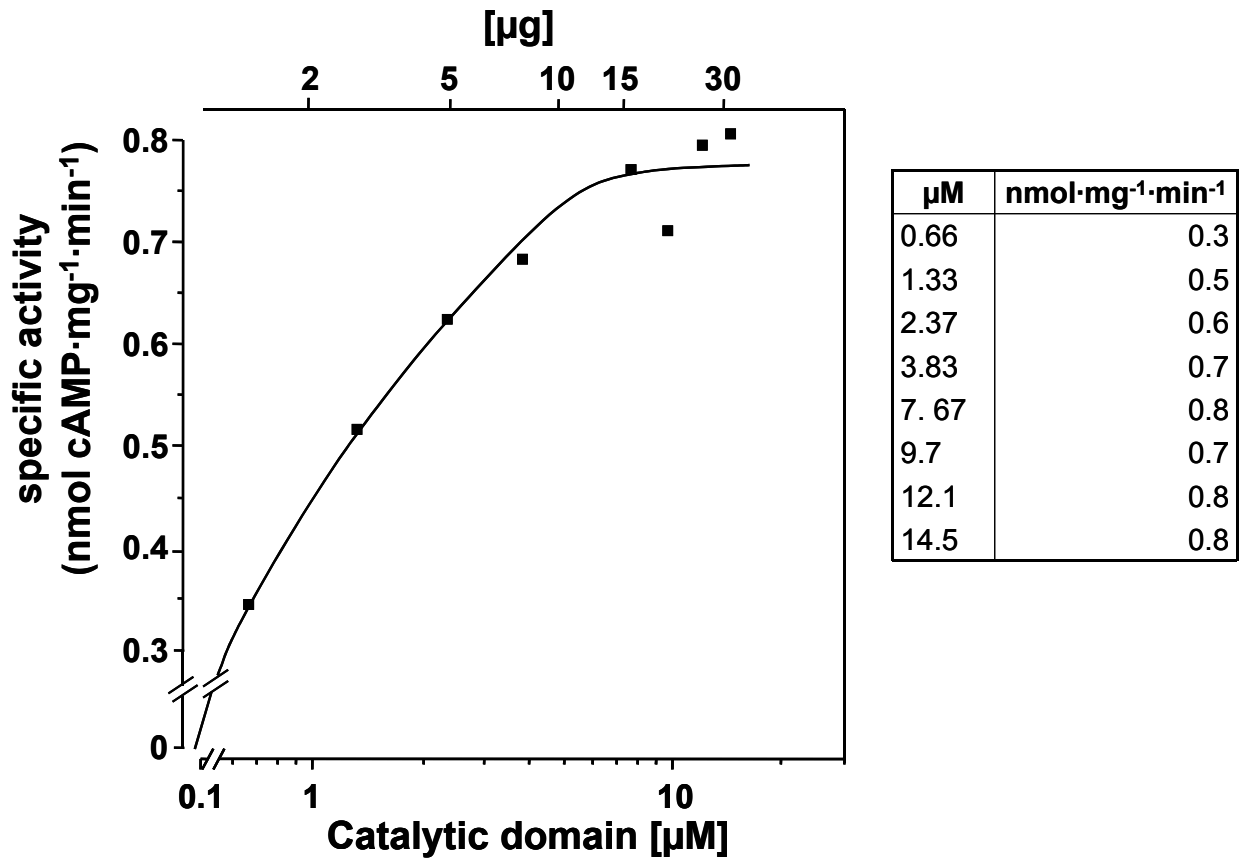
Fig. 4.21: Protein dependence with GTP as a substrate. Assay conditions: 850 µM GTP, 5 mM Mn^{2+}, MOPS pH 7.5, 30 °C and 20 min.

4.2.2.3.8 Enzyme kinetics

The substrate dependency was assayed with 5 mM Mn^{2+} as a cofactor (Fig. 4.22a). The K_m was 299 µM, the V_{max} was 16.1 pmol/min. The Hill coefficient of 0.8 indicates no cooperativity.

a
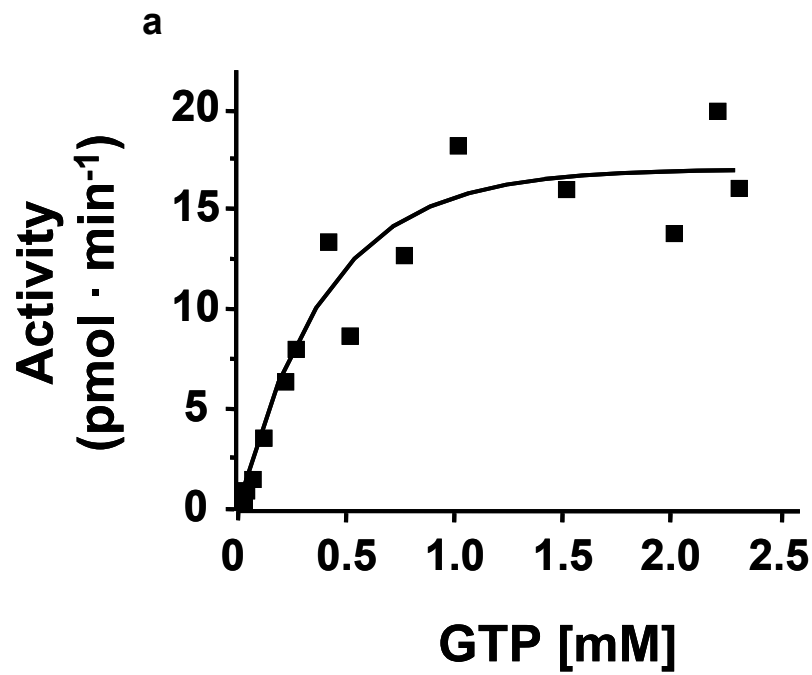
mM	pmol·min⁻¹
0.01	0.5
0.02	1.1
0.05	1.7
0.1	3.7
0.2	6.5
0.25	8.2
0.4	13.5
0.5	8.8
0.75	12.8
1	18.3
1.5	16.1
2	13.9
2.2	20
2.3	16.2

b
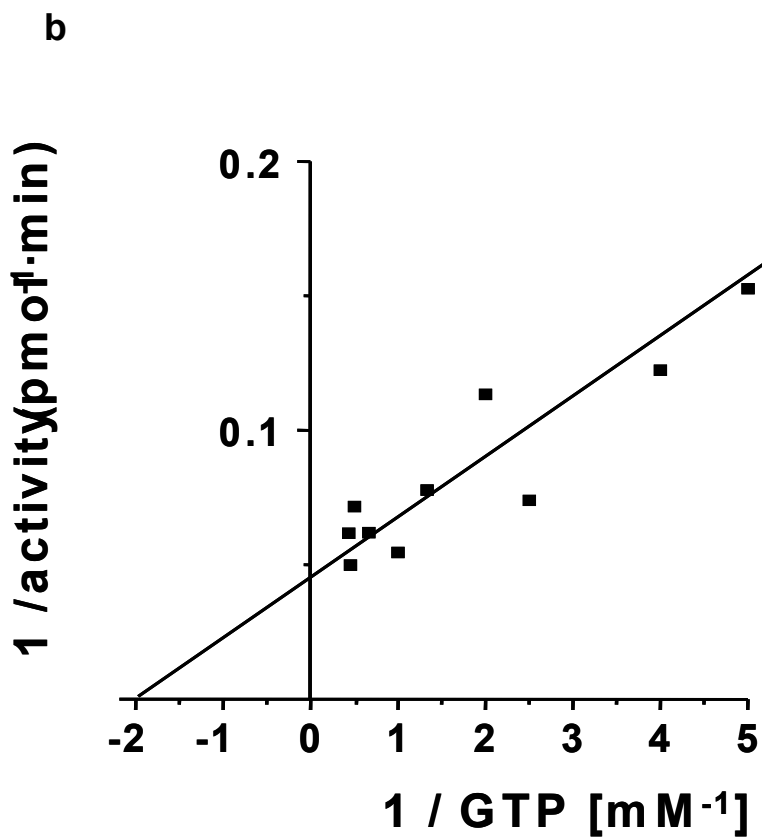
1/activity	1/ATP
100	1.9
50	0.9
20	0.6
10	0.3
5	0.15
4	0.12
2.5	0.07
2	0.11
1.3	0.08
1	0.05
0.66	0.06
0.5	0.07
0.45	0.05
0.43	0.06

78
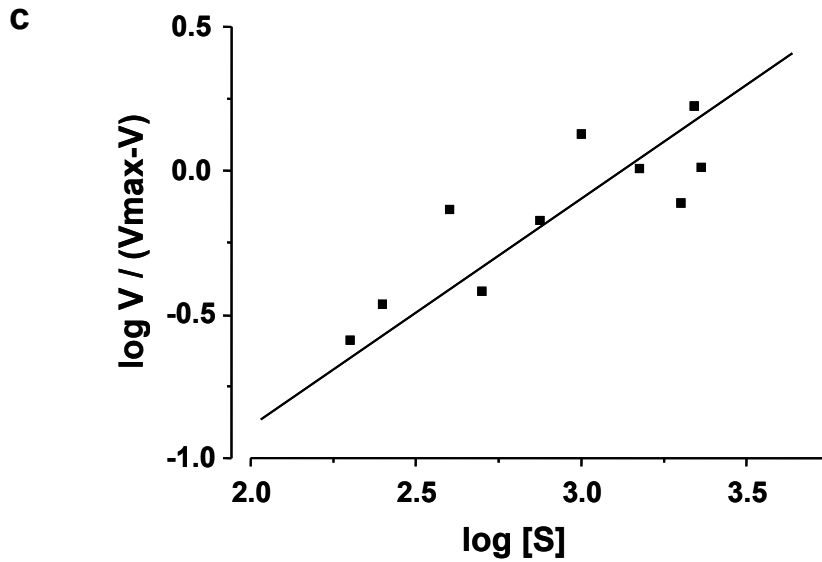
Fig. 4.22: **a**) Michaelis-Menten kinetic for GTP as a substrate. Assays were conducted at 4.85 μM of protein, MOPS pH 7.5, 30°C, 5 mM Mn^{2+} and 10 min; **b**) corresponding Lineweaver-Burk linear fit; **c**) corresponding Hill-Plot (R^2= 0.9456; y= 0.7953x - 2.4837). **b** and **c**) the four smallest values were not drawn for graph clarity but taken for calculation of K_M and V_{max}. V= activity (pmol·min^{-1}); [S]= GTP.

UTP did not affect GC activity but other nucleotides did, presumably because of allosterical interactions or because they were used as substrates, e.g. ATP inhibited the GC activity as expected, since the enzyme uses it as a substrate (Fig. 4.23).
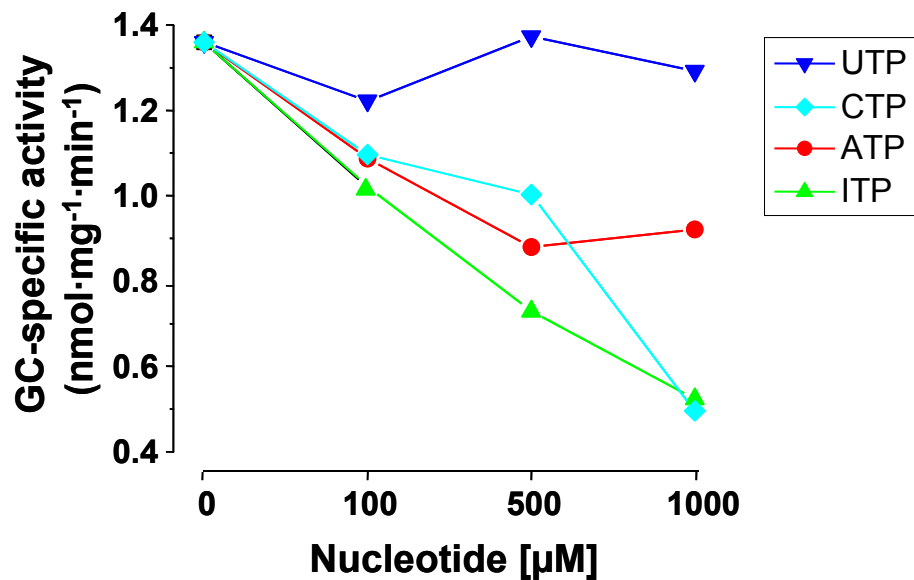
Fig. 4.23: Influence of nucleotides on the GC activity. Assay conditions: 850 μM GTP, 30 °C, 5 mM $MnCl_2$, MOPS pH 7.5, 20 min, 4.6 μM (9.6 μg) protein.

4.2.2.4 Sensitivity of the antibodies anti-KD0386

BioGenes GmbH Berlin produced antibodies against the catalytic domain (KD0386; see chapter 3). The immune reactivity of the antigen-affinity purified antibodies from two immunized rabbits was tested individually against different protein concentrations by Dot Blotting (Fig. 4.24). With an antibody dilution of 1:10 000 it was possible to detect 10 ng protein (rabbit 4555). Effect of addition of anti-KD0386 on the AC activity of the catalytic domain was tested (Table 4.11). Inhibition of the enzyme activity results obvious since antibody bound to the protein brings it to precipitation.
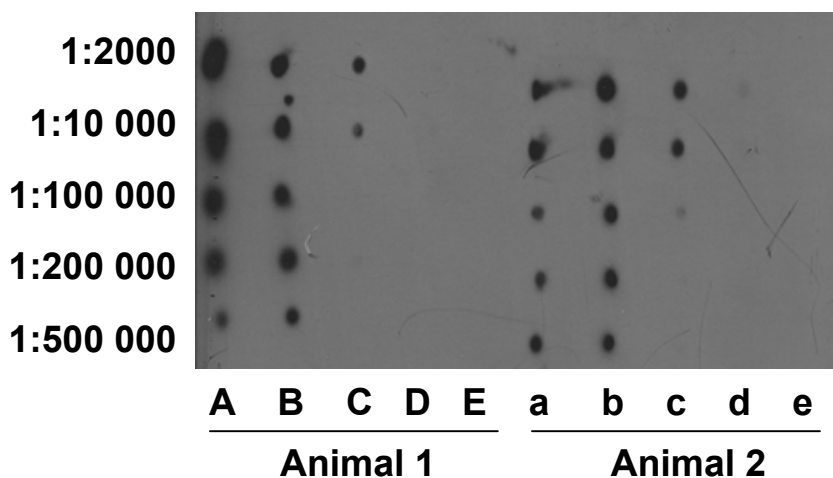
Fig. 4.24: Control of the KD0386 antibodies by Dot Blot. 5 different antibody dilutions (1:2000 to 1:500,000) as well as protein concentrations were used (rabbits 4554: animal 1; and 4555: animal 2). Protein concentrations were 1 µg (A), 100 ng (B), 10 ng (C), 1 ng (D) and 0.1 ng (E).

Anti-KD0386 [µg]	specific activity $(nmol \cdot mg^{-1} \cdot min^{-1})$	% residual activity
No addition	5.2	100
0.8	4.5	86
1.6	4.5	86
2.4	4.1	77
3.2	3.7	71
5	1	20

Table 4.11: Inhibition of the AC activity by addition of antibody anti-KD0386. Assay conditions: 500 µM ATP, 30°C, 20 min, 5 mM Mn^{2+}, MOPS pH 7.5, 8.5 µg (4.1 µM) protein.

4.2.2.5 Multimerization of the catalytic domain

On a Coomassie-stained SDS-PAGE the Ni^{2+}-NTA purified protein was visible as a single band (Fig. 4.13). Using 3 µg protein/lane it was tested whether multimerization could be detected in a Western blot (Fig. 4.25). The protein sample (3 µg, 1.45 µmol, purified, dialyzed, 20% glycerol, at –20°C for about 2 months) was blotted from a 15% SDS-PAGE gel following the standard Western Blot method described in chapter 3. AC and GC activities of the sample were 3.7 and 1.1 nmol/mg/min, respectively (tested with 500 µM ATP or GTP; 10 min; MOPS pH 7.5; 5 mM Mn^{2+}). The formation of oligomers presumed from the protein dependence curve (Fig. 4.21) was proved as well as the difference in sensitivity between anti-KD0386 and Coomassie blue.
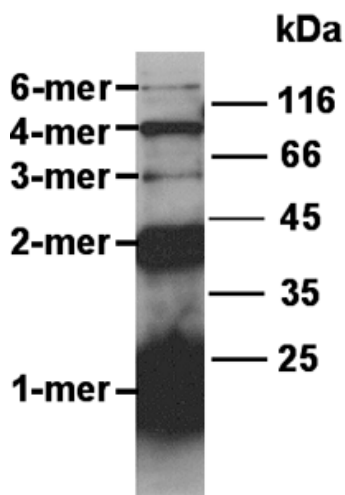
Fig. 4.25: Western blot analysis of the AC domain. Primary antibody diluted 1:200 000 and secondary antibody (Goat anti-rabbit antibodies conjugated with horseradish peroxidase) 1:5000. Exposure time was 15 s.

4.2.2.6 Determination of cross-reactivity between anti-KD0386 and other ACs

The 15 mycobacterial ACs (present work; Guo et al., 2001; Linder et al., 2002; Wetterer, Hammer, Motaal, Zeibig, Weber, Luban unpublished data) were analyzed with the specific antibodies anti-KD0386 for determination of possible cross-reactivity (see Table 4.12 and Fig. 4.26 a to d). At a dilution of 1:10000 of the antibodies all ACs with exception of Rv3645c, Rv1318c, Rv1319c and Rv1320 showed crossreactivity after a 15 s exposure. Based on these results, this antibody will not be a suitable tool for immunodetection studies of Rv0386.

Adenylyl cyclase	Construct	Sample type	Molecular weight (kDa)	Protein amount applied (µg)
Rv1625c	Holoenzyme	Purified	47	2
Rv2435c	Catalytic domain	Purified	26	4
Rv1264	Holoenzyme	Purified	43	5
Rv2212	Holoenzyme	Purified	40	3
Rv1900 (LipJ)	Holoenzyme	Purified	51	6
Rv0891	Holoenzyme	Cell homogenate	33	16
Rv1647	Holoenzyme	Cell homogenate	37	30
Rv1358	Catalytic domain	Cell homogenate	29	16
Rv1359	Holoenzyme	Cell homogenate	28	22
Rv2488	Catalytic domain	Cell homogenate	29	16
Rv3645c	Holoenzyme	Cell homogenate	60	19
Rv1318c	Holoenzyme	Cell homogenate	60	17
Rv1319c	Holoenzyme	Cell homogenate	60	20
Rv1320c	Holoenzyme	Cell homogenate	60	20

Table 4.12: Samples of the mycobacterial ACs used for a Western blot with antibodies anti-KD0386.
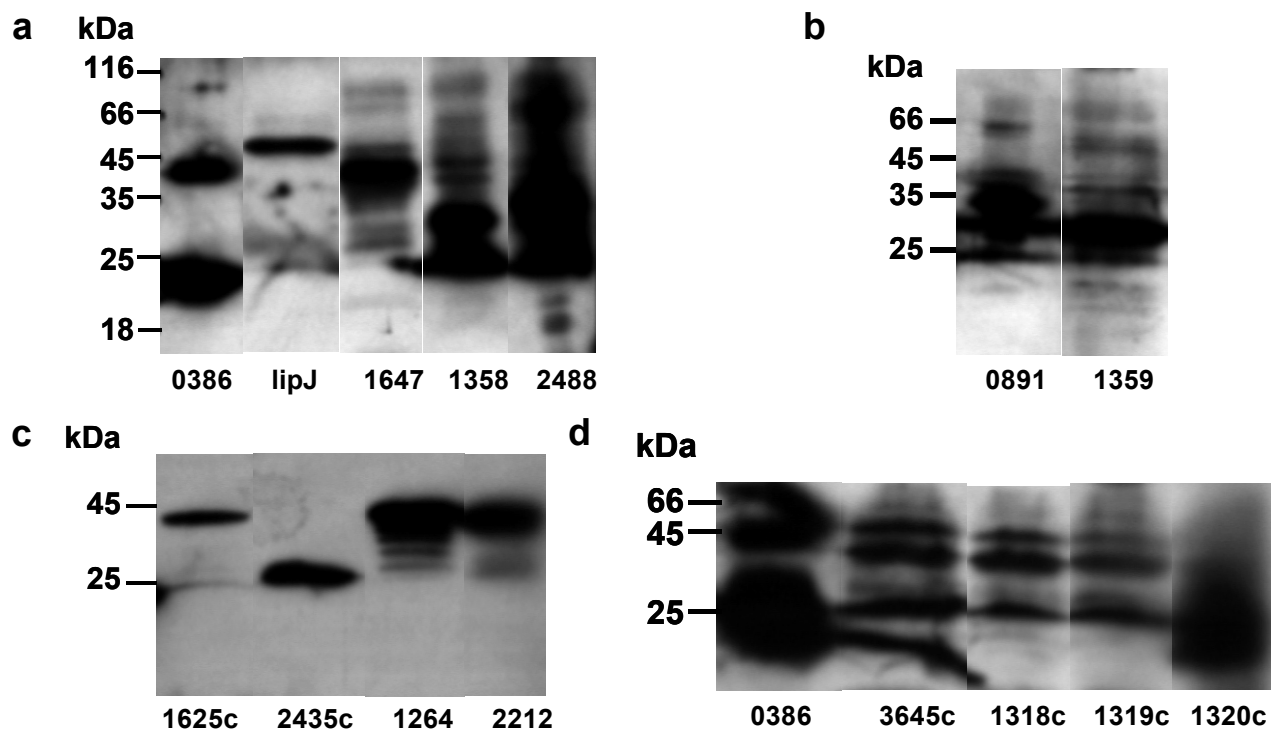
Fig. 4.26 a to d: Western blot of the 15 ACs of *M. tuberculosis* with antibodies anti-KD0386. According to the molecular weight of each protein (see table 4.21), only ACs of figure **d** did not show cross-reactivity with anti-KD0386.

4.2.2.7 Expression, purification and characterization of mutants Q57K, Q57A, N106D, N106A and Q57K/N106D

These mutants were generated to study the importance of residues Q_{57} and N_{106} for the AC activity of Rv0386 on the basis of the alignment with mammalian cyclases and with Rv1625c (Fig. 4.27).


```
                       Pu
                       ⇩
Rv1625c     259 :DELVDQ---HGLEKIKVSGDSYMVVSGVPRPRPD-----------HTQAL
AC2_C2_RAT  923 :DDLLSKPKFSGVEKIKTIGSTYMAATGLSAIPSQEHAQEPERQYMHIGTM
ParaGC_C1  1669 :D-LLCQQ--HEVQKIETVGKTYMAAAGLKIHVSQKSNP--------VNKV
Rv0386       46 :IAAHDG---V-RPVEQGEGDSFVLAF------------------------

                                                      Pu
                                                      ⇩
Rv1625c     295 :ADFALDMTNVAAQLKDPRGN-P-VPLRVGIATGPVVAGVVGSRRFFYDVW
AC2_C2_RAT  973 :VEFAYALVGKLDAINKHSFN-D-FKLRVGINHGPVIAGVIGAQKPQYDIW
ParaGC_C1  1708 :ISLALDMKRYVMSNE------T-FQIKIGIHYGNVIAGVIGHHKPQFSLI
Rv0386       68 :--ACASDAVAAALDLQRARL-APIRLRIGVHTGEVALRDEG------NYA
```

Fig. 4.27: Alignment of Rv0386 AC domain with the cytosolic domains of mammalian AC type 2, *Paramecium* GC and Rv1625c. Marked in boxes: purine-specifying residues.

All mutants were expressed in BL21 cells (60 µM IPTG, RT, 4 h). They were purified with Ni-NTA-agarose and dialyzed (20% glycerol; Fig. 4.28a,b,c). Mutants Q57K, N106A and Q57K/N106D were well purified. From 400 ml culture the recoveries of Q57K and N106A were 3 and 0.7 mg protein, respectively. From 600 ml culture the recovery of Q57K/N106D was 0.8 mg protein. Purification of mutants N106D and Q57A was incomplete. The presence of a 25 kDa protein after purification and dialysis could not be avoided. From 600 ml culture the recovery of N106D was only 80 µg protein. Recovery of mutant Q57A was 0.1 mg protein from 400 ml culture. No further purification was attempted with these proteins. Western blot analysis with antibodies anti-KD0386 was more sensitive than Coomasie blue showing dimerization of all mutants and degradation of N106D (Fig. 4.28d).
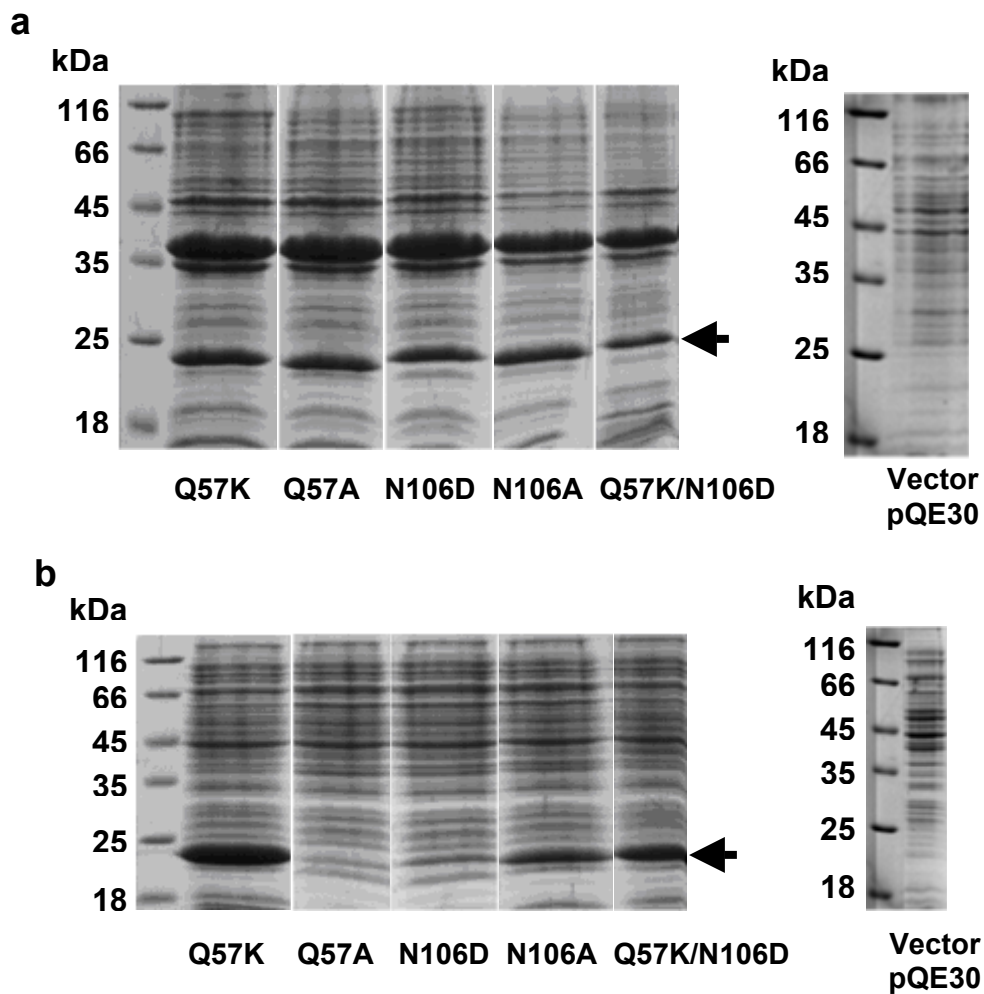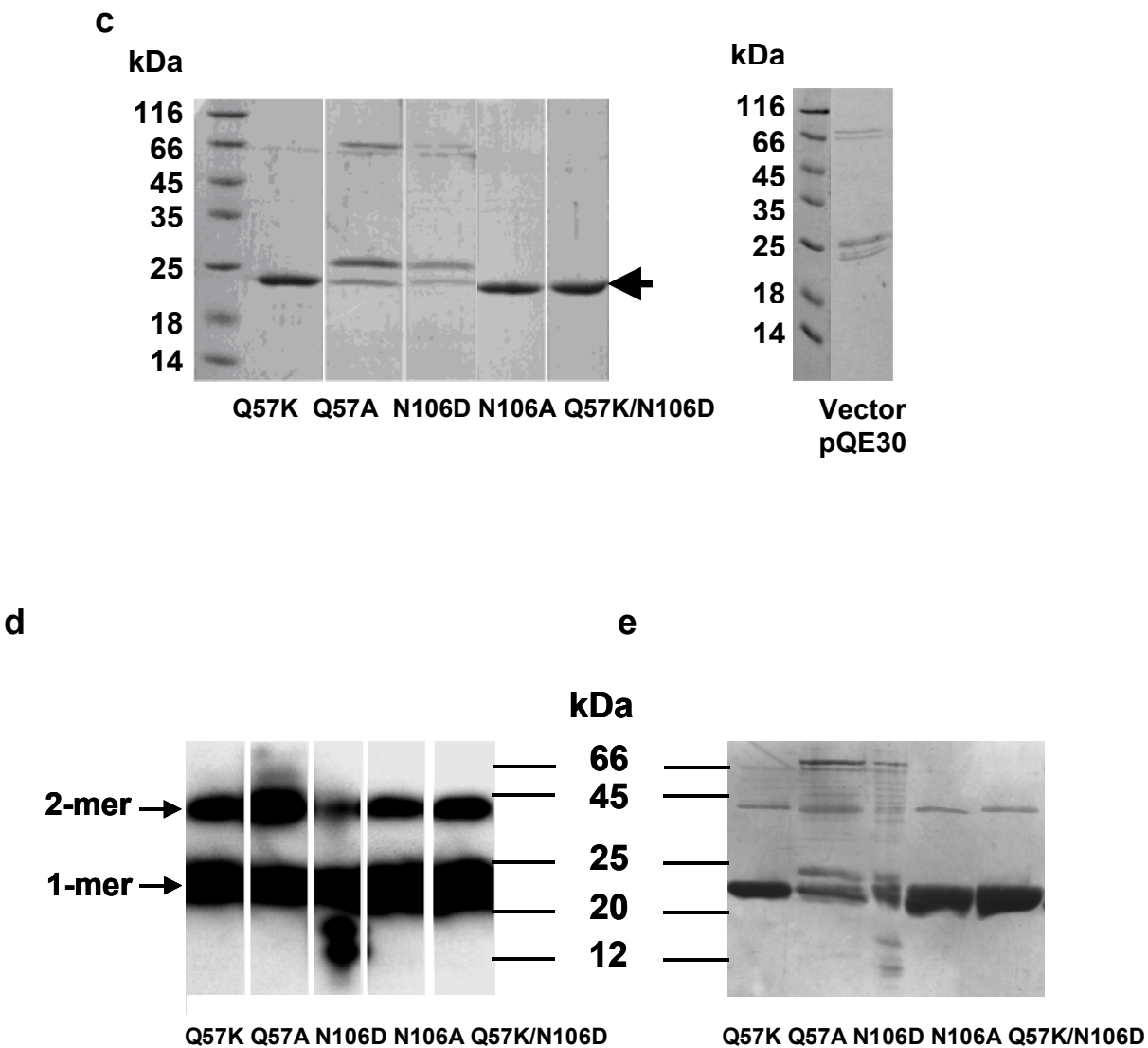
Fig. 4.28: 15% SDS-PAGE analysis of five mutants in pellet and supernatant fractions and after Ni-NTA purification with further dialysis (Coomassie staining). About 20 µg protein were applied in each lane from the pellets (**a**) and supernatants (**b**). Purified and dialyzed proteins (**c**) were applied in amounts of about 1-1.5 µg. Controls of vector pQE30 also in pellet, supernatant and purification's eluate are shown. Note proteins of the vector control that could also bind to Ni-NTA-agarose (**c**). (**d**) Western blot from 15% SDS-PAGE of 2 µg/lane of each mutant. Antibodies anti-KD0386 were used (5 s exposure time).

4.2.2.7.1 AC activity of the mutants

Substrate specificity in class III ACs is determined by a lysine and aspartic acid residues (e.g. K296 and D365 in Rv1625c), in Rv0386, these residues are glutamine and asparagine. Therefore, it was already surprising, that Rv0386 had AC activity at all. Now I investigated by a mutational approach the importance of the substrate-defining amino acids for catalysis in Rv0386. In all mutants, activity was below 10% of the wild-type level (Table 4.13) indicating the critical role of Q57 and N106 for substrate recognition in Rv0386 which could not be improved or replaced when mutating to the canonical corresponding amino acids.

Mutant	[µM]	cpm	Specific activity (pmol·mg^{-1}·min^{-1})		(%) activity of corresponding basal	[ATP] in assay (µM)
Q57K	2	182	18	(363)	5	75
	5	294	16	(289)		
	9.5	562	16	(433)		
Q57A	3.9	145	66	(3905)	1.4	1000
	5.8	120	35	(3281)		
N106D	1.9	62	0	(569)	0	75
	4.9	68	0	(538)		
	9.7	76	0	(381)		
N106A	1.9	125	13	(569)	1.5	75
	4.9	121	5	(538)		
	9.7	245	6.4	(381)		
Q57K/N106D	2.4	290	136	(1830)	6	500
	4.8	397	95	(2080)		

Table 4.13: AC activities of the five mutants. Specific activities in parenthesis correspond to the catalytic domain wild type under the same assay conditions (MOPS pH7.5, 10 min, 30°C, 5 mM Mn^{2+}). Detection limit was 5 pmol·mg^{-1}·min^{-1}.

4.2.2.7.2 pH dependence

Each mutant was tested with different buffer systems from pH 3 to 9 (Fig. 4.29 a and b). Activity was strongly affected by the pH. Optimal buffers were: for Q57K and N106A Glycin/NaOH buffer pH 8.4 and for N106D and Q57K/N106D Glycin/NaOH pH 9.1.

Q57A did not show activity under these assay conditions. Activity of Q57A was actually only detectable with Tris/HCl pH 8.3 and MOPS pH 7.5 when testing at higher ATP concentrations (Tables 4.13 and 4.14). Since activities of Q57A and N106D are rather low it resulted not clear if they show a large buffer effect or the observations are due only to the substrate concentration in the different assays (see Tables 4.13 and 4.14, Fig. 4.29a and b for comparison). A slight displacement of the pH optimum compared with the catalytic domain wild type was observed only for N106D and Q57K/N106D (Fig. 4.17 and 4.29).

b
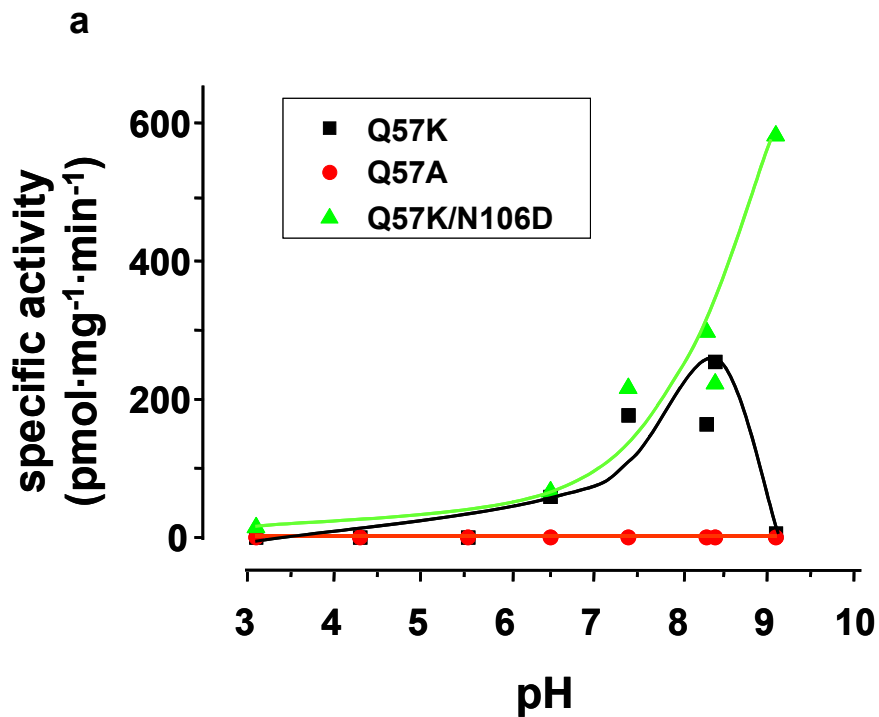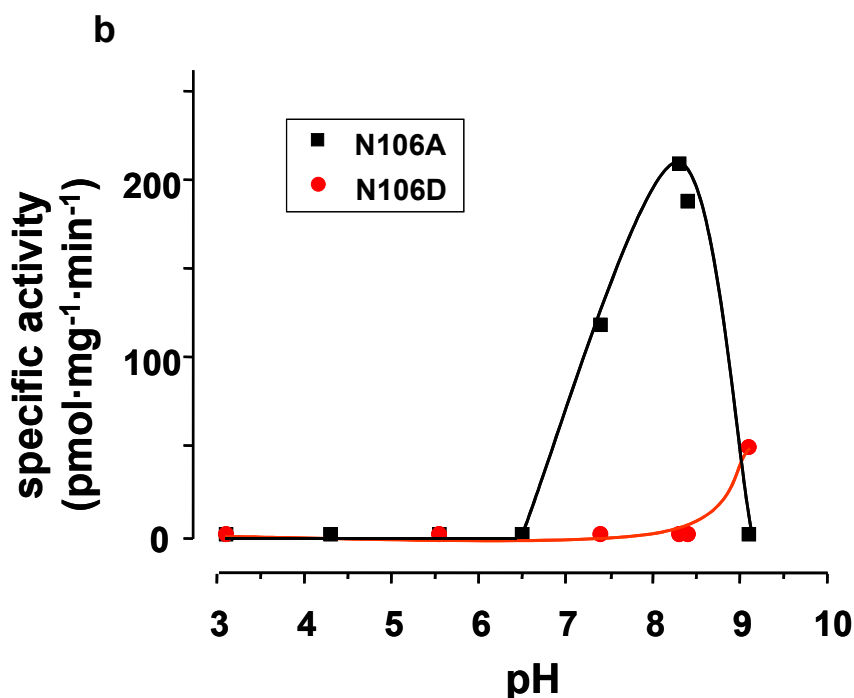
Fig. 4.29: Dependence on pH of the mutants. Assay conditions: 500 µM ATP, 10 min, 30°C, 5 mM Mn^{2+}, 6 µM Q57K, 4 µM Q57A, 5 µM Q57K/N106D (**a**), 3 µM N106D and 9 µM N106A (**b**).

Mutant	[µM]	cpm	Specific activity (pmol·mg^{-1}·min^{-1})		% activity of corresponding basal	ATP in assay [µM]	pH
Q57K	1.9	634	512	(7787)	**6.5**	850	8.4
Q57A	1.9	290	182	(4577)	**4**	850	8.3
N106D	3.9	155	45	(1872)	**2.4**	850	9.1
N106A	2.4	176	161	(3168)	**5**	500	8.4
Q57K/N106D	2.9	648	399	(6437)	**6**	850	9.1

Table 4.14: AC activities of the mutants measured at their pH optimum. The pH values correspond to the final pH measured after mixing all components of the AC-test mixture. Assays conditions: 30°C, 20 min, 5 mM Mn^{2+}. Data are from separate tests. Specific activities in parentheses correspond to the catalytic domain wild type under the same assay conditions.

4.2.2.7.3 Protein dependence

Mutants Q57K, Q57A and Q57K/N106D showed a notable decrease in activity at increasing protein concentration (Fig. 4.30a,b,d). The Kd values could not be calculated because at lower concentrations AC activity was reliably unmeasurable. It is possible that the mutants have a Kd similar or lower than that calculated for the wild type (0.14 µM) and that less active multimers are formed upon increasing protein concentrations. Activity of N106A was relatively constant at increasing protein concentrations (Fig. 4.30c). For mutant N106D it was impossible to determine protein dependency because of its lack of activity at concentrations lower than 1.9 µM, and its low recovery after purification made it impossible to test it at concentrations higher than 5 µM (Fig. 4.30e).

a

µM (µg)		nmol·mg^{-1}·min^{-1} (cpm)	
0.14	(0.3)	1.7	(189)
0.29	(0.6)	0.9	(194)
0.39	(0.8)	0.8	(210)
0.48	(1)	0.9	(281)
1.45	(3)	0.6	(465)
4.4	(9)	0.3	(641)
7.3	(15)	0.2	(827)

b
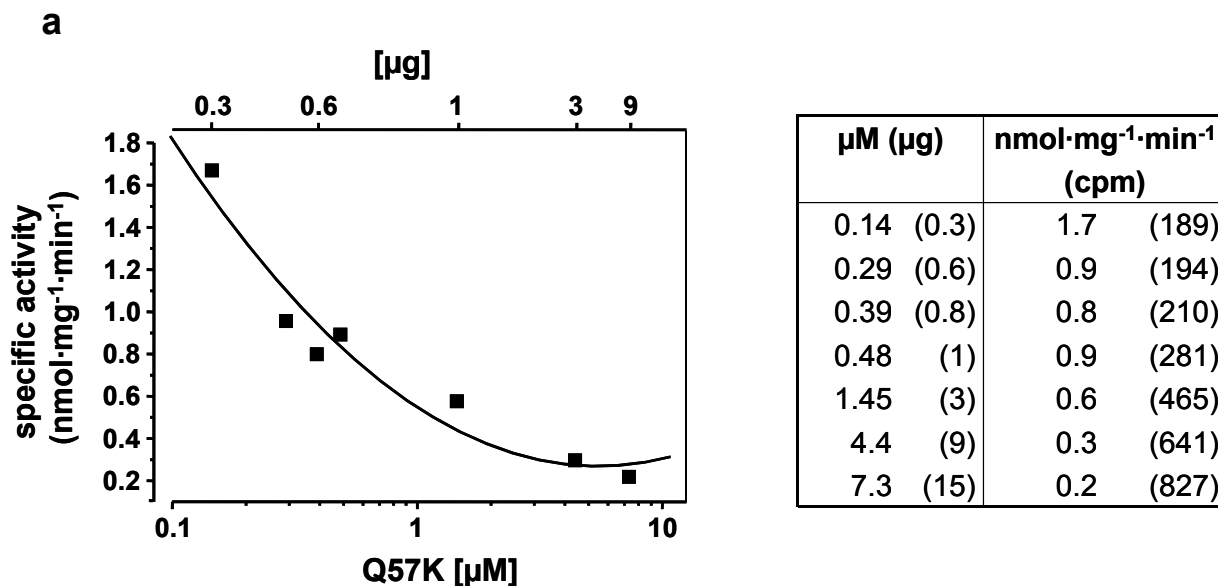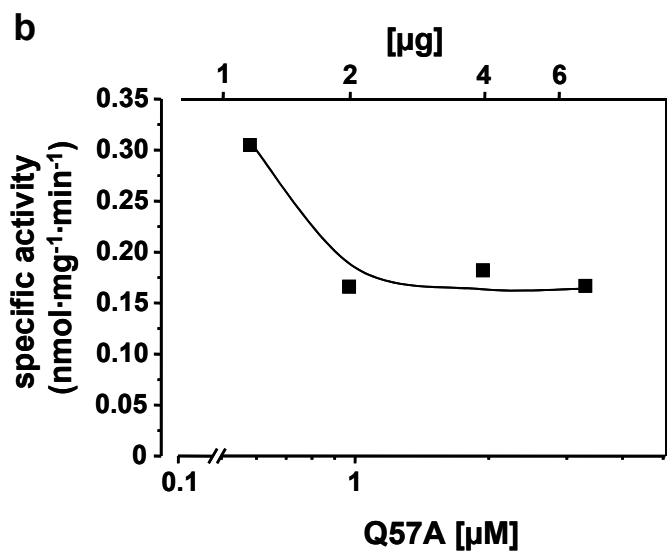
μM (μg)		nmol·mg⁻¹·min⁻¹ (cpm)	
0.58	(1.2)	0.30	(191)
0.97	(2)	0.17	(189)
1.94	(4)	0.18	(290)
3.3	(6.8)	0.17	(419)

c
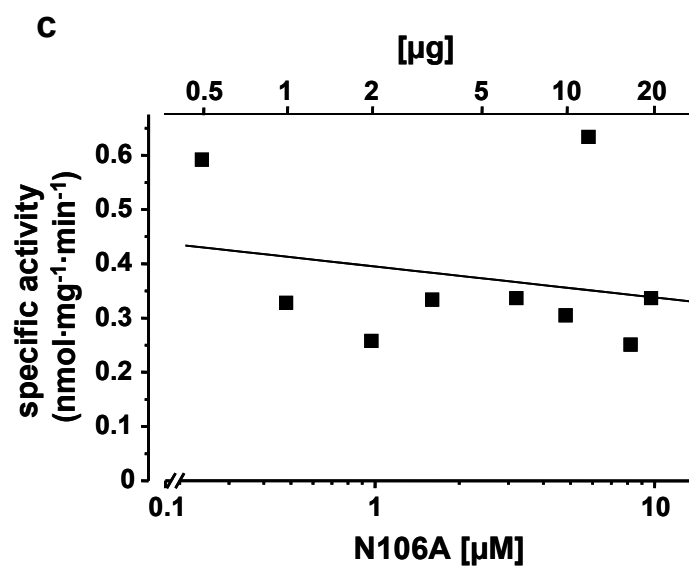
μM (μg)		nmol·mg⁻¹·min⁻¹ (cpm)	
0.24	(0.5)	0.6	(102)
0.48	(1)	0.3	(111)
0.97	(2)	0.2	(136)
1.6	(3.3)	0.3	(243)
3.2	(6.7)	0.3	(453)
4.8	(10)	0.3	(618)
5.8	(12)	0.6	(1430)
8.2	(17)	0.2	(859)
9.7	(20)	0.3	(1216)

d
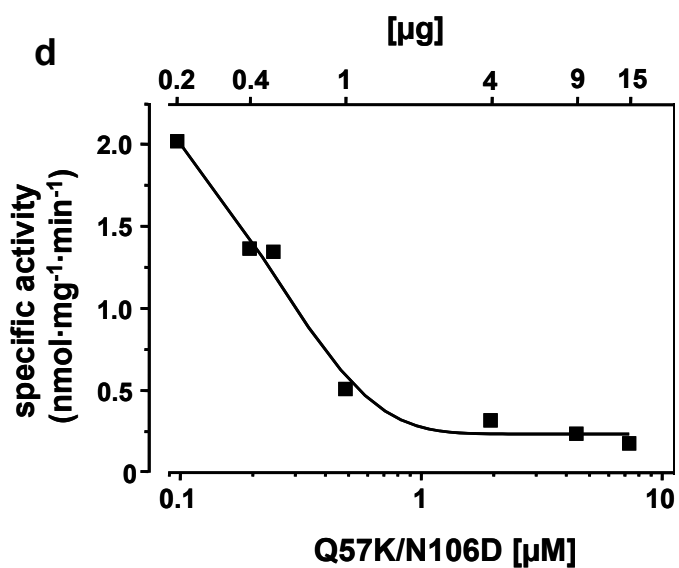
μM (μg)		nmol·mg⁻¹·min⁻¹ (cpm)	
0.097	(0.2)	2	(164)
0.194	(0.4)	1.4	(178)
0.243	(0.5)	1.3	(226)
0.485	(1)	0.5	(174)
1.94	(4)	0.3	(303)
4.4	(9)	0.2	(400)
7.3	(15)	0.2	(622)

e

μM (μg)		nmol·mg⁻¹·min⁻¹ (cpm)	
0.5	(1)	0	(71)
1	(2)	0	(63)
2	(4)	0	(66)
3.9	(8)	45.1	(155)
5	(10.4)	47.7	(179)
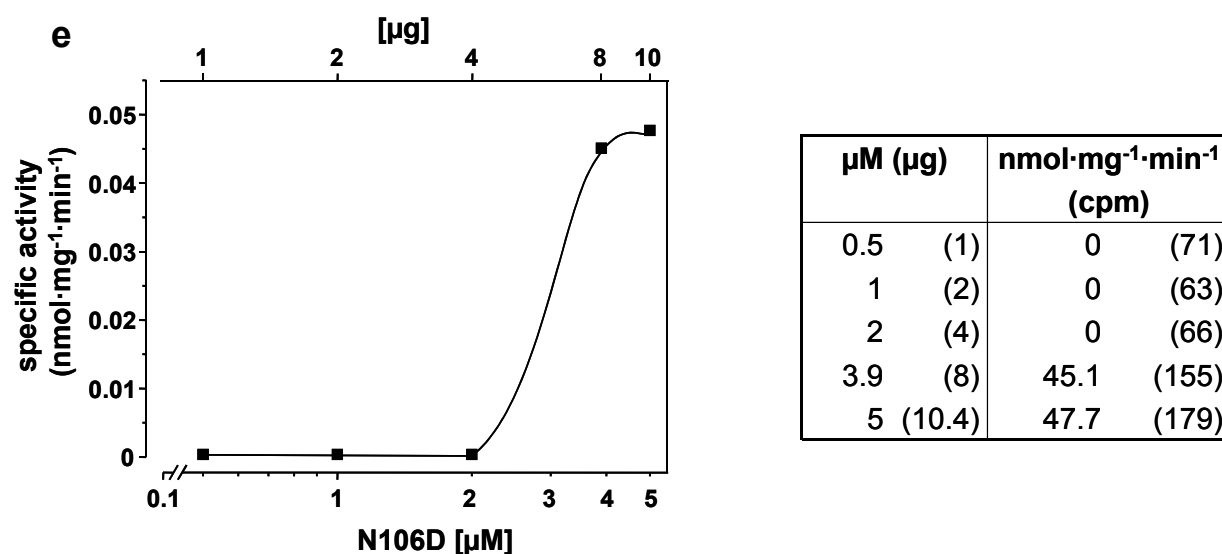
Fig. 4.30: Protein dependence of the Rv0386 catalytic domain mutants. For the curves of Q57K (**a**), Q57A (**b**), Q57K/N106D (**d**) and N106D (**e**) conditions were 850 μM ATP, 5 mM Mn²⁺, 30°C, 20 min. Assay conditions for N106A (**c**) were 500 μM ATP, 5 mM Mn²⁺, 30°C, 10 min. Each mutant was tested at the optimal pH. Data are from a single representative experiment (n=2 with similar results).

4.2.2.7.4 GC activity of the mutants

Direct comparison of AC and GC activities of the mutants showed a considerably attenuation in the substrate discrimination for mutants Q57K/N106D and N106A (Table 4.15). Mutant Q57K discriminate the substrate in the same proportion that the wild type did (30% residual activity). No GC activity was detectable for mutants N106D and Q57A. Activity of lower or higher protein concentrations of these mutants was impossible to be detected. It is possible that residues Q57 and N106D in conjunction are responsible for preferring ATP as a substrate.

Mutant	μM Protein (μg)	AC activity pmol · mg⁻¹ · min⁻¹ (cpm)		GC activity pmol · mg⁻¹ · min⁻¹ (cpm)		% GC residual activity	GTP in assay [μM]
Q57K	1.9 (4)	512	(634)	154	(245)	30	850
Q57A	1.9 (4)	182	(290)	0	(184)	0	850
N106D	3.9 (8)	45.1	(155)	0	(161)	0	850
N106A	2.4 (5)	161	(176)	109	(176)	67.7	500
Q57K/N106D	4.4 (9)	357	(918)	331	(801)	92.7	850

Table 4.15: GC activities of the mutants of the catalytic domain. Assay conditions: 30°C, 20 min for Q57A, Q57K, N106D and Q57K/N106D and 10 min for N106A. Each mutant was tested at its pH.

4.2.2.8 Expression and characterization of the mutant N106S

Mutant N106S was generated to turn the substrate specificity of the catalytic domain toward GTP, according to the alignment of the sequences of the AC domain of Rv0386 and the C1 domain of the *Paramecium* GC (Fig. 4.31). It was assumed that residue E56 (next neighbor to Q57) in Rv0386 could bind the purine corresponding to E1681 in ParaGC. Therefore only N106 was mutated to S.
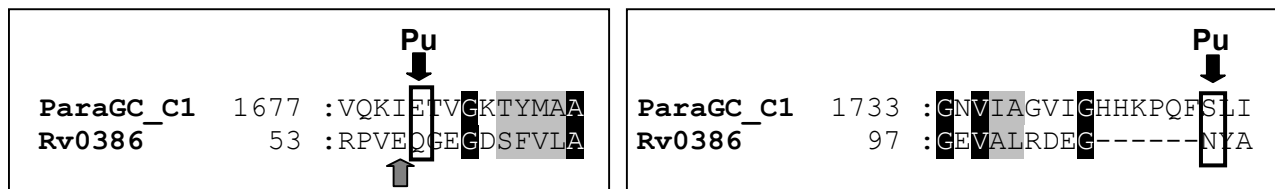
Fig. 4.31: Short alignment of Rv0386 with *Paramecium* GC showing their corresponding purine-specifying residues. Residues E_{1681} and S_{1748} confer ParaGC its GTP-specificity (Linder et al., 2000).

N106S was expressed in BL21 cells (5 h, 60 µM IPTG, RT), purified and dialyzed as usual and stored at –20°C with 20% glycerol. From 3 x 600 ml bacterial culture approx. 1 mg of purified and dialyzed protein was obtained. Expression and purification grade of N106S were analyzed by SDS-PAGE (Fig. 4.32a). Dimerization of the mutant was shown with a Western blot with anti-KD0386 (Fig. 4.32b).
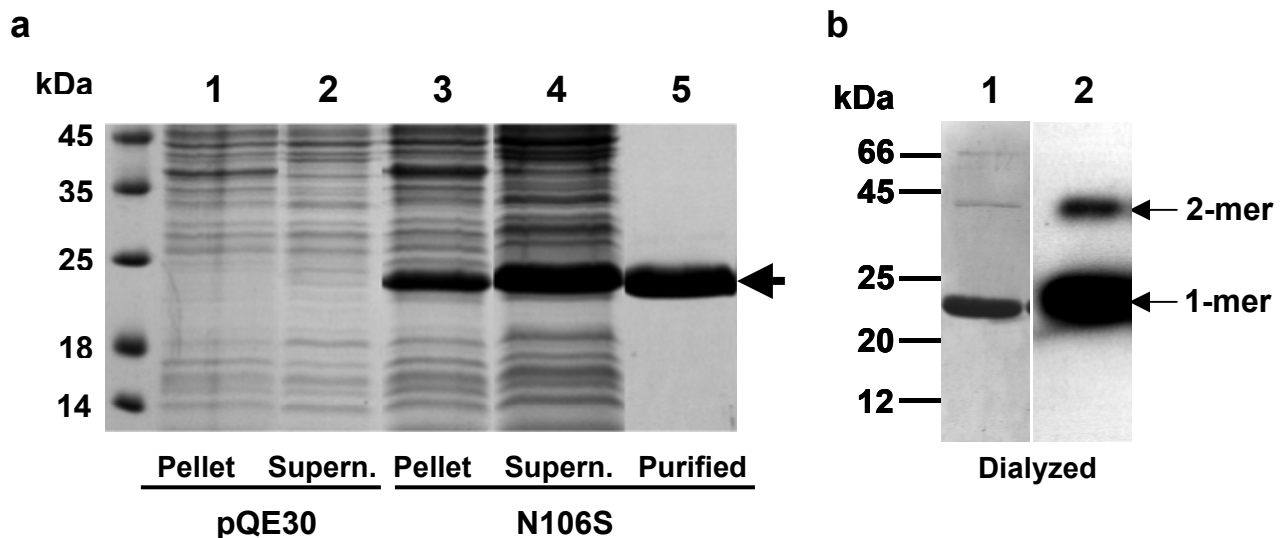
N106S showed an AC residual activity of 43% with respect of the wild type. The GC activity corresponded to 8% of its AC activity (Table 4.16).

Protein [µM]	AC activity $nmol \cdot mg^{-1} \cdot min^{-1}$ (wild type)		% activity of the wild type	GC specific activity ($nmol \cdot mg^{-1} \cdot min^{-1}$)	% GC vs. AC activity
2.4	2.1	(5.4)	38.9	0.2	9.5
3.8	1.7	(3.7)	46	0.1	5.9

Table 4.16: AC and GC activities of N106S. Wild-type AC activity at the same assay conditions are in parentheses. Assay conditions were: 850 µM ATP or GTP, 20 min, 30°C, buffer MOPS pH 7.5.

Protein and substrate dependence of N106S were investigated. Like wild type, the mutant N106S AC activity increased with increasing protein concentrations (figure 4.33). That indicates dimerization already observed in Western blot (see above). The graphically derived apparent Kd value is 0.3 µM (2-fold wild type). The enzyme kinetics of N106S showed a K_m value of 337 µM and V_{max} of 9.3 pmol/min (figure 4.34). The Hill coefficient of 0.9 indicated no cooperativity.

µM	nmol·mg^{-1}·min^{-1}
0.1	0.9
0.3	1.6
0.5	2.2
1.1	2
1.4	2.9
2.8	2.1
5.5	1.5
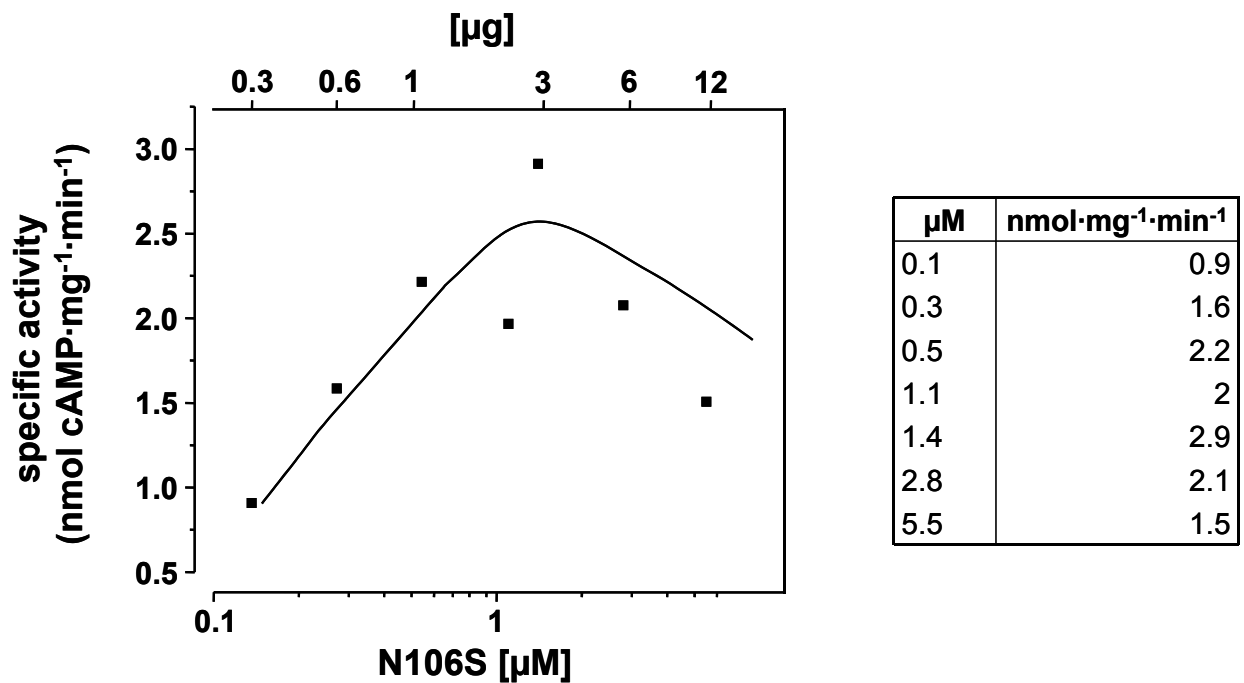
Fig. 4.33: Protein dependence of N106S with ATP as a substrate. Assay conditions: 850 µM ATP, 5 mM Mn^{2+}, MOPS pH 7.5, 30 °C, 20 min. Protein concentrations were from 136 nM to 5.5 µM. An EC$_{50}$ value of 0.34 µM could be derived from a graphical analysis.

a

mM	pmol·min^{-1}
0.08	1.9
0.17	3.1
0.25	3.6
0.42	4.7
0.59	4.9
0.76	7.2
1	8.1
1.4	10.1
1.7	7.9
2.1	8.3
2.4	7.8

b

1/activity	1/ATP
11.8	0.52
5.9	0.32
3.9	0.28
2.3	0.21
1.7	0.20
1.3	0.14
1	0.12
0.7	0.10
0.6	0.13
0.5	0.12
0.4	0.13

c
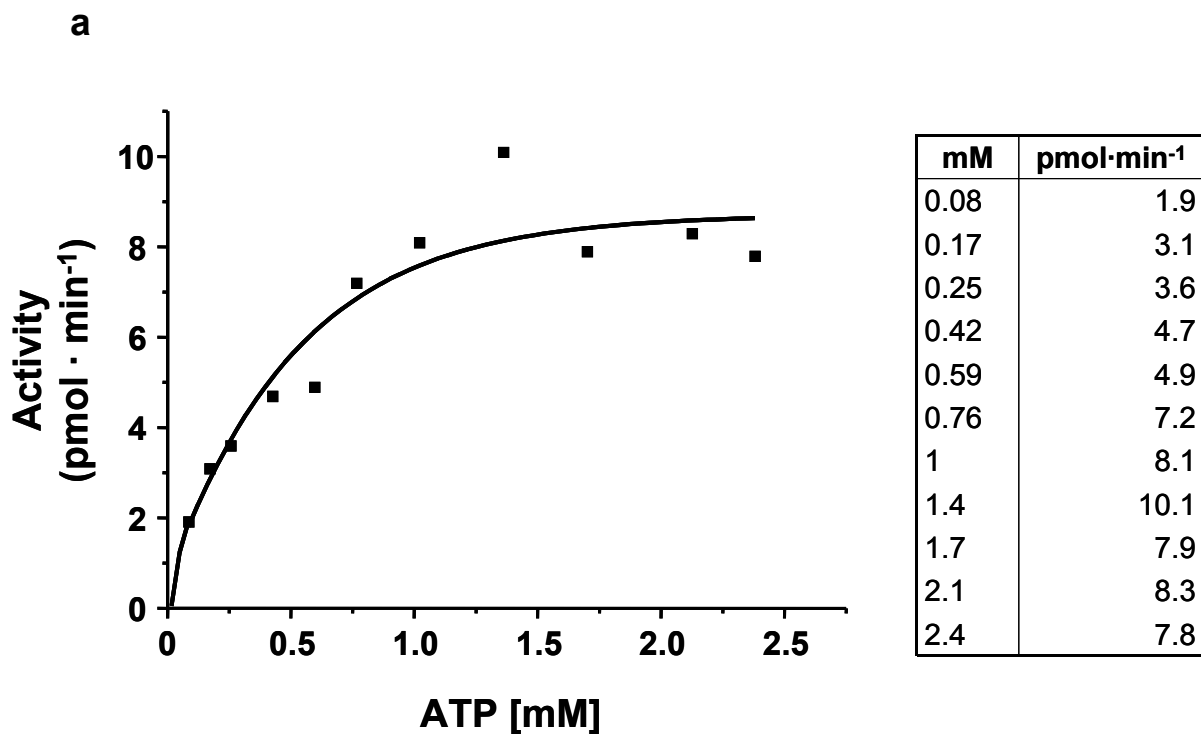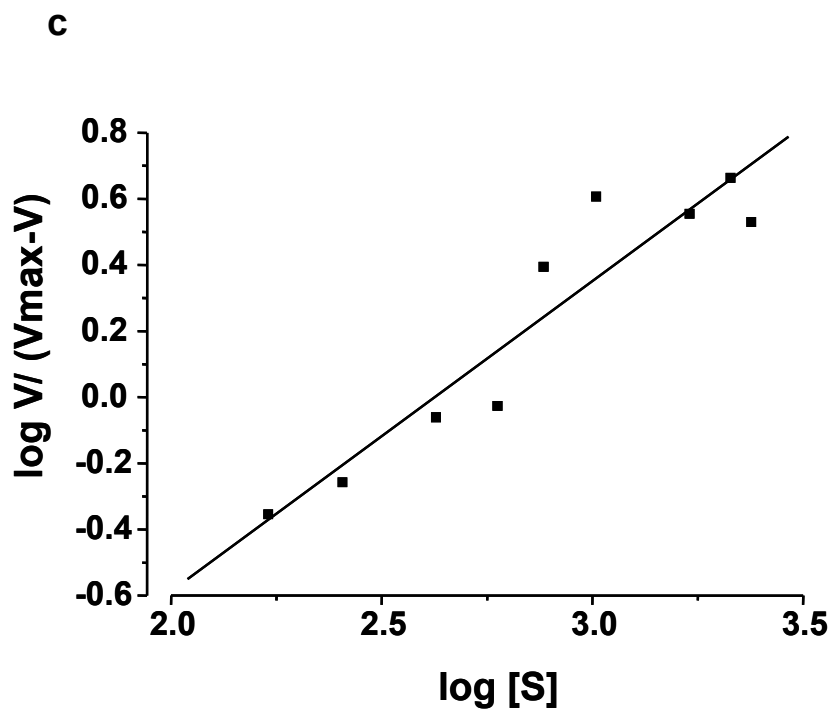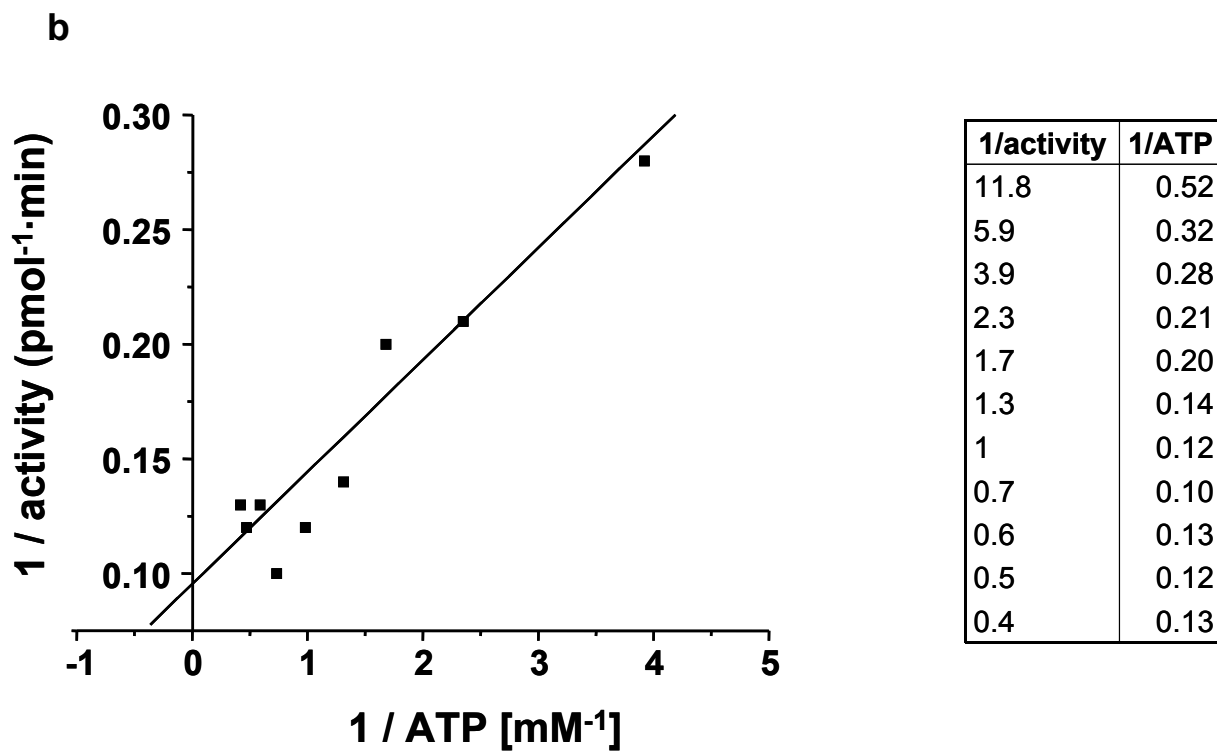
Fig. 4.34: **a**) Michaelis-Menten kinetic for ATP as a substrate. Assays were conducted at 1.4 µM of protein, MOPS pH 7.5, 30°C, 5 mM Mn^{2+} and 10 min; **b**) corresponding Lineweaver-Burk linear fit; **c**) corresponding Hill-Plot (R^2= 0.9229; y= 0.92213x - 2.42055). **b** and **c**) the two smallest values were not drawn for graph clarity but taken for K_M and Vmax calculation. V= activity (pmol·min^{-1}); [S]= ATP.

4.2.2.9 Expression and characterization of an N-terminally elongated AC domain

This protein corresponds to the Rv0386 AC domain elongated at the N-terminus with 7 amino acids (MRLSGAG) and is also called AC domain MT0399 (see 3.6.3.7 and 3.6.3.8 on chapter 3 for details). Purpose of its characterization was to determine its utility for crystallization experiments. Protein was expressed with N-terminal as well as with C-terminal His-tag in BL21 cells (5 h, 60 µM IPTG, RT), purified and dialyzed as usual. Recoveries of 0.6 mg of N-terminally His-tagged protein (from 3 x 600 ml culture) and 0.3 mg of C-terminally His-tagged protein (from 400 ml culture) were obtained. Expression and purification steps were controlled by SDS-PAGE (Fig. 4.35).

Both proteins showed an AC activity of 30% of the wild type. The GC activities correspond to 27% of the AC activity for N-His tag MT0399 and 21% of the AC activity for C-His tag MT0399 (Table 4.17). The proportion AC/GC activity of both proteins is similar to that of wild type indicating no change in the substrate discrimination. Nevertheless the low activities point to a critical role of the N-terminus of the protein for catalysis in Rv0386. Both proteins were used for crystallization experiments but without success.
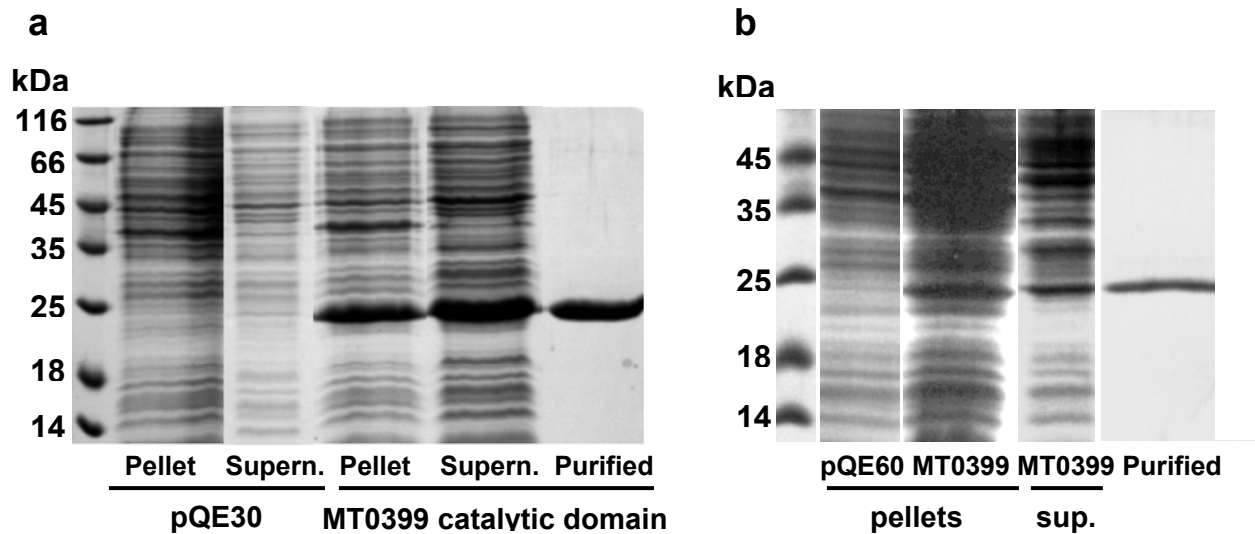
Fig. 4.35: 15% SDS-PAGE analysis of the N-terminally elongated AC domain (equivalent to MT0399 AC domain). **a)** Protein with N-terminal His-tag and **b)** with C-terminal His-tag. Empty vectors pQE30 and pQE60 were also expressed as controls. 20 µg of pellets and 10-15 µg of supernatants were applied. 1-2 µg of purified and dialyzed protein were applied.

Protein	[µM]	AC activity nmol · mg^{-1} · min^{-1} (wild type)		% AC activity of the wild type	GC activity nmol · mg^{-1} · min^{-1}	% GC vs. AC activity
N-His tag	1.4	2.3	(6.7)	34.3	n.d.	-
MT0399	2.4	1.4	(5.4)	25.9	0.4	28.6
	3.8	1.2	(3.7)	32.4	0.3	25
	5.2	1.2	(4.1)	29.2	n.d.	-
C-His tag	1.9	0.9	(4.2)	21.4	0.2	22
MT0399	3.9	1	(3.4)	29.4	0.2	20

Table 4.17: AC and GC activities of MT0399 AC domain with N-terminal and C-terminal His-tag. Activities of both proteins were examined in separated assays (AC domain wild type at same assay conditions in parentheses). Assay conditions: 850 µM ATP or GTP, 20 min, 30°C, buffer MOPS pH 7.5. n.d.= not determined.

Protein and substrate dependence of MT0399 with N-His tag were investigated. No apparent Kd value could be determined (Fig. 4.36). Probably the dimerization of the protein occurred at protein concentrations <214 nM. That could not be further investigated due to the detection limit. The enzyme kinetics of this protein showed a K_m value of 2.3 mM and V_{max} of 13.6 pmol/min (Fig. 4.37). The Hill coefficient of 0.9 pointed to no cooperativity. Such characterizations were not made for MT0399 with C-terminal His-tag.

µM	nmol·mg^{-1}·min^{-1}
0.21	1.3
0.43	1.2
0.73	1.3
1	1.9
3.1	1
6.1	0.8
10.2	0.7
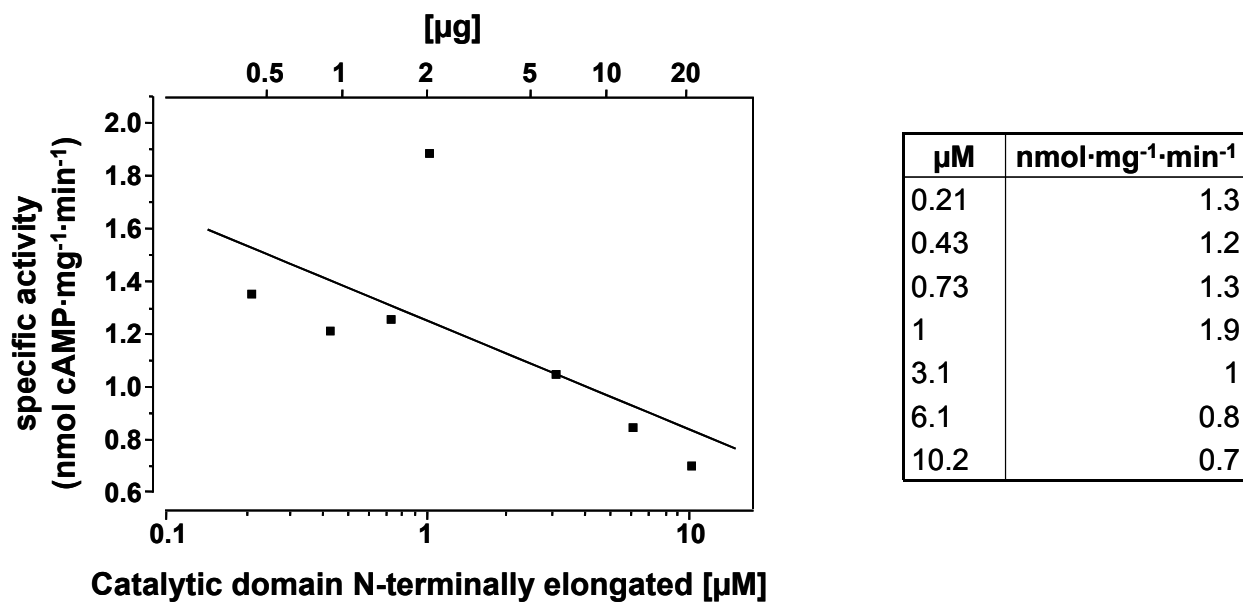
Fig. 4.36: Protein dependence of MT0399 (N-His tag) with ATP as a substrate. Assay conditions: 850 µM ATP, 5 mM Mn^{2+}, MOPS pH 7.5, 30 °C, 20 min. Protein concentrations tested were from 214 nM to 10.2 µM.

a
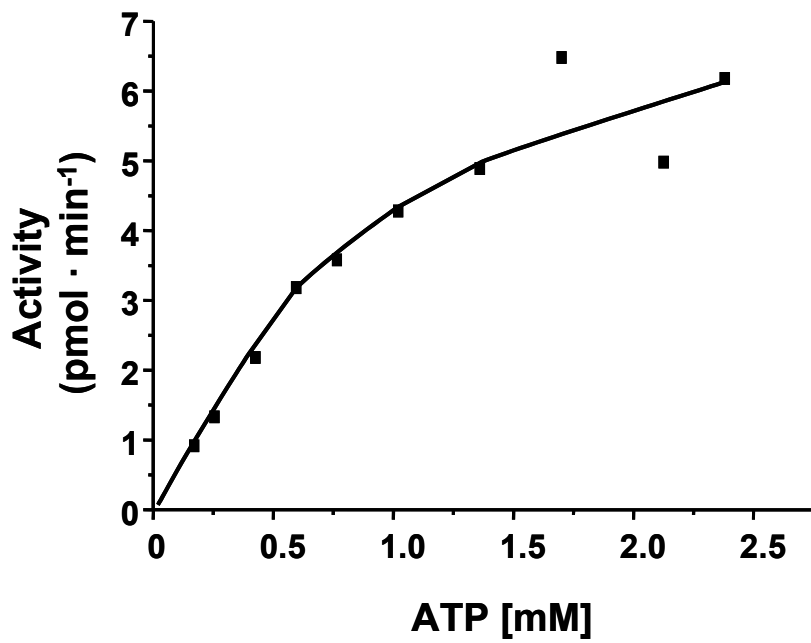
mM	pmol·min^{-1}
0.2	0.9
0.25	1.3
0.4	2.2
0.6	3.2
0.8	3.6
1	4.3
1.4	4.9
1.7	6.5
2.1	5
2.4	6.2

b
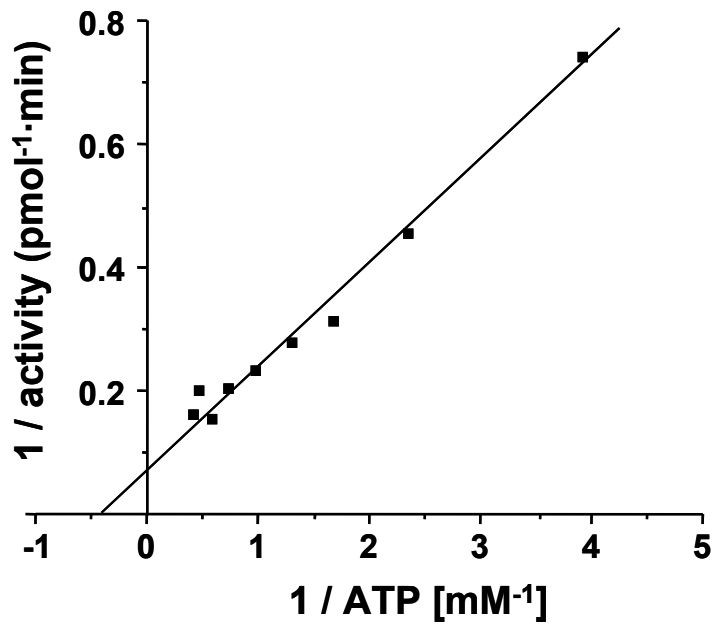
| 1/activity	1/ATP
5.9	1.1
4	0.7
2.4	0.4
1.7	0.3
1.3	0.3
1	0.2
0.7	0.2
0.6	0.1
0.5	0.2
0.4	0.2

c
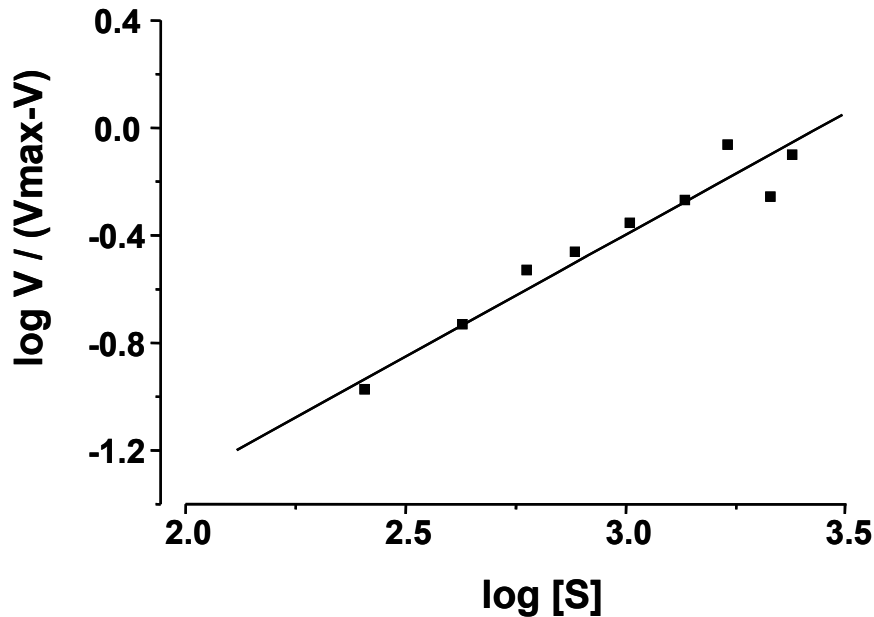
Fig. 4.37: a) Michaelis-Menten kinetic for ATP as substrate. Assays were conducted at 1.5 µM protein, MOPS pH 7.5, 30°C, 5 mM Mn^{2+} and 10 min; **b**) corresponding Lineweaver-Burk linear fit; **c**) corresponding Hill-Plot (R^2= 0.9540; y= 0.90744x − 3.11893). **b** and **c**) the smallest value were not drawn for graph clarity but taken for K$_M$ and Vmax calculation. V= activity (pmol·min^{-1}); [S]= ATP.

4.2.2.10   Expression and characterization of the C-terminally His-tagged AC domain

The C-terminally His-tagged AC domain of Rv0386 was expressed in BL21 cells (5 h, 60 µM IPTG, RT) with a protein recovery of 0.7 mg pro 400 ml culture after Ni^{2+}-NTA-agarose purification (Fig. 4.38). The protein was dialyzed (20% glycerol) and stored at − 20°C. The calculated molecular weight was 20 kDa including the C-terminal His-tag. This protein showed 81% of the AC activity of the N-terminally His-tagged AC domain.
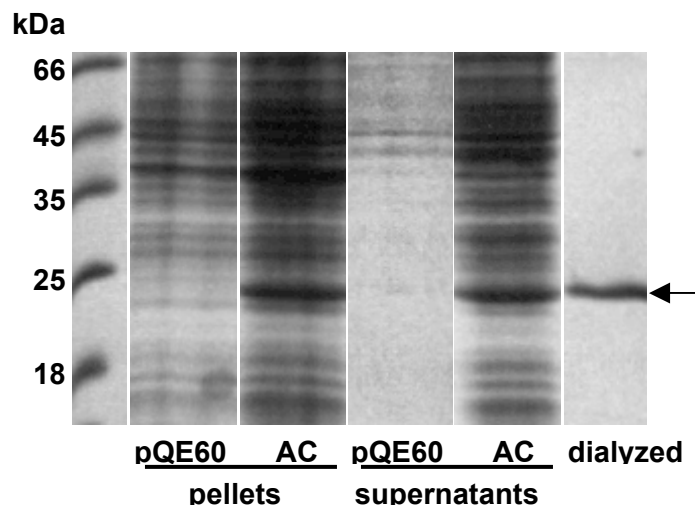
Fig. 4.38: 15% SDS-PAGE of the C-terminally His-tagged AC domain. An empty vector control was carried in parallel. 20 µg of pellet proteins were applied. From supernatants, 5 µg of pQE60 and 20 µg of AC domain were applied. After purification and dialysis 2 µg of protein were applied.

Protein and substrate dependence of the C-terminally His-tagged AC domain were examined. A considerably slowdown of the dimerization process of this protein was observed. The graphically derived apparent Kd value was 1.5 µM (Fig. 4.39). Surprisingly up to 15 µg protein no reduction in the activity was observed. After kinetic characterization the K_m value was 454 µM and V_{max} was 34.8 pmol of cAMP/min (3.9 nmol of cAMP·mg^{-1}·min^{-1}). The Hill coefficient of 0.9 indicated no cooperativity. A slight inhibition of the activity upon 2 mM ATP was observed (Fig. 4.40).
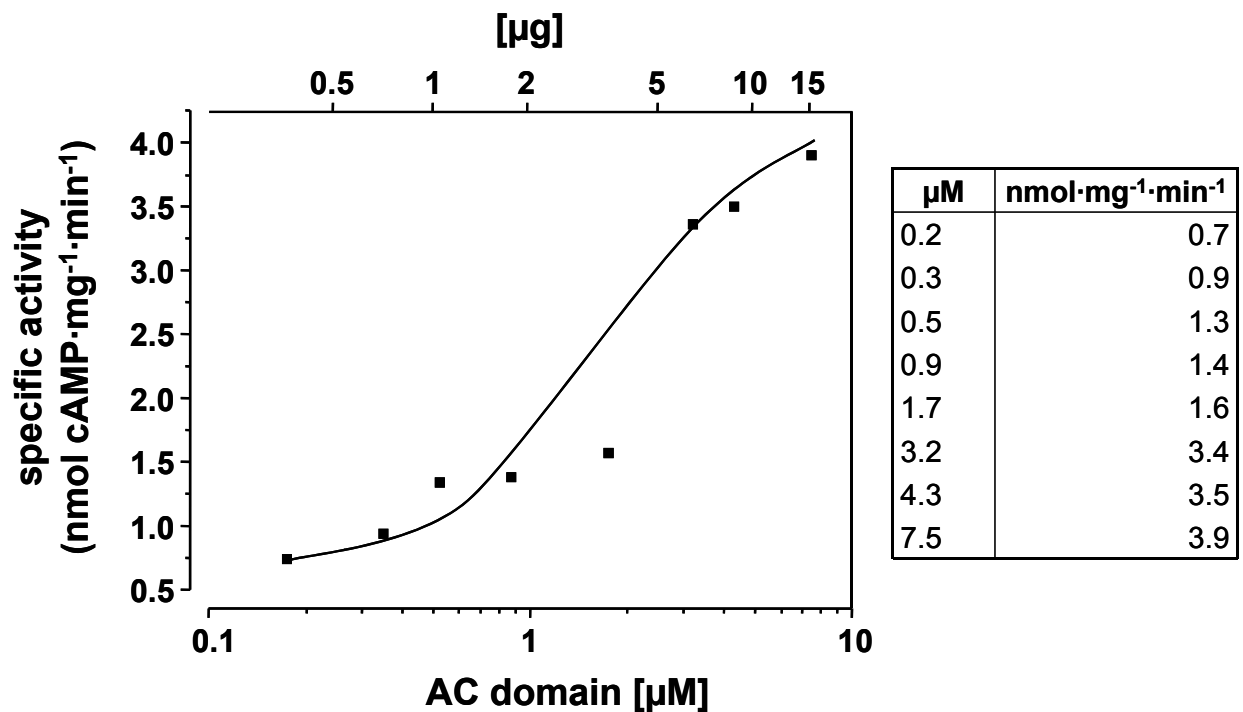
Fig. 4.39: Protein dependence of the AC domain with an C-terminal His-tag. Assay conditions: 850 µM ATP, 5 mM Mn^{2+}, MOPS pH 7.5, 30°C, 20 min. Protein concentrations tested were from 175 nM to 7.5 µM. An EC_{50} value of 1.5 µM could be derived graphically.

µM	nmol·mg^{-1}·min^{-1}
0.2	0.7
0.3	0.9
0.5	1.3
0.9	1.4
1.7	1.6
3.2	3.4
4.3	3.5
7.5	3.9

a

mM	pmol·min^{-1}
0.1	5.7
0.2	8.2
0.25	12.1
0.4	23.2
0.6	24.8
0.8	28.2
1	28.2
1.4	28.9
1.7	27.2
2.1	26.3
2.4	22.8
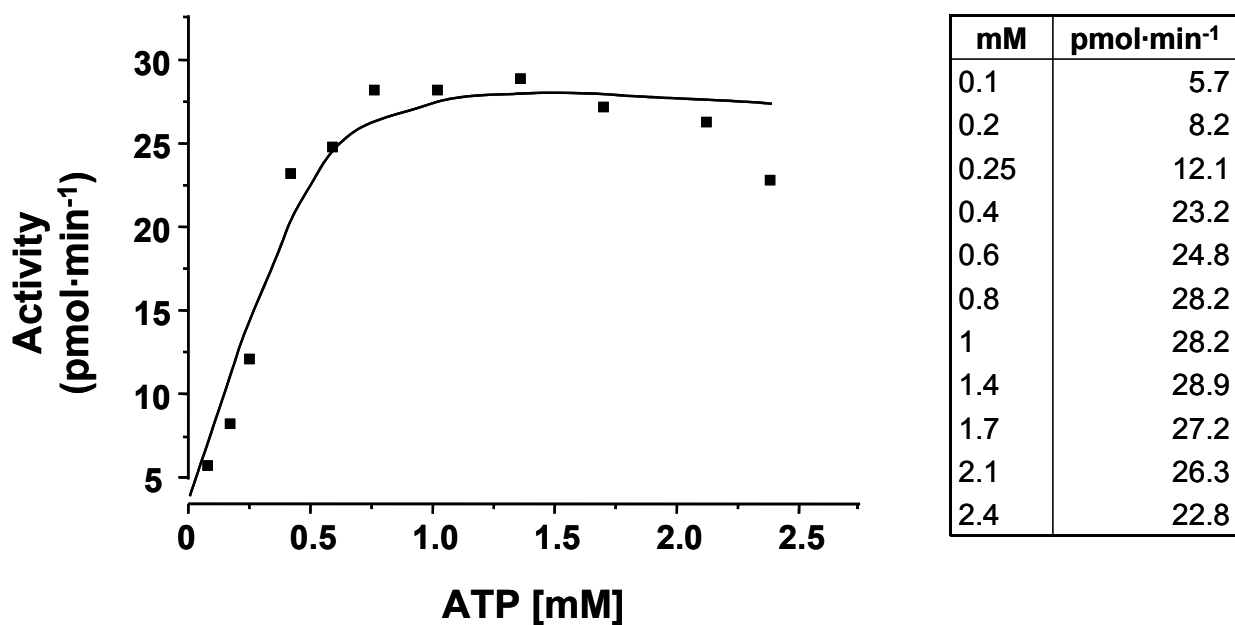
b
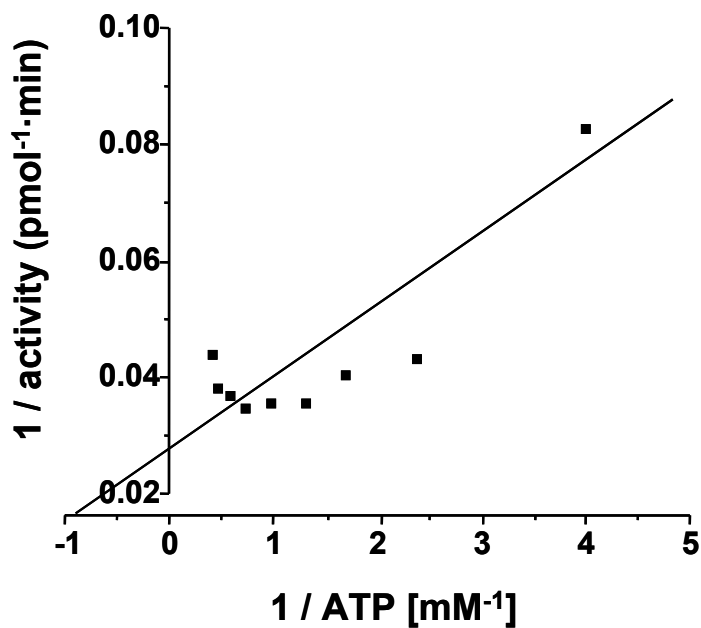
1/activity	1/ATP
12.5	0.17
5.9	0.12
4	0.08
2.4	0.04
1.7	0.04
1.3	0.03
1	0.03
0.7	0.03
0.6	0.04
0.5	0.04
0.4	0.04

c
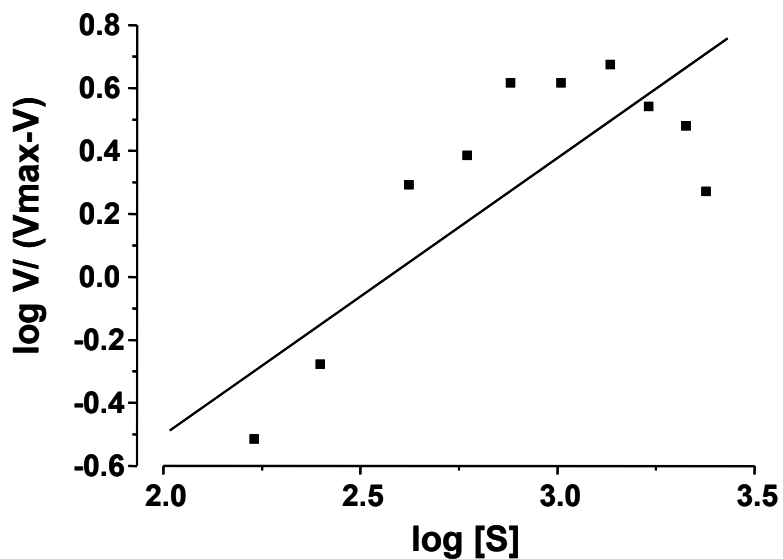
Fig. 4.40: a) Michaelis-Menten kinetic of AC domain with C-terminal His-tag. Assay was conducted at 4.3 µM of protein, MOPS pH 7.5, 30°C, 5 mM Mn^{2+} and 10 min; **b)** corresponding Lineweaver-Burk linear fit; **c)** corresponding Hill-Plot (R^2= 0.8225; y= 0.9085x – 2.3927). **b)** the two smallest values were not drawn for graph clarity but taken for K_M and Vmax calculation. V= activity (pmol·min^{-1}).

4.2.2.11 Expression and AC assay of mutant R7G

This mutant was generated due to a cloning error (see 3.6.3.10 for details). It was expressed in BL21 cells (60 µM IPTG, 4 h, RT), pellet and supernatant were separated, analyzed by SDS-PAGE and tested (Fig. 4.41). AC assay conditions were 75 µM ATP, 37°C, 2 mM Mn^{2+}, 10 min, Tris-HCl pH 7.5. Activity of the supernatant fraction (0.6 pmol min^{-1}) was identical to an empty vector control (0.5 pmol min^{-1}). Activity of the pellet was 0.3 pmol min^{-1} (control pellet 0.1 pmol min^{-1}). After Ni-NTA-agarose purification the specific activity was 26 pmol mg^{-1} min^{-1} (4 µM protein, 30°C, 75 µM ATP, 10 min, Tris pH 7.5). Under identical conditions the wild type AC showed a specific activity of 460 pmol mg^{-1} min^{-1}. Since the activity of this mutant was rather low it is possible that this N-terminally positioned arginine residue in Rv0386 AC domain is critical for catalysis. For Rv1625c it is reported a significant loss of the activity when two N-terminally located arginine residues i.e. R43 and R44 were mutated to alanine and glycine, respectively (Reddy et al., 2001).
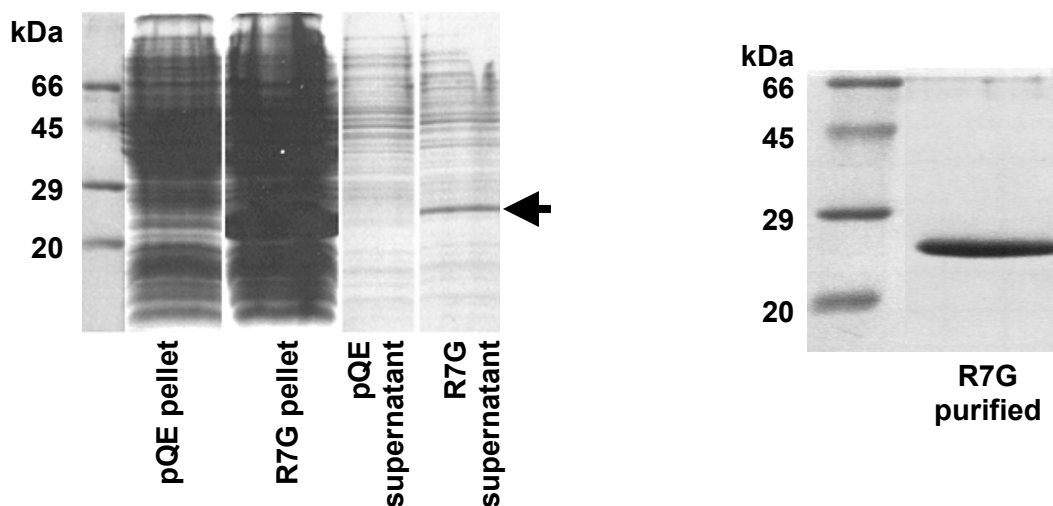
Fig. 4.41: 15% SDS-PAGE analysis of the mutant R7G in pellet and supernatant and in eluate after Ni-NTA purification. Applied were about 40 µg protein of pellets and about 20 µg protein of supernatants. About 5 µg of purified R7G were applied.

4.2.2.12 Crystallization of the AC domain

First crystals were obtained with the N-terminally His-tag construct with buffers CS#22 and CS2#1 (Hampton Research kit). Variations of these conditions and of the protein

solution components and concentration were made to optimize size and quality of the crystals (Table 4.18). With the C-terminally His-tagged construct four different conditions corresponding to buffers CS#6, CS2#30, CSL#22 and CSL#25 were found (Table 4.19). See appendix for pictures of the best crystals obtained.

Protein: N-terminally His-tagged Rv0386$_{(1-175)}$				
Buffer components	Protein solution components	Protein concentration	Crystal form	Remarks
30% PEG 4000, 0.1 M Tris HCl pH 8.5, 0.2 M sodium acetate (CS#22*)	50 mM Tris HCl pH 8.5, 20% glycerol, 10 mM NaCl, 2 mM β-mercaptoethanol	9 mg/ml	Needles (clusters)	After 3 weeks at 12 °C.
10% PEG 6000, 2 M NaCl (CS2#1*)			Cubes (very small)	after 8 days at 12°C
Optimization based on buffer CS2#1*				
10% PEG 6000, 2 M NaCl	50 mM Tris HCl pH 8.5, 10% glycerol, 10 mM NaCl, 2 mM β-mercaptoethanol	16 mg/ml	Rectangles 40 x 25 µm	after 6 days at 12°C
		30 mg/ml	Rectangles 115 x 30 µm	after 2 weeks at 12°C
		22 mg/ml + 1.7 mM α,β-CH$_2$ ADP	Rectangles 150 x 35 µm	
10% PEG 6000, 2 M NaCl, 1 mM Dibutyryl cAMP		22 mg/ml	Rectangles 200 x 75 µm	
12% PEG 6000, 2 M NaCl		30 mg/ml	Rectangles 115 x 30 µm	after 2 weeks at 12°C
10% PEG 5000, 2 M NaCl		16 mg/ml	Rectangles 50 x 20 µm	after 8 days at 12°C
10% PEG 4000, 2 M NaCl			Rectangles 200 x 175 µm	
10% PEG 3350, 2 M NaCl			Rectangles 200 x 175 µm	

Table 4.18: Summary of conditions in which crystals of N-terminally His-tagged Rv0386$_{(1-175)}$ were grown. * Name according to the Crystal Screen Buffer Kit of Hampton Research (CS= crystal screen, CS2= crystal screen 2, CSL= crystal screen lite).

Protein: C-terminally His-tagged Rv0386$_{(1-175)}$					
Buffer name	Buffer components	Protein solution components	Protein conc.	Crystal form	Remarks
CS#6*	30% PEG 4000, 0.1 M Tris HCl pH 8.5, 0.2 M MgCl$_2$	50 mM Tris HCl pH 8.5, 5 % glycerol, 2 mM β-mercaptoethanol, 10 mM NaCl, incubated 12-14 h at 0°C with 1 mM ATP	9 mg/ml	Needles (clusters)	after 10 days at 16 °C
CS2#30	10 % PEG 6000, 0.1 M Hepes pH 7.5, 5% MPD			Needles 40 x 5 µm	
CSL#22	15% PEG 4000, 0.1 M Tris HCl pH 8.5, 0.2 M sodium acetate			Half-moon shaped 50 x 50 µm	after 24 h at 16 °C
CSL#25	0.5 M sodium acetate, 0.1 M imidazole pH 6.5			Needles** 40 x 5 µm	

Table 4.19: Summary of conditions in which crystals of N-terminally His-tagged Rv0386$_{(1-175)}$ were grown. * Name according to the Crystal Screen Buffer Kit of Hampton Research (CS= crystal screen, CS2= crystal screen 2, CSL= crystal screen lite). ** Diffraction-quality crystals; see preliminary data in appendix.

4.2.3   Expression of Rv0386 holoenzyme

4.2.3.1 Expression in BL21 (DE3) [pREP4] cells

After expression (60 µM IPTG, 5 h, RT) cells were sonificated, pellet and supernatant were separated (14000 x g; 30 min; 4°C) and tested for AC activity. BL21 with empty vector served as a control (Fig. 4.42a). The expected MW including His-tag was 117 kDa. The holoenzyme was in the pellet (presumably inclusion bodies). For better lysis a French Press was used (20,000 psi). Pellet and supernatant were separated as usual, protein purified with Ni-NTA-agarose (Fig. 4.42b) and tested. Only degradation products (because AC activity was detectable) were found in the eluate after purification.

a

kDa	A B C D
116	
66	
45	
35	
25	

A	B	C	D
pQE sup.	pQE pellet	Holo sup.	Holo pellet
0	0	0	158

Activity (pmol min^{-1})

b

A B C	kDa
	116
	66
	45
	35
	25

A	B	C
Holo pellet	Holo sup.	Holo eluate
11.23	0.27	2.74

Activity (pmol min^{-1})
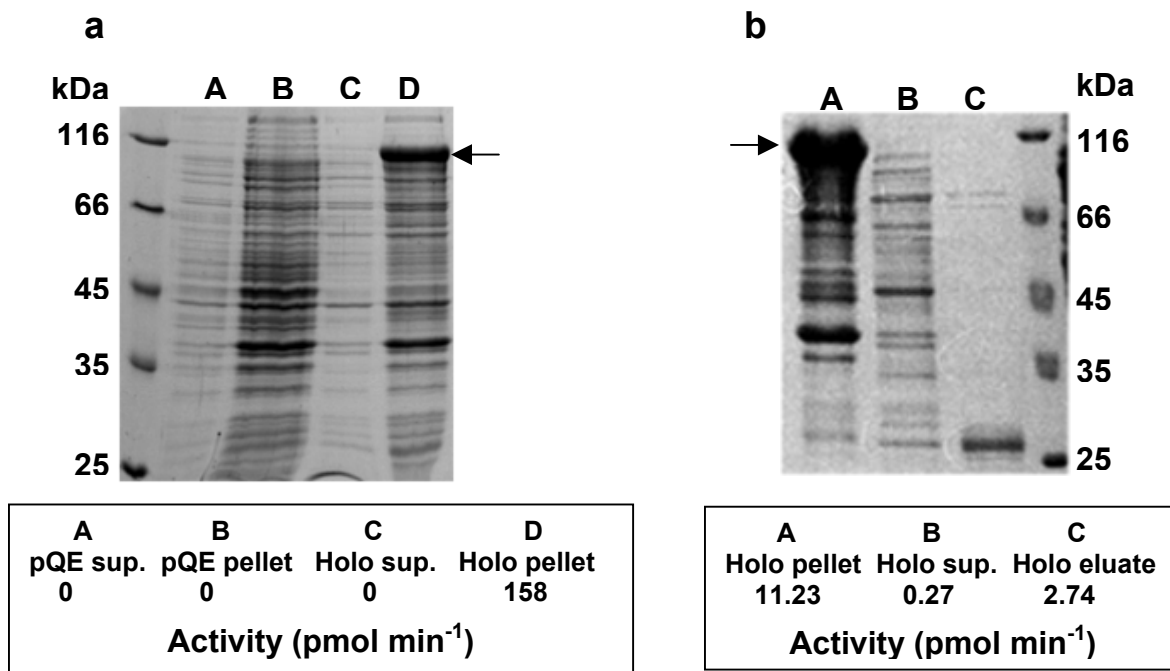
Fig. 4.42: 12.5% SDS-PAGE of the holoenzyme Rv0386 using (**a**) sonification and (**b**) French Press for lysis. (**a**) Applied were: about 1.5 µg protein of supernatants, 4 µg of pQE30 pellet and 7 µg of holoenzyme pellet. Activities correspond to: 5 µg protein of supernatants, 32 µg of pQE30 pellet and 55 µg of holoenzyme pellet. (**b**) Applied were: 8 µg of pellet, 5 µg of supernatant and 1 µg of purified protein. Activities correspond to 10.8 µg protein. Specific activity of the purified protein (0.9 µM) was 254 pmol cAMP·mg^{-1}·min^{-1} (assay conditions: 75 µM ATP, 10 min, 30°C, 2 mM Mn^{2+}, Tris-HCl pH 7.5).

Incubation with DNAse (30 min, 0°C, 20 µg/ml) direct after cell lysis with French Press was performed in case that the holoenzyme bound to the DNA. Pellet and supernatant were as usual separated and the supernatant was further purified with Ni-NTA-agarose (Fig. 4.43). An increment of soluble holoenzyme in the supernatant was not observed.

kDa	A B C D E
116	
66	
45	
35	
25	
18	

A	B	C	D	E
pQE30 sup.	pQE30 pellet	Holo pellet	Holo sup.	Holo eluate
0.06	0.12	40	0.2	0.72

Activity (pmol min^{-1})
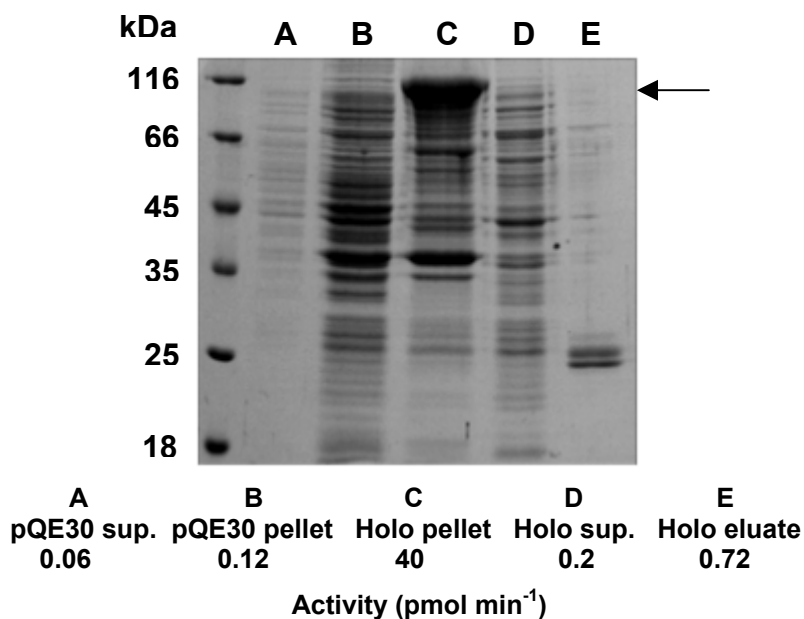
Fig. 4.43: 12.5% SDS-PAGE analysis. Applied were : 24 µg of pellet proteins, 5 µg of supernatant and 1 µg of eluate. AC activities correspond to 2 µg pQE30 supernatant, 54 µg pQE30 pellet, 72 µg holoenzyme pellet, 40 µg holoenzyme supernatant and 4 µg (0.3 µM) of eluate. Specific activity of the purified protein is 181 pmol cAMP·mg^{-1}·min^{-1}. Assay conditions: 75 µM ATP, 10 min, 2 mM Mn^{2+}, Tris/HCl pH 7.5, 30°C, 60 mM imidazole (from purified samples).

Furthermore protease inhibitors (Complete tablets) with and without EDTA (1 mM) were added to the lysis buffer before French Press lysis. Pellets and supernatants were separated and analyzed by SDS-PAGE together with the eluates after protein purification (Fig. 4.44). In both cases intact holoenzyme could be recovered in the eluates.

a

A	B	C
Holo sup.	Holo pellet	Holo eluate
0.4	38.6	1.1
Activity (pmol min^{-1})		
without EDTA		

b

A	B	C
Holo pellet	Holo sup.	Holo eluate
81.3	0.9	1.8
Activity (pmol min^{-1})		
with EDTA		
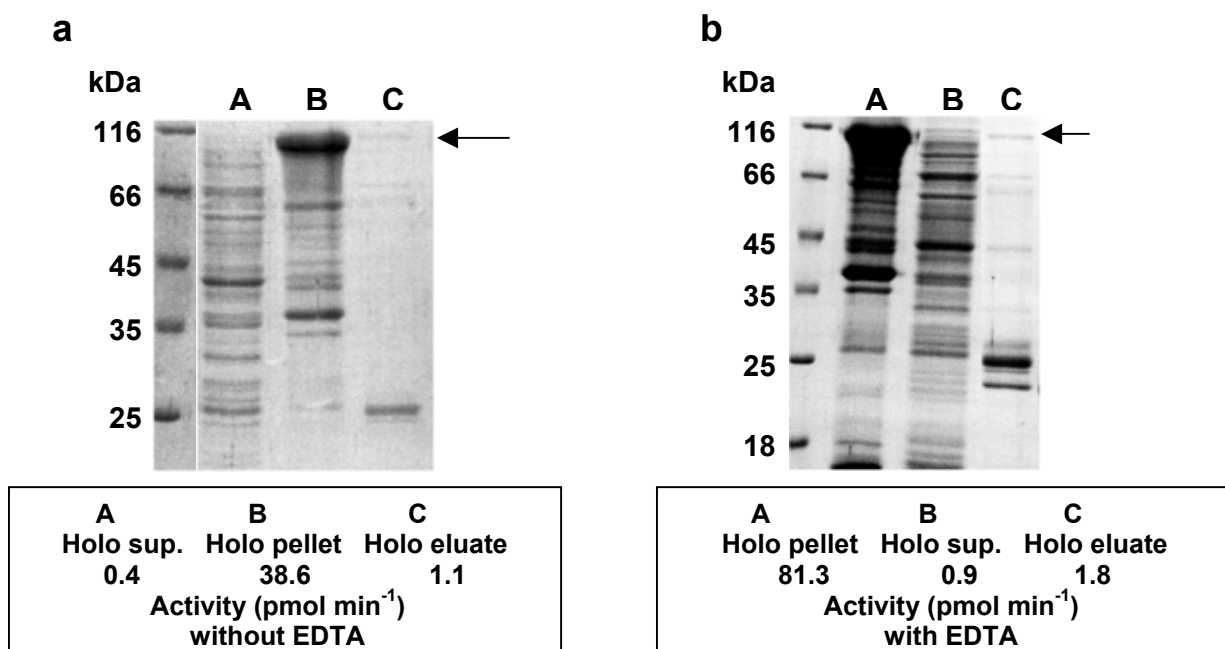
Fig. 4.44: 12.5% SDS-PAGE analysis after addition of (**a**) protease inhibitors only or (**b**) together with EDTA. Applied were 20 µg pellets, 15 µg supernatants and 1.5 µg eluates. AC activities are from (**a**) 50 µg pellet proteins, 35 µg supernatant, 4.8 µg eluate (0.4 µM; 235 pmol cAMP·mg^{-1}·min^{-1}) and (**b**) 90 µg pellet proteins, 52 µg supernatant, 6.8 µg eluate (0.6 µM; 262 pmol cAMP·mg^{-1}·min^{-1}). Assay conditions: 75 µM ATP, 30°C, 2 mM Mn^{2+}, Tris/HCl pH 7.5, 10 min, 60 mM imidazole (from purified samples).

Expression of the holoenzyme with and without IPTG induction (60 µM, 3 h, RT) was investigated. Cell lysis was with addition of protease inhibitors and French Press, pellet and supernatant were separated as usual and the holoenzyme was further purified with Ni-NTA-agarose (Fig. 4.45). The majority of the expressed holoenzyme was found in the

pellet. From the expression without IPTG a little more soluble protein could be purified, but presumably IPTG induction is not directly responsible for the formation of inclusion bodies.

without IPTG			with IPTG		
A	**B**	**C**	**D**	**E**	**F**
Sup.	Pellet	Eluate	Sup.	Pellet	Eluate
5.9	31	1.4	3.4	79	0.1
		Activity (pmol min^{-1})			
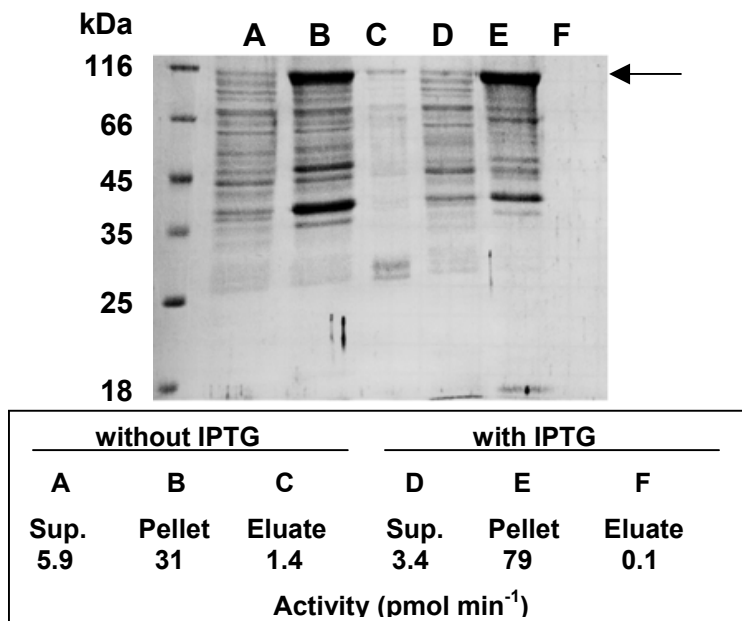
Fig. 4.45: 12.5 % SDS-PAGE of the holoenzyme expression with and without IPTG induction. Applied were: about 4 µg of pellet and supernatant proteins together with 0.5 µg of eluates. AC activities were from 80 µg supernatants, 54 µg pellet –IPTG, 90 µg pellet +IPTG, 3.2 µg eluate –IPTG (0.3 µM; 443 pmol·mg^{-1}·min^{-1}) and 0.8 µg eluate +IPTG (68 nM; 174 pmol·mg^{-1}·min^{-1}). Assay conditions: 100 µM ATP, 30°C, 10 min, 2 mM Mn^{2+}, MOPS pH 7.5, 60 mM imidazole (from purified samples).

4.2.3.2 Membrane preparations and attempts of solubilization

A methodology normally used for solubilizing membrane proteins by addition of detergents was used for the holoenzyme (see chapter 3, purification of insoluble proteins). Detergents polidocanol (1%; Table 4.20; Fig. 4.46) and CHAPS (2%; Fig. 4.47 and 4.48) solubilized good quantities of intact holoenzyme. Other detergents like SDS and Nonidet P-40 were of no use for the solubilization (Fig. 4.47). Degradation products were present in all cases.

SAMPLE		SDS-PAGE	AC-TEST	
		µg protein	µg protein	Activity (pmol min^{-1})
A	Pellet after centrifugation at 3000 g	100	412	185
B	Supernatant after centrifugation at 3000 g	-	74	7.4
C	Supernatant after centrifug. At 100,000 g	14	54	0.7
D	Pellet after incubation with detergent	12	49	15.3
E	Supernatant after 2nd centrifug.100,000 g	12	48	0.6
F	Pellet after 2nd centrifug, at 100.000 g	62	249	113.5
G	Wash fraction of Ni-NTA purification	4	16	0
H	Eluate after Ni-NTA purification	3	16	4.3

Table 4.20: Solubilization and purification of the holoenzyme using 1% Polidocanol. Samples of each step of the process were analyzed by SDS-PAGE and AC-test. Assay conditions: 75 µM ATP, 30°C, 10 min, MOPS pH 7.5. Eluate after purification showed a specific activity of 274 pmol·mg^{-1}·min^{-1} for 16 µg protein (7.6 µM).
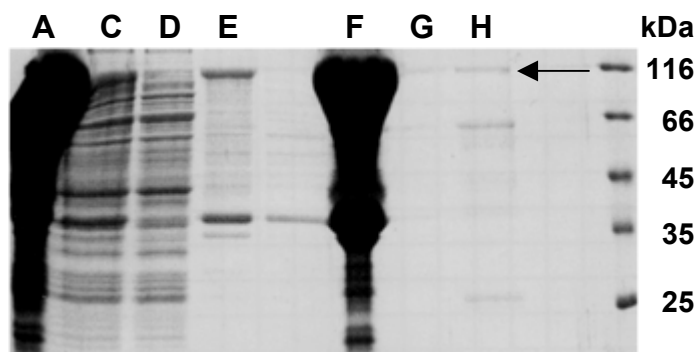
Fig. 4.46: 12.5% SDS-PAGE analysis of the solubilization with 1 % Polidocanol. Sample and protein amounts applied on each lane are specified above in table 4.17.

To investigate the solubilization with 2% CHAPS, 1% TRITON X-100, 1% SDS and 1% NONIDET P-40, pellets before incubation with the detergent and the Ni-NTA-purification eluates were analyzed by Western blot (antibodies anti-RGS-His$_4$). For CHAPS and TRITON, the presence of more degradation products in the eluates than in the pellets was noticeable. With SDS it was impossible to purify detectable amounts of protein.
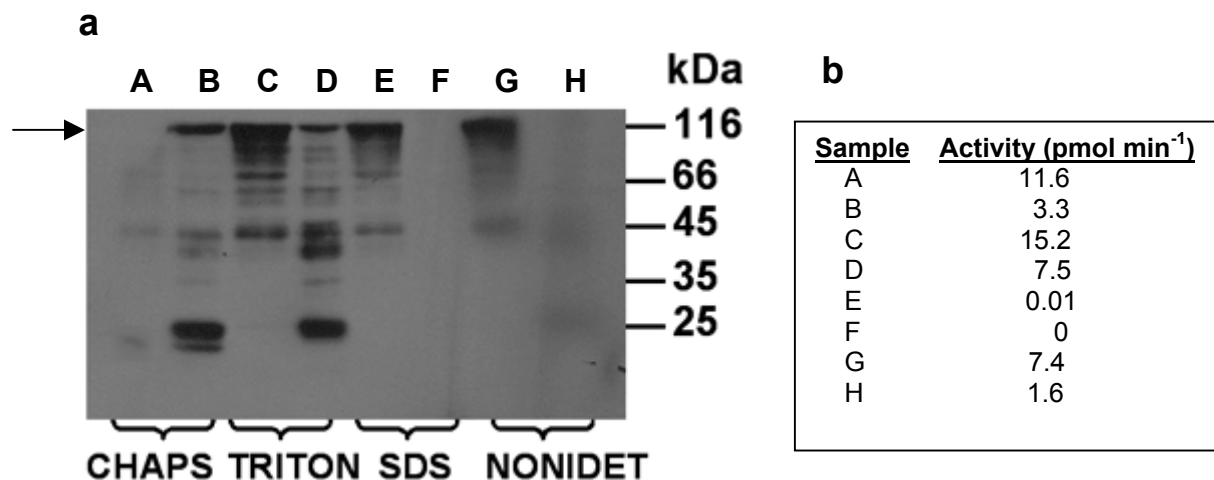
Fig. 4.47: a) Western blot analysis of the pellets (33,000 x g) before incubation with detergent (lanes A, C, E and G each about 4 µg of protein) and of the Ni-NTA-purification eluates (lanes B, D, F and H each 0.2-1 µg of protein). Samples were analyzed with antibodies anti-RGS-His$_4$ diluted 1:2000. Time of exposure: 5 s. **b**) AC-test of the blotted samples: 75 µM ATP, MOPS pH 7.5, 30 °C, 10 min, with ATP-regenerating system. Specific activities of the eluates were: 555 (CHAPS), 1871 (Triton), 0 (SDS) and 311 (Nonidet) pmol mg^{-1} min^{-1}.

Solubilization with CHAPS (2%) was examined in detail. Cell pellets were processed as usual. 8-15 µl of each step were analyzed by Western blot (no determination of protein concentration was made; Fig. 4.48). Degradation products of the holoenzyme were detected in most of the samples with exception of the supernatant after the second centrifugation step at 33,000 x g. A protein concentration of 80 ng was calculated for it by Western blot through a calibration curve (catalytic domain as a standard, anti-RGS-His$_4$). An AC-test using 320 ng protein showed an activity of 0.9 pmol min^{-1} (assay conditions: 500 µM ATP, MOPS pH 7.5, 30 °C, 4 min, with ATP-regenerating system). It corresponds to a specific activity of 2.9 nmol mg^{-1} min^{-1}.

Lane	Sample
A:	French Press homogenate
B:	Pellet (3000 x g)
C:	Supernatant (3000 x g)
D:	Supernatant (33,000 x g)
E:	Pellet (33,000 x g)
F:	Suspension after 1 h with detergent
G:	Pellet 2nd time at 33,000 x g
H:	Supernatant 2nd (33,000 x g)
I:	Eluate after Ni-NTA purific.
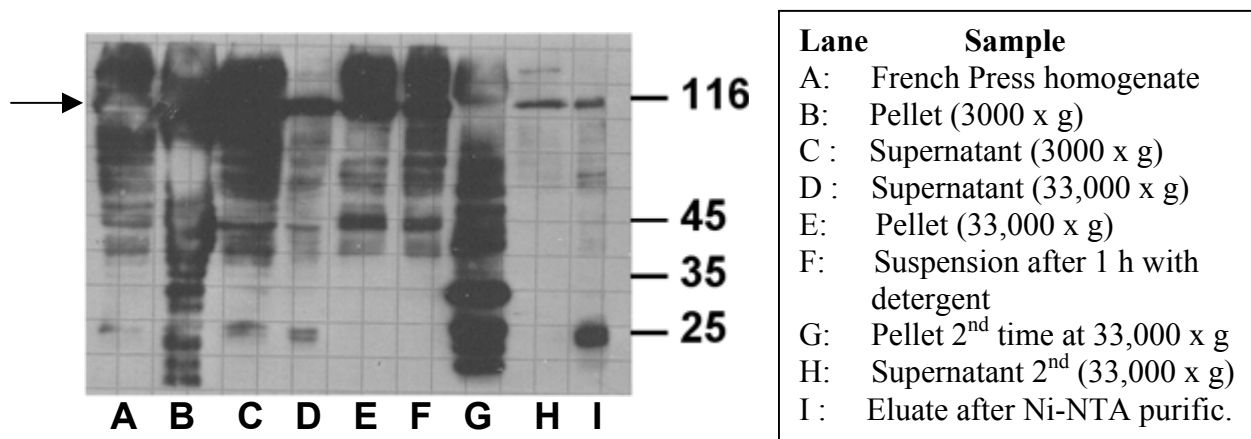
Fig. 4.48: Western blot analysis of the solubilization with 2% CHAPS. Antibodies anti-RGS-His$_4$ were used. Time of exposure was 60 s. 8 µl of pellet samples as well as 15 µl of supernatant and eluate samples were applied.

4.2.3.3 Expression in BL21 STAR and ROSETTA cells

4.2.3.3.1 Expression in BL21 STAR (DE3) [pREP4] cells

Expression was performed with and without IPTG induction (30 µM, O/N, 15°C). Cells were sonicated in presence of protease inhibitors, lysates were incubated with lysozyme and DNAse, pellets and supernatants were separated as usual and protein was purified with Ni-NTA agarose (Fig. 4.49). From 3 x 600 ml of uninduced culture, 20 µg purified protein were obtained and from an induced culture 12 µg protein. AC activities of pellets, supernatants and eluates were measured (Table 4.21). Differences between holoenzyme expression with or without induction with IPTG were not observed. In both cases eluates after purification showed degradation products.
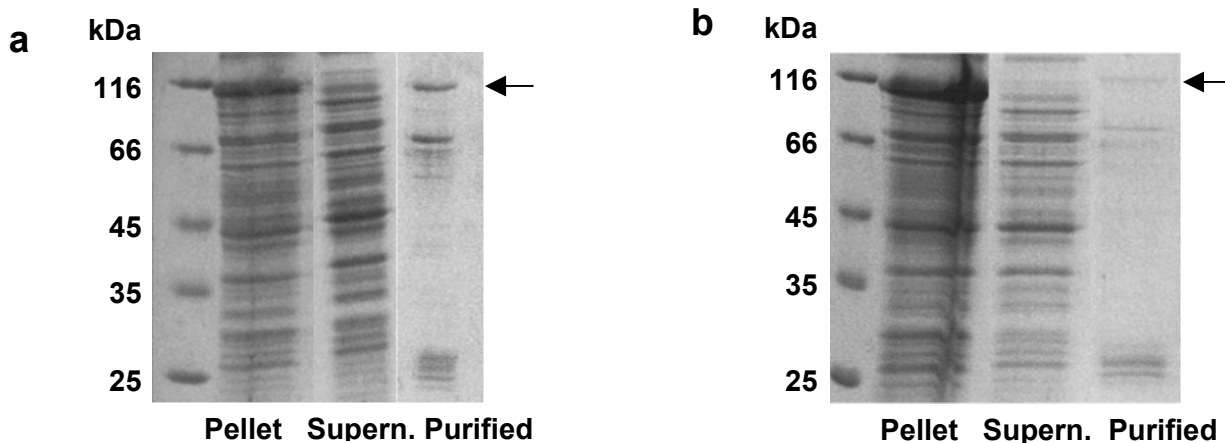
Fig. 4.49: 12.5% SDS-PAGE analysis of the expression in BL21 STAR cells. **a)** Cells were not induced; applied: 20 µg pellet, 15 µg supernatant and 1 µg eluate. **b)** Cells induced with 30 µM IPTG; applied: 20 µg pellet, 9 µg supernatant and 0.6 µg eluate.

Sample		Protein (µg)	Activity (pmol·min⁻¹)	Specific activity (nmol·mg⁻¹·min⁻¹)
Pellet		40.3	59	1.4
Supernatant	uninduced	14.8	0.7	0.05
Eluate		2	7.3	3.7
Pellet		35.6	77	2.2
Supernatant	+30 µM IPTG	8.6	0.2	0.02
Eluate		1.2	6.7	5.6

Table 4.21: AC activities of pellets, supernatants and eluates of expression in BL21 STAR cells. Specific activites of eluates: 3.7 nmol·mg⁻¹·min⁻¹ without induction and 5.6 nmol·mg⁻¹·min⁻¹ with induction (30 µM IPTG). Assay conditions: 500 µM ATP, 10 min, 30°C, 5 mM Mn^{2+} and MOPS pH 7.5. Detection limit was 20 pmol·mg⁻¹·min⁻¹.

An expression was carried out (60 µM IPTG, 16°C, 21 h). Cells were lysed with French Press with prior addition of protease inhibitors (Complete EDTA-free), and the cell homogenate was incubated with lysozyme and DNAse. Pellet and supernatant were separated as usual and protein was purified from with Ni-NTA-agarose (19 h binding). From 3 x 600 ml culture 0.4 mg protein could be purified. Analysis by SDS-PAGE (Fig. 4.50), AC assay (Table 4.22) and Western blot of the pellet, supernatant and eluate were performed (Fig. 4.51).

Sample	Activity (pmol·min⁻¹)	Specific activity (nmol·mg⁻¹·min⁻¹)
Pellet	21	4.3
Supernatant	1.1	0.2
Eluate	31	6.3

Table 4.22: AC activities from expression in BL21 STAR cells. Protein amount tested of each sample was 5 µg. Specific activity of the purified protein: 6.3 nmol·mg⁻¹·min⁻¹. Assay conditions: 500 µM ATP, 10 min, 30 °C, 5 mM Mn^{2+} and MOPS pH 7.5.

112

a

kDa

116 —
66 —
45 —
35 —
25 —
18 —

Pellet Supern. Purified

b

kDa

116 —
66 —
45 —
35 —
25 —
18 —
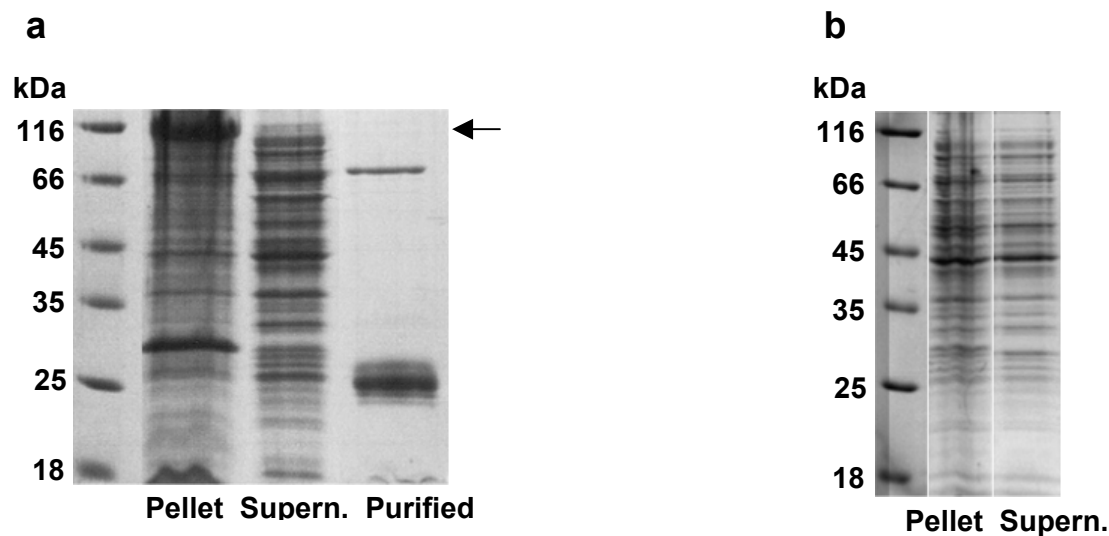
Pellet Supern.

Fig. 4.50: 12.5% SDS-PAGE analysis of (**a**) expression of holoenzyme in BL21 STAR cells; applied were: 20 μg protein of pellet and supernatant and 3.2 μg of eluate and (**b**) expression of empty vector pQE30; applied were: 10 μg of pellet and supernatant.

A   B   C

— 58
— 39
— 24
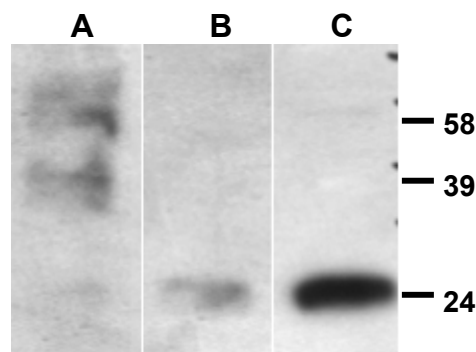
Fig. 4.51: Western Blot analysis of the expression in BL21 STAR cells. Lanes A and B correspond to the proteins of the pellet (5 μg) and eluate (1.4 μg) respectively, analyzed with anti-KD0386. Lane C corresponds to the eluate analyzed with anti-DB0386 (antibodies against the DNA-binding domain). Time of exposure: 15 s. Molecular weight of the signals were calculated with the marker peqGold.

After SDS-PAGE and Western blot analysis a band of approximately 25 kDa was identified as the main degradation product of the holoenzyme after purification. A considerable amount of non-degraded protein could be purified after expression in STAR cells when cell lysis was performed with sonification, but with the unavoidable presence of degradation products.

4.2.3.3.2 Expression in BL21 ROSETTA [pREP4] cells

Expression was carried out (12 h, 12°C, 60 µM IPTG), cells were lysed with French Press with prior addition of protease inhibitors (Complete EDTA free), cell homogenate was incubated with lysozyme and DNAse, pellet and supernatant were separated as usual and the protein was purified with Ni-NTA agarose (19 h binding). SDS-PAGE analysis (Fig. 4.52), AC assay (Table 4.23) and Western blot (Fig. 4.53) were performed.
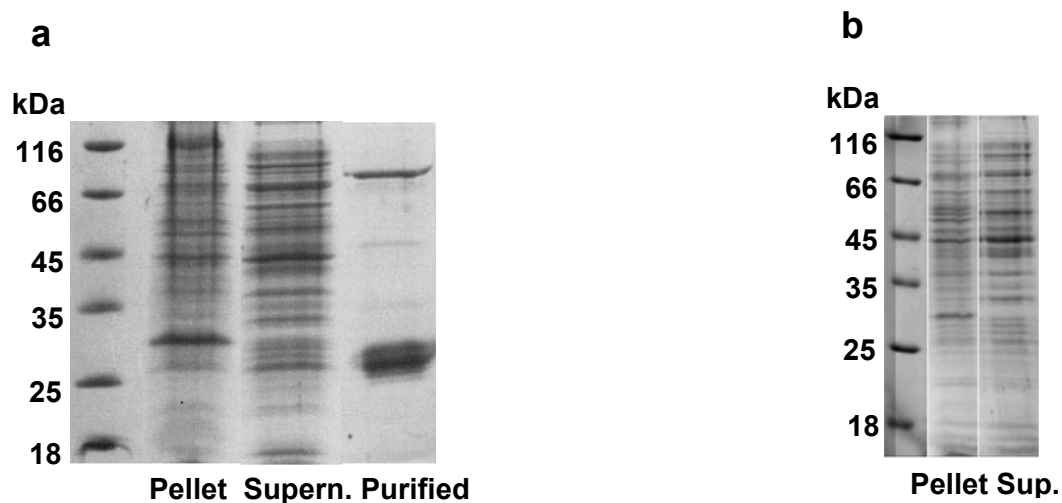
Fig. 4.52: 12.5% SDS-PAGE analysis of expression in BL21 ROSETTA cells of (**a**) the holoenzyme; applied were: 20 µg protein of pellet and supernatant and 3.8 µg of eluate and (**b**) the empty vector pQE30; applied were: 10 µg protein of pellet and supernatant.

Sample	Activity (pmol·min^{-1})
Pellet	13.2
Supernatant	0.8
Eluate	23.8

Table 4.23: AC activity of the holoenzyme expressed in BL21 ROSETTA cells. Protein amount tested was 5 µg of each sample. Specific activity of the purified protein: 4.8 nmol·mg^{-1}·min^{-1}. Assay conditions: 500 µM ATP, 10 min, 30 °C, MOPS pH 7.5.
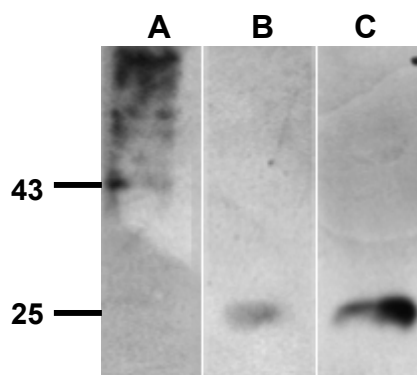
Fig. 4.53: Western Blot of the holoenzyme expressed in ROSETTA cells. Lanes A and B: pellet (5 µg) and eluate (1.2 µg) analyzed with anti-KD0386. Lane C correspond to the eluate analyzed with anti-DB0386 (antibodies against the DNA-binding domain, see below). Time of exposure: 15s.

4.2.3.4 Anion exchange chromatography of the purified holoenzyme

The holoenzyme was expressed in BL21 STAR cells (1.2 L culture, 12°C, 15 h, 60 µM IPTG). Cell lysis was with protease inhibitors (Complete tablets, EDTA free) and French Press, lysates were incubated with lysozyme and DNAse, after centrifugation (33,000 x g, 1 h) protein purification was carried out as usual, 20% glycerol was added. Sample for chromatography was 800 µl of Ni-NTA eluate (about 280 µg protein; Fig. 4.54a). Chromatography was described in chapter 3 more exact. A 40 µL sample of fractions with UV absorption was tested. An SDS-PAGE analysis and Western Blot (Fig. 4.55) were performed only for those fractions which showed AC activity.
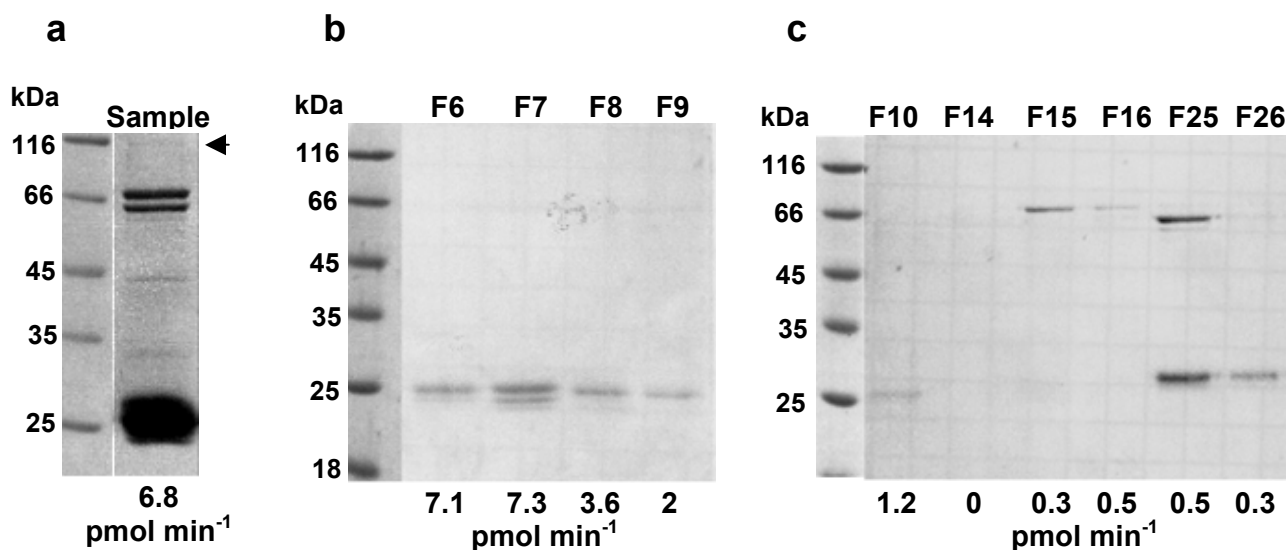
Fig. 4.54: a) 12.5% SDS-PAGE analysis of the Ni-NTA eluate sample applied for chromatography; **b** and **c**) fractions collected with 100mM NaCl (6 to10), 200 mM NaCl (14 to16) and 300 mM NaCl (25 and 26). Activities in AC-test (850 µM ATP, 30 °C, MOPS pH 7.5, 5 mM MnCl$_2$, 20 min) are shown below.
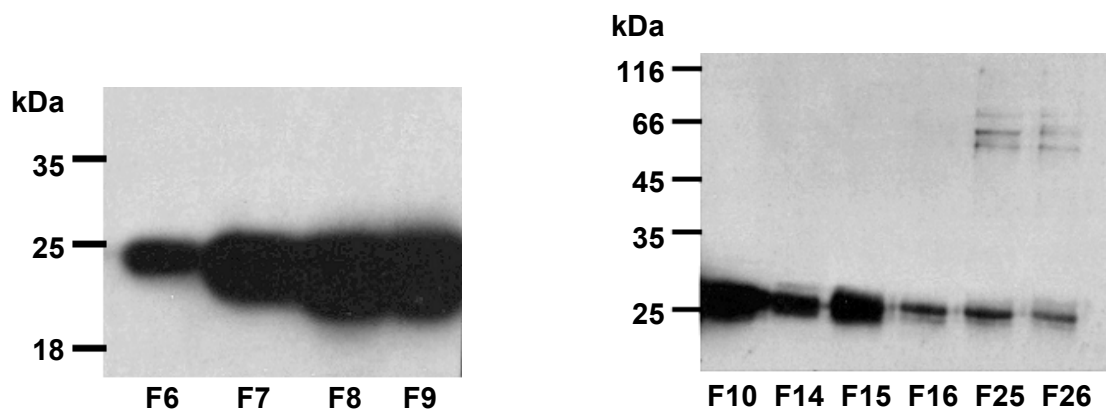
Fig. 4.55: Western blot analysis of the fractions analyzed in figure 4.54. Antibodies anti-KD0386 diluted 1:10000 were used. Time of exposure was 60 s.

All fractions which showed AC activity contained a degradation product of approximately 25 kDa containing the AC catalytic domain of Rv0386. Another degradation product of about 60 kDa contained also the catalytic domain but had low activity presumably because of the high NaCl concentrations (fractions 25 and 26, 300 mM NaCl).

4.2.3.6 Determination of Rv0386 orthologs in *M. bovis* BCG and *M. smegmatis*

Mycobacterium bovis and *Mycobacterium smegmatis* cell cultures (about 3 days old) were obtained from Priv. Doz. Dr. Peter Sander (Institut für Medizinische Mikrobiologie, Universität Zürich). 50 ml cultures were centrifuged (5,000 x g, 10 min). Pellets were frozen with liquid nitrogen, then suspended in 3 ml of 50 mM Tris-HCl pH 7.5 and lysed with French Press. Cell homogenates (1 ml) were centrifuged (20,000 x g, 30 min), supernatants were separated from pellets, and pellets were suspended in 100 µl of 50 mM Tris-HCl pH 7.5. Samples were analyzed by Western blot with specific antibodies anti-KD0386 and anti-DB0386. The presence of an ortholog of Rv0386 was shown for *M. bovis* (detected in the soluble fraction only) but not in *M. smegmatis* (Fig. 4.56). These results are in line with genomic data which indicate the presence of a Rv0386 ortholog in *M. bovis*, but not in *M. smegmatis*. With anti-KD0386 additionally to the ~116 kDa band detected, other more intense protein signals could be detected, maybe corresponding to other cyclases which cross-reacted with the antibodies. With the antibodies anti-DB0386 only a protein of about 64 kDa was detected, possibly another protein of *M. bovis* with a similar DNA-binding domain.
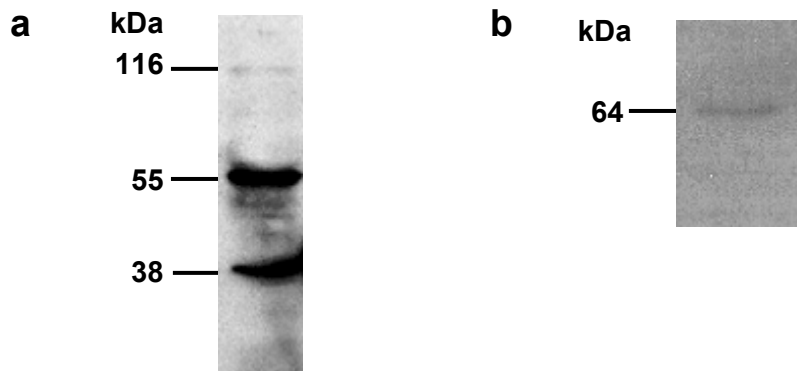
Fig. 4.56: Western blot analysis of the supernatant proteins found in *M. bovis BCG* with specific antibodies anti-KD0386 (**a**) and anti-DB0386 (**b**). 11 and 7 µg protein were applied in **a** and **b** respectively. Antibodies anti-KD0386 were diluted 1:10000 and the anti-DB0386 1:5000. Time of exposure was 60 s (**a**) and 90 s (**b**). Molecular weight of the proteins detected were calculated with the help of the protein marker peqGold.

4.2.4 Expression of the putative ATPase domain of Rv0386

This domain (351 aa, 39 kDa) was expressed in BL21 cells (30 µM IPTG, 25°C, 3 h). Cells were sonificated, pellet and supernatant were separated and analyzed by SDS-PAGE (Fig. 4.57a). After Ni-NTA purification the yield of protein was 0.1 mg protein from 400 ml culture. Most of the protein was found in the pellet and after Ni-NTA purification the protein could be detected only by Western blot (Fig. 4.57b). Molecular weight of this protein is about 39 kDa (including N-terminal His-tag).
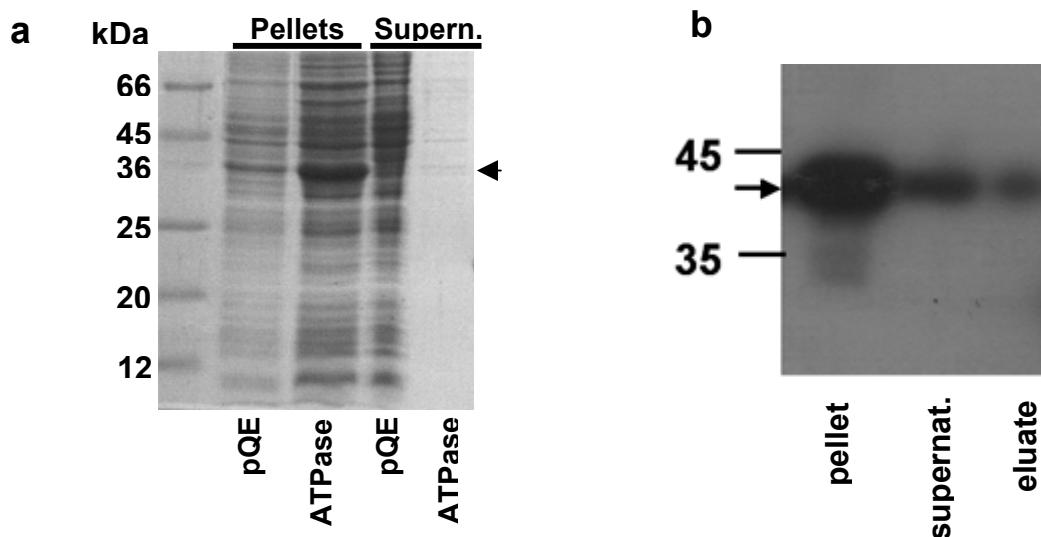
Fig. 4.57: a) 15% SDS-PAGE analysis of empty vector pQE30 and ATPase domain expressed in BL21 cells (Coomassie staining). Applied were: 20 µg of pellets and pQE30 supernatant and 8 µg of ATPase supernatant. **b)** Western blot of pellet, supernatant and eluate from Ni^{2+}-NTA of the ATPase domain with anti-RGS-His$_4$. Applied were: 2 µg of pellet and supernatant and 1 µg of purified protein. Time of exposure: 5 seconds.

The ATPase domain was also expressed in BL21 STAR cells (6 µM IPTG, 20°C, 3 h). Cells were sonificated, pellet and supernatant were separated and analyzed by SDS-PAGE (Fig. 4.58a). For protein purification and Western blot analysis a second expression was carried out (30 µM IPTG, 22°C, 3.5 h). A sample of pellet and supernatant was separated and the rest of the supernatant was purified as usual. From 600 ml culture it was impossible to determine protein with BIORAD in the Ni-NTA eluate. All samples were analyzed by Western blot (Fig. 4.58b). Since only small amounts of ATPase domain were soluble, no ATPase tests were attempted.
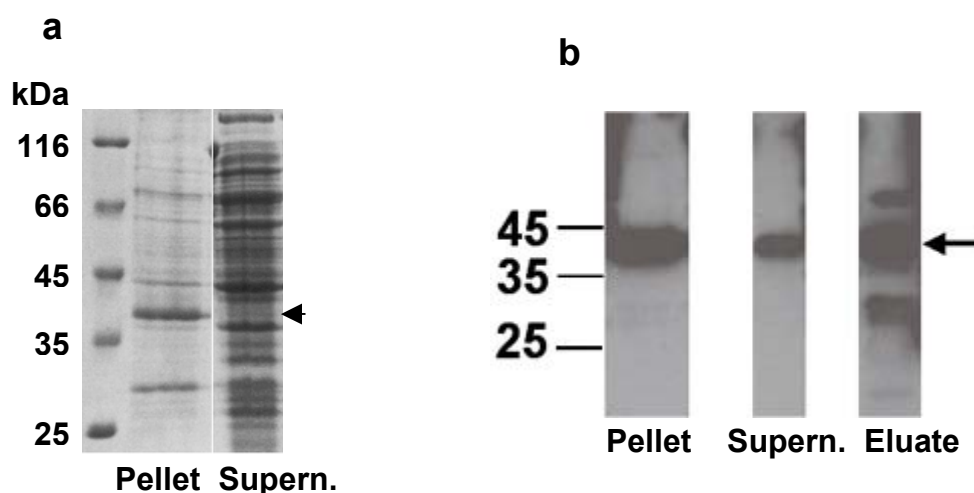
Fig. 4.58: a) 12.5% SDS-PAGE analysis of the ATPase domain in BL21 STAR cells. About 20 µg protein of both pellet and supernatant were applied. **b)** Western blot analysis. Pellet (1.2 µg), supernatant (0.5 µg) and 15 µl eluate after Ni-NTA purification were analyzed with anti-RGS-His$_4$. Time of exposure: 15 s.

4.2.5 Expression of the putative transcription factor domain of Rv0386

This domain (929 aa, 100 kDa including His-tag) was expressed in BL21 and BL21 STAR cells (60 µM IPTG, 23 °C, 3 h) and analyzed by SDS-PAGE (Fig. 4.59). Only the pellet of BL21 STAR cells showed an overexpressed protein compared with the control.
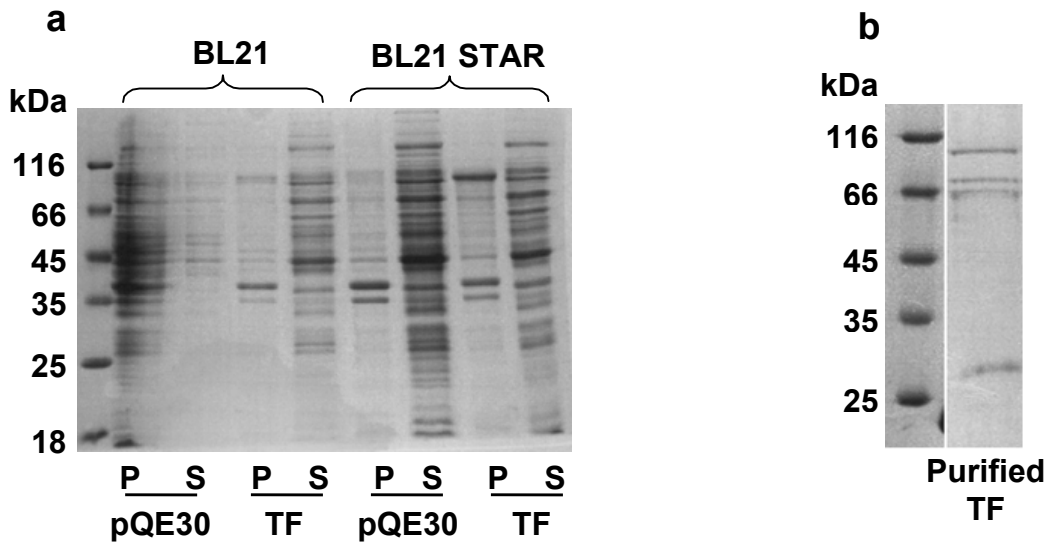
Fig. 4.59: 12.5% SDS-PAGE analysis of **a)** empty pQE30 and transcription factor (TF) domain expressed in BL21 and BL21 STAR cells. Applied were: 15-20 µg of pQE30 pellet (BL21), pQE30 supernatant (STAR) and TF supernatants (BL21 and STAR); 8-12 µg of pQE30 and TF pellets (STAR), pQE30 supernatant and TF pellet (BL21). **b)** TF domain purified with Ni-NTA-agarose after expression in STAR cells. 15 µl of the eluate were applied. P=pellets and S=supernatants.

For Western blot analysis expression was carried out again (30 µM IPTG, 3 h, 22 °C). Cells were sonicated (with protease inhibitors and lysozyme). Pellet, supernatant and eluate after Ni-NTA-agarose purification were analyzed by Western blot. The presence of degradation products in pellets and eluates was observed.
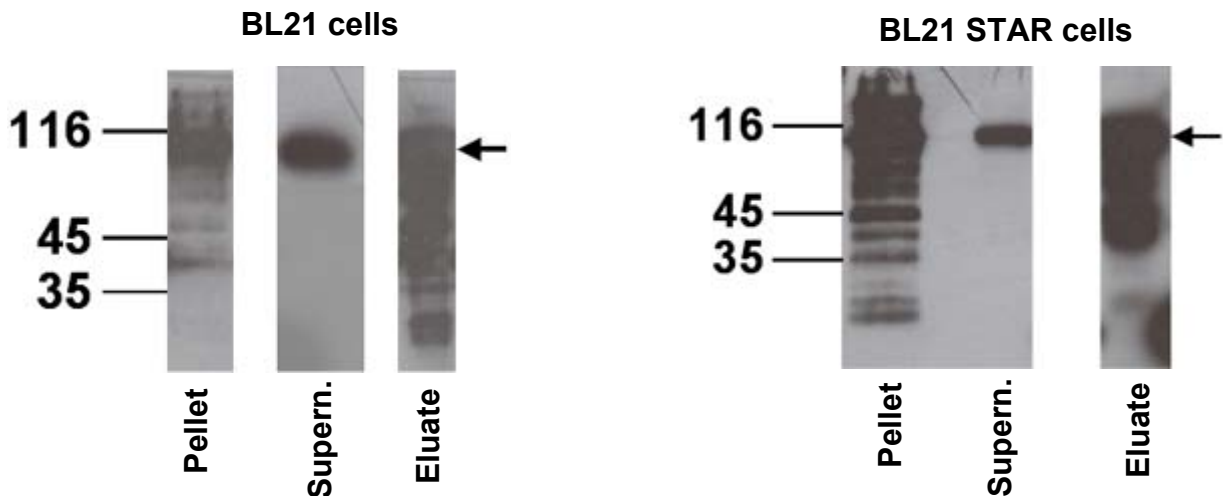
Fig. 4.60: Western blot of the transcription factor domain expressed in BL21 and BL21 STAR cells. Applied were: about 1 µg of pellets, 0.5 µg of supernatants and 15 µl of the Ni-NTA-agarose eluate. Antibodies anti-RGS-His$_4$ were used. Time of exposure: 15 s.

4.2.6   Expression of the putative DNA-binding domain of Rv0386

This domain (103 aa, 12 kDa including His-tag)) was expressed in BL21 cells (4 h, 25°C, 60 μM IPTG). Pellet, supernatant and eluate after Ni-NTA purification were analyzed by SDS-PAGE and Western blot (Fig. 4.61 a, b, c). This protein tended to dimerize and multimerize.
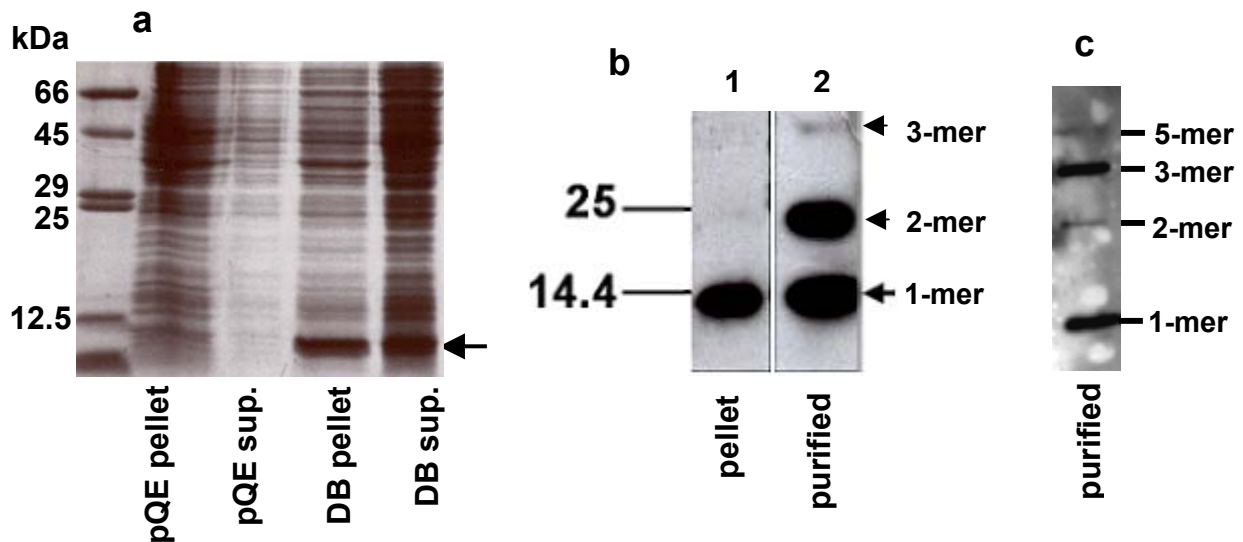
Fig.4.61: (**a**)15% SDS-PAGE analysis of the DNA-binding domain (DB) expressed in BL21 cells (pQE30 empty vector as a control). (**b**) Western blot of 0.3 μg of pellet and eluate from Ni-NTA analyzed with anti-RGS-His$_4$ antibodies. Time of exposure: 15 seconds. (**c**) Western blot of the purified DB domain (1 μg) after dialysis with phosphate buffer and concentration. Time of exposure: 30 s. Protein of lane **b2** was purified without DNAse and that of lane **c** with addition of it.

4.2.6.7   Sensitivity and specificity of the antibodies anti-DB0386

A protein sample with a concentration of 1-2 μg/μl (Fig. 4.62a) was used by BioGenes to produce affinity purified antibodies against the DNA-binding domain of Rv0386 (see chapter 3). The reactivity of the antibodies from two immunized rabbits was tested against different concentrations of DNA-binding domain by Dot Blot (Fig. 4.62b). With an antibody dilution of 1:10 000 it was possible to detect 10 ng protein (rabbit 5081).
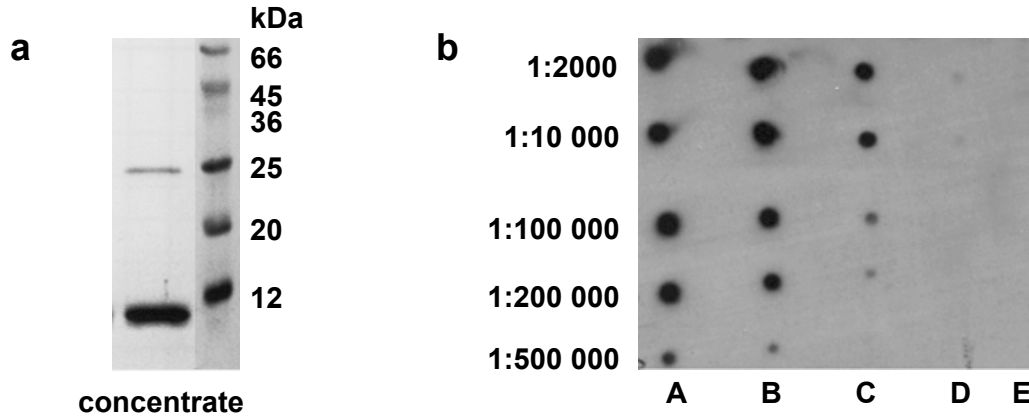
Fig. 4.62: (**a**) 15% SDS-PAGE analysis of the protein used for production of anti-DB0386. 2 µg protein were applied. (**b**) Dot Blot. 5 different antibody dilutions (1:2000 to 1:500,000) as well as protein concentrations were used for testing purified antibodies from rabbit 5081. Protein concentrations were: 300 ng (A), 100 ng (B), 10 ng (C), 1 ng (D) and 0.1 ng (E). Time of exposure: 30 s.

With anti-DB0386 it was possible to detect cross-reactivity with proteins of the three *E. coli* strains used for expression within this work (Fig. 4.63). These proteins have a MW (derived from standards on the blotted membrane) of 68 and 25 kDa and presumably contain a DNA-binding domain very similar to that of Rv0386. Based on these results, this antibody seems to recognize many DNA-binding proteins of *E. coli* and probably also of *M. tuberculosis*. Its utility for immunodetection of Rv0386 might be only possible simultaneously with antibody anti-KD0386.
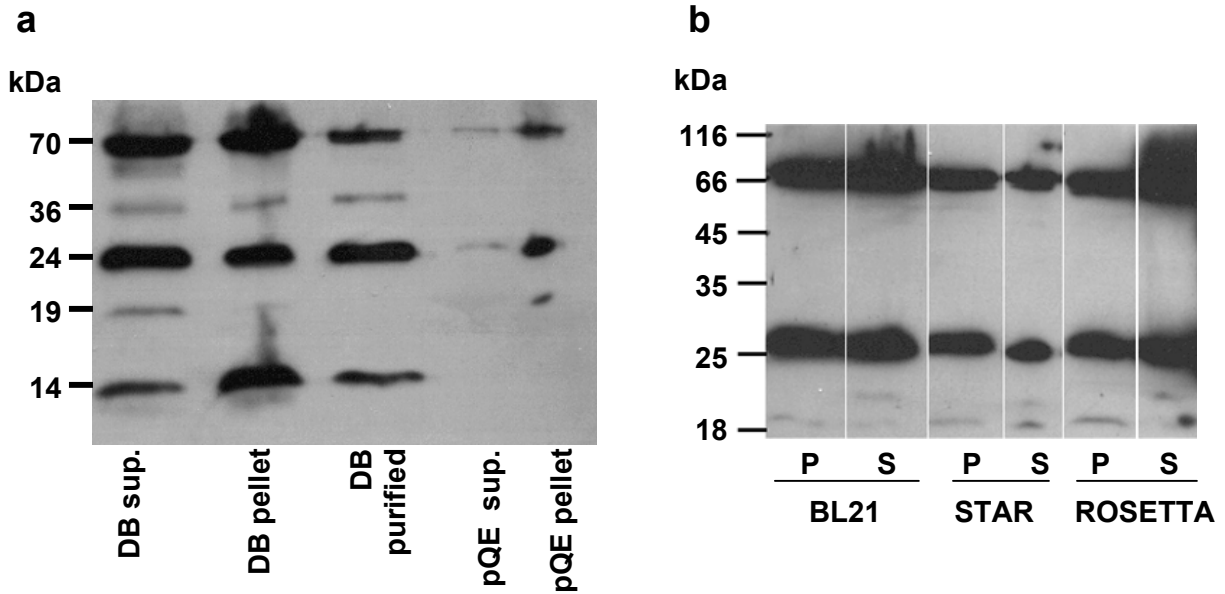
Fig. 4.63: (**a**) Western blot of the expressed DNA-binding domain and empty vector pQE30 in BL21 cells. Antibodies anti-DB0386 1:5000 were used. Time of exposure: 30 s. Molecular weights were calculated with a marker. Applied were: 20 µg of DB supernatant, pellet and pQE30 pellet. From pQE30 and DB purified, 3 and 1 µg respectively were applied. (**b**) Western blot of expression of empty vector pQE30 in BL21, STAR and ROSETTA cells analyzed with antibodies anti-DB0386 (diluted 1:10000). 10 µg protein were applied in each lane. Time of exposure was 60 s.

5   Discussion

5.1 Chimeras of mycobacterial Rv1625c mutants with soluble mammalian and *Paramecium* cyclases

As a complementary study to the already published data regarding the expression and characterization of Rv1625c (Guo et al., 2001), additional point mutants N372A, N372T and D300S were generated here. The idea was to check a) whether a threonine might be able to take over the function of an asparagine in transition state stabilization and b) whether a serine might substitute for aspartate in substrate definition. This was not the case. The mutants were essentially inactive. This was further proof that the six canonical amino acids identified in mammalian ACs as critical for catalysis and conserved in Rv1625c, are also absolutely essential in the latter.

Since soluble mammalian AC domains C1 and C2 from different isoforms have shown a restored catalytic activity when mixed i.e. IC1/IIC2 (Tang and Gilman, 1995; Whisnant et al., 1996; Yan et al., 1996), VC1/IIC2 (Désaubry et al., 1996; Dessauer et al., 1997; Sunahara et al., 1997; Tesmer et al., 1997) and IXC1/IIC2 (Hoffmann, 1999), the possibility to get active chimeras by mixing mycobacterial soluble catalytic domains with mammalian ones was enticing. The mycobacterial Rv1625c inactive mutant D300A lacking its metal binding function and consequently their mammalian-like C1-domain function, were barely able to reconstitute enzymatic activity when mixed with mammalian IC1, VC1, VIIC1 or IXC1 domains. Similarly inactive mutants R376A, N372T and N372A having no mammalian-like C2-function were not really capable to complement inactive mammalian IIC2 and IXC2 domains.

The observed specific AC activity of these chimeras was very small, actually tiny compared to Rv1625 wild type. This fact indicates that although the substrate binding and some catalysis may have taken place, no complementation of the C1/C2 domains occurred. One explanation could be the differences of dimerization epitopes between Rv1625c and mammalian ACs, e.g. the "arm region" (proposed in Linder et al., 2002) where the motif KWQ is replaced by RFF in Rv1625c. This KWQ motif is invariant in ACs I-VIII and specially the residue Q (Q_{584} in AC type V from rabbit) was identified as crucial on the dimerization interface (Zhang et al., 1997; Sunahara et al., 1998; Tucker et al., 1998). Although recent studies showed that in the chimera VC1a/IIC2 the KWQ

motif is not involved in substrate binding (Tesmer et al., 1999), it could be possible that its direct participation depends on the cyclase isoform. The lack of the KWQ motif in Rv1625c and also the conformational changes due to the mutations in the mycobacterial monomers could explain the lack of a robust reconstitution.

Surprisingly, the C2-positioned domain from the *Paramecium* GC and its triple mutant ParaGC-C2KWQ reconstituted a soluble AC when mixed with mammalian IIC2 domain (Linder et al., 2000) and with the mycobacterial mutant D300A. The topological inversion of the cytosolic domains in the *Paramecium* GC and the fact that the KWQ motif is an AC-specific stabilizing motif of the dimerization between C1 and C2, was in agreement with Linder et al. (2000). The C2-positioned *Paramecium* GC domain contributed the metal binding function while IIC2 or D300A contributed the substrate specificity function upon mixing. Additionally, the mutant ParaGC-C2KWQ stabilized and/or enhanced the interaction of the C1 and C2 equivalent domains since the specific activity of ParaGC-C2KWQ/IIC2 was about 3-fold higher than ParaGC-C2/IIC2. That was not the case with the chimera ParaGC-C2KWQ/D300A which was equally active as the ParaGC-C2/D300A dimer. Dimerization of the chimera ParaGC-C2/D300A was substrate dependent, no activity with Mg^{2+} was supported, the diterpene forskolin did not stimulate and P-site inhibitors did not inhibit. It seems that the individual catalysis and regulation properties possess enough structural complementarity to allow formation of a functional chimera. Kinetic characterization of ParaGC-C2/D300A showed an ATP affinity (K_m= 201 µM) similar to that of Rv1625c AC domain (K_m= 150 µM; Guo et al., 2001) with about a 70-fold reduced V_{max} (29.5 nmol cAMP·mg^{-1}·min^{-1}).

An open question concerning the Rv1625c AC was why was it not stimulated by forskolin, a character shared among all but type IX of the mammalian ACs. The alignments in chapter 4 (Fig. 4.1 a, b) clearly indicate the amino acid differences which may be responsible for this lack of a forskolin effect on Rv1625c. While in mammalian ACs the binding of forskolin is shared between C1 and C2 domains (the C1 domain provides a threonine and C2 a serine, e.g., T_{426} in ACI-C1 and S_{927} in ACII-C2), in the mycobacterial AC Rv1625c those residues are substituted by an asparagine (N372) and an aspartate (D300), respectively, both directly implicated in catalysis. Rv1625c acts as a homodimer with two ATP binding sites in contrast to the mammalian ACs in which one site has evolved to bind forskolin instead of ATP. Therefore, it was speculated that the

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mutants N372T and D300S may reconstitute an active heterodimer with a single ATP-binding site and a forskolin-binding site.

Somewhat surprisingly mutants N372T and D300S subserving C1 and C2 functions, respectively, did complement each other quite well to form an active enzyme (Table 4.6, Fig. 4.10). A forskolin stimulation, however, was not observed. This is evidence that those two amino acids implicated in forskolin stimulation are required but not sufficient to confer diterpene sensitivity. Obviously, the binding pocket for forskolin at the C1/C2 interface is more elaborate and probably requires additional structural features absent in Rv1625c. Similarly, chimeras N372T/IIC2 and N372T/IXC2 were not activated by addition of forskolin. The observed weak effects were solely due to the solvent DMSO. Reconstitution experiments with the mutants N372T, N372A and D300S were carried out. Chimeras of these mutants did not show Mg^{2+} supported activity in agreement with the observations for the wild type (Guo et al., 2001). By titration of N372T, N372A and R376A with increasing amounts of D300A or D300S it was determined that they folded correctly and are capable of forming productive heterodimeric complexes in analogy to a mammalian C1-C2 arrangement. Nevertheless, the apparent dissociation constants derived from the titration curves showed lower affinity of the monomers to each other in comparison with the wild type (Guo et al., 2001). Conformational changes due to mutations may explain this effect on the dimerization of these chimeras.

5.2 Adenylyl cyclase catalytic domain of Rv0386

The gene product of Rv0386 was not originally predicted as an AC but denoted as a transcriptional regulator containing an ATP/GTP-binding site motif A, a luxR family signature and an helix-turn-helix motif. After a functional classification of nucleotide cyclase superfamilies in *M. tuberculosis* through computational methods and phylogenetic analysis, the cyclase homology domain (CHD) of Rv0386 was described as a cytoplasmic class III AC with similarity to CHDs detected in other Gram-positive bacteria, e.g. in *Streptomyces* (McCue et al., 2000). Following subclassification of class III ACs, Rv0386 was assigned to class IIIc ACs in which the frequency of substitutions in the six canonical catalytic residues is remarkably high (Linder and Schultz, 2003). In the CHD of Rv0386 a substrate-defining glutamine and an asparagine instead of the canonical lysine and aspartate were found. Since enzymatic activities have been

demonstrated for unorthodox cyclases having variations in the canonical residues and since all known subclass IIIc enzymes appear to function as homodimers (Linder and Schultz, 2003), two major questions were addressed: Has AC Rv0386 an AC activity in spite of the changes of both amino acids involved in defining the substrate specificity? And if active, is AC Rv0386 functioning as a homodimer like mammalian-type AC Rv1625c?

The recombinant CHD Rv0386$_{(1-175)}$ was used for answering these questions. This AC was capable of converting ATP to cAMP, using Mn^{2+} as a cofactor. Yet it displayed a rather low activity (4.8 nmol of cAMP·mg^{-1}·min^{-1}), corresponding to only 1/400 to 1/250 of AC activities characterized in other mycobacterial ACs, e.g. Rv1625c (Guo et al., 2001) and Rv1264 (Linder et al., 2002). Most surprisingly, Rv0386$_{(1-175)}$ had also a 30% GC side activity strictly dependent on Mn^{2+} as divalent cation cofactor. No AC nor GC activities were detectable with Mg^{2+} as a cofactor. A preference for Mn^{2+} was also shown for Rv1625c and Rv1264 (Guo et al., 2001, Linder et al., 2002). It was reported that macrophages are able to import Mn^{2+} providing a suitable environment for resident *M. tuberculosis*. The mycobacterial gene product Rv0924c was recently reported to be a homologue of the macrophage Nramp Mn^{2+} transporter protein (Agranoff et al., 1999; Reddy et al., 2001). The kinetic properties of Rv0386$_{(1-175)}$ such as temperature optimum of 30-37°C, activation energy of 76 kJ/mol and pH optimum of 8.3 were within the usual range reported for the mammalian AC catalysts. No activation by forskolin and no inhibition by the P-site inhibitor 2'd3'-AMP were observed, as expected. Substances like α,β-CH$_2$-ADP (ADP-analog) and cordycepin 5'-triphosphate (ATP-analog) inhibited AC activity as did cAMP derivates like monobutyryl cAMP and dibutyryl cAMP. Dimerization of the catalytic domain of Rv0386 was demonstrated by the fact that AC specific activity increased almost 5-fold by increasing protein concentration and an apparent association constant of 0.1 µM was derived indicating a high affinity of the monomers to each other. That was further substantiated by western blotting, where with the specific antibody anti-KD0386, the presence of monomers, dimers, tetramers together with higher multimers was observed. Using GTP as a substrate, the specific activity increased only 3-fold upon increasing protein concentration and the calculated apparent association constant was 0.9 µM. The fact that the dimerization process occurs slower with GTP than with ATP points to an important role played by the substrate during the formation of the

homodimeric catalytic complex. AC kinetic parameters were K_m= 417 µM and V_{max}= 4.8 nmol of cAMP·mg^{-1}·min^{-1}. For GC activity K_m and V_{max} values of 299 µM and 1.6 nmol of cAMP·mg^{-1}·min^{-1} were obtained.

To determine if the amino acid residues Q_{57} and N_{106} which deviate from the canonical K and D, are critical for catalysis in Rv0386, they were mutated to A. Residual AC specific activities of less than 2% for mutants Q57A and N106A demonstrated the important role played by Q and N when recognizing ATP as a substrate. GC residual activities of these mutants were also lower showing that the same residues are involved in recognizing GTP as a substrate. Whether the AC activity of Rv0386 is so low because of the substituted canonical K and D residues was examined with the mutants Q57K, N106D and Q57K/N106D. A reduction of more than 90% of the AC activity was observed by these three mutants. The GC activity was also affected as expected. Interestingly, comparison of the AC and GC activities of the mutants showed a significant reduction in the substrate discrimination for mutants Q57K/N106D and N106A. These results strongly argue that completely different substrate-specifying mechanisms exist in Rv0386 in comparison with canonical class III ACs.

Through an alignment of the sequences of the AC domain of Rv0386 and the *Paramecium* GC (see appendix and alignment in section 4.2.2.8, chapter 4) the substitution of Q_{57} and N_{106} (Rv0386) by E_{1681} and S_{1748} (in ParaGC) was identified. Assuming that E_{56} (Rv0386) might functionally correspond to E_{1681} of ParaGC, the mutation of N_{106} to S was examined for its substrate specificity. This mutant had no increased GC activity, on the contrary it was reduced >80%. The AC activity of N106S was also reduced but to a lesser extent. Considering that the AC activity of Rv0386 is lower in comparison with the mycobacterial AC's Rv1625c and Rv1264, its low but detectable GC activity points to a certain plasticity of the substrate-defining mechanism. A hypothetical substrate binding mechanism for AC Rv0386 is shown in figure 5.1. This way of binding of ATP or GTP is drawn according to the mode of binding of cAMP or cGMP within the phosphodiesterase (PDE) superfamily based on the X-ray structure of the catalytic domain of PDE 4B2B (Xu et al., 2000).
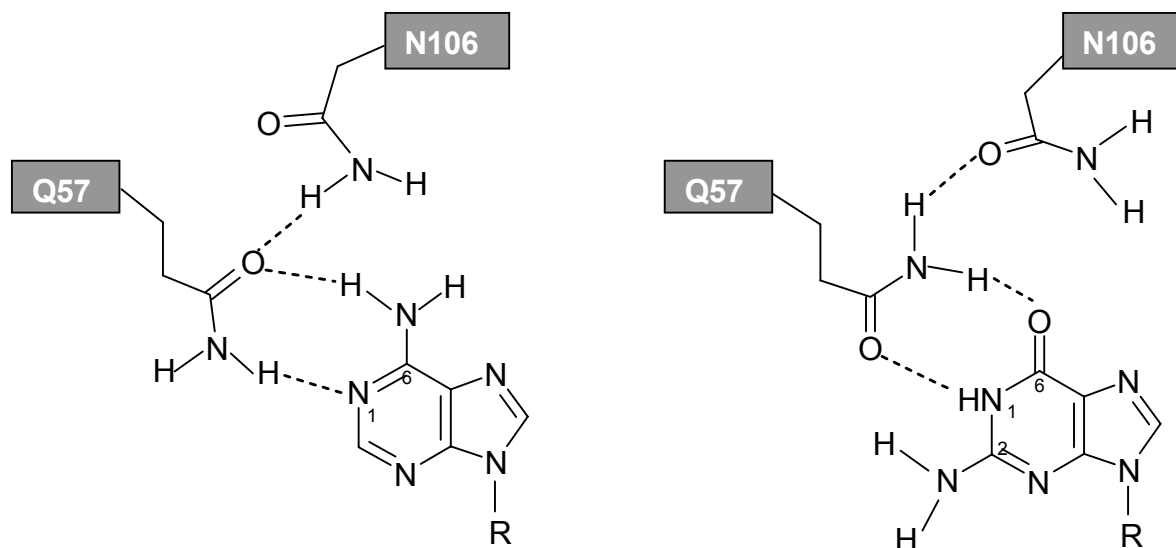
Fig. 5.1: Hypothetical substrate recognition mechanism proposed for Rv0386 as adenylyl cyclase (left) and as guanylyl cyclase (right). See explanation in the text.

According to the model of cAMP binding in PDE4, the side chain of a glutamine residue present in the active site forms hydrogen bonds with the N_1 and N^6 groups of adenine, but this glutamine could also accommodate the N_1 and O^6 groups of guanine in cGMP-binding by rotation of its amide group by 180° (Xu et al., 2000). Compared with that, in Rv0386 Q_{57} could be responsible for the dual specificity and N_{106} could form a hydrogen bond with Q_{57} affecting the correct orientation of the substrate towards the transition state. In PDE4 this role is taken by a tyrosine side chain. Unfortunately, the low AC and GC activities of mutants Q57K, Q57A, N106D, N106A and Q57K/N106D did not permit kinetic studies to determine if their diminished turnover was due to reduced substrate-affinity, conformational changes affecting the transition state or both. However, the kinetic parameters obtained for N106S using ATP as a substrate somewhat support the hypothetical substrate-binding mechanism proposed above. Since N106S showed a reduced V_{max} but not reduced K_m in comparison with the catalytic domain of the wild type, it is clear that N_{106} is less important for coupling the substrate than for positioning it. Such an argument was previously made for the *Anabaena* AC cyaB1, where a threonine functionally replaced the substrate-specifying aspartate and its mutation to alanine resulted also in a V_{max}- but not a K_m-change (Kanacher et al., 2002). Unfortunately, it was not possible to make a kinetic study for N106S to determine its specificity for GTP as a substrate because of its low GC activity. Finally, when the

differences in length of the respective side chains between Q and K, and N and D are considered, the variation in length of the "arm region" or "dimerization region" in Rv0386 (see chapter 1 for explanation and alignments in appendix) compared with this sequence segment in mammalian ACs (8 vs. 14 residues, respectively) could be also responsible for the changes in the fine structure of the purine-binding pocket in Rv0386 as a result of a conformational adjustment and compensation. Within this arm region, the triad KWQ (in mammalian ACs) or LVR (in *Paramecium* GC) is found. Particularly, the Q of this triad was claimed to assist K and D in adenine binding (Tesmer et al., 1997; Sunahara et al., 1998) while its correspondent R in the *Paramecium* GC is supposed to assist the E and S in guanine binding (Linder and Schultz, 2002; see fig. 4.1 for sequence alignments). On the contrary, structural data concerning substrate binding of the AC VC1a/IIC2 chimera showed that the KWQ motif appeared uninvolved (Tesmer et al., 1999). In any case this motif stabilizes AC-specifically the dimerization and its residues are absent in the shortened arm region of Rv0386. The unexpected reduction in the GC activity upon mutation of N_{106} to S highlights again the different substrate-specifying mechanism in Rv0386.

The effect on the catalytic properties of $Rv0386_{(1-175)}$ by modifications of its N-terminal region was also remarkable. Mutant R7G showed only 6% residual activity and the Rv0386 catalytic domain-equivalent of MT0399 (with 7 amino acid residues elongation before the M_1 of Rv0386) showed only 30% residual activity. Kinetic parameters of R7G were not investigated but the N-terminally elongated catalytic domain showed a 5-fold reduction in ATP-affinity (K_m= 2.3 mM) in comparison with $Rv0386_{(1-175)}$ (K_m= 417 µM). Notably, small C-terminal modifications of $Rv0386_{(1-175)}$ like changing the His-tag to the C-terminal position were well tolerated and did not modify the substrate affinity (K_m= 454 µM) and caused only an insignificant reduction (20%) in the specific activity, but compromising significantly the dimerization process.

It can be concluded that amino acid additions or charge changes in the N-terminal end of Rv0386 considerably affected the substrate binding and/or catalysis, maybe because of important conformational changes. These results together with the mutational studies show how important the determination of the three-dimensional structure of the unorthodox catalytic domain of Rv0386 might be to establish the individual architecture of its purine-binding pocket. Within the scope of this study, crystals of the AC catalytic

domain of Rv0386 were obtained. N-terminal as well as C-terminal His-tagged Rv0386$_{(1-175)}$ were successfully used for these purposes. Using the vapor diffusion method, a wide variety of crystallization conditions and crystal forms were found for these proteins. Until now diffraction-quality crystals were obtained only with the C-terminal His-tagged catalytic domain. Since Rv0386$_{(1-175)}$ has a high crystallization potential, optimization experiments are continuing in collaboration with the Biochemie-Zentrum in Heidelberg.

5.3 Holoenzyme Rv0386

Rv0386 seems to be a very interesting enzyme in terms of its multi-domain composition, the possible evolutionary origin of its modular architecture and, of course, its demonstrated unorthodox activity as an AC. In Rv0386, the N-terminally located cyclase homology domain is succeeded by a putative transcription factor domain comprising an ATPase domain and a HTH-DNA-binding domain.

The Rv0386 holoenzyme could be expressed in three different *E. coli* strains (BL21, BL21 STAR and BL21 ROSETTA all with pREP4 plasmid), but it ended up mostly in inclusion bodies. Small amounts of full length protein remained in the soluble fraction and could be purified using Ni-NTA-agarose including all kinds of degradation products, possibly originating during the purification process. It is likely that the protein was degraded instead of prematurely terminated, since the full length protein was still present in the insoluble material (inclusion bodies). It was attempted to optimize expression varying external conditions (time, temperature, concentration of the inducer) or using special host strains for improvement of mRNA stability (STAR cells) or enhancing the expression of proteins containing rare codons (ROSETTA cells). The use of protease inhibitors and detergents for protein solubilization after cell lysis were also tried. DNAse was added after cell disruption in case that protein was bound to DNA through its DNA-binding domain. These steps did not improve recovery of the holoenzyme. The conditions found to be best for obtaining more soluble protein and less degradation products were expression in BL21 STAR cells without induction with IPTG (expression at 15°C for 16 h) and addition of lysozyme and DNAse after cell disruption. The protein obtained under these conditions showed an AC activity of 3.7 nmol of cAMP·mg^{-1}·min^{-1}. Unfortunately, the eluate contained at least five additional main degradation products which, if containing AC catalytic domain, could also contribute to

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the AC activity observed. Therefore, it is impossible to make any conclusions or comparisons between the AC activity of the holoenzyme and the AC domain.

With the antibodies anti-KD0386 and anti-DB0386 it was possible to identify the principal degradation products of Rv0386. Identified with anti-KD0386 were products of 25-28 kDa, 40-45 kDa and 60-70 kDa as main bands. With anti-DB0386, a main product of about 25 kDa was detected. There were also degradation products observed on the SDS-PAGE gels that were not detected either with anti-KD0386 nor with anti-DB0386, probably corresponding to holoenzyme fragments that did not contain the AC catalytic domain nor the DNA-binding domain. It could be speculated that the folding of the holoenzyme Rv0386 was defective and that the most probable sites for rupture were interdomain, mainly after the AC catalytic domain. Until now for most studied transcription factor proteins, binding of their cognate signals destabilized them for proteolysis (sometimes autoproteolysis) (Little et al., 1980; Becker et al., 1999), but recently it was also reported that the transcription factor TraR of *Agrobacterium tumefaciens* must bind its inducing ligand (N-3-oxooctanoyl-L-homoserine lactone) to acquire its native conformation and protease resistance when expressing in *E. coli* cells. This TraR signaling ligand is critical for the folding of nascent protein into its mature tertiary structure (Zhu and Winans, 2001). Both aspects could be the reason why Rv0386 full length protein was found mainly degraded. Either an *E. coli* signaling molecule is inducing proteolysis of Rv0386 in some manner or a particular ligand for Rv0386 could exist in *M. tuberculosis* and not in *E. coli* expression cells, which could be responsible for attaining the Rv0386 native conformation and conferring resistance to cellular proteolysis only when synthesized in the presence of this signal molecule.

The *in vivo* expression of Rv0386 was shown using *Mycobacterium bovis* cell cultures (BCG strain), which is reported to contain a homologous protein denoted as Mb0393 (Garnier et al., 2003). A very slight signal of the holoenzyme could be detected with the help of antibodies anti-KD0386 in the soluble cellular fraction. Since the antibodies anti-KD0386 were able to recognize 10 of the other 14 class III AC's present in *M. tuberculosis* (probably because of the high conformation similarity between their cyclase homology domains), the detection of two additional stronger signals (about 55 and 38 kDa) with these antibodies in the soluble fraction of *M. bovis* cells could be explained as the recognition of other cyclases. It is possible to suggest that Rv0386 as a

transcription factor could show variable expression levels and was expressed in very low amounts when the *M. bovis* cells were disrupted for analysis. With the antibodies anti-DB0386 only a signal of about 64 kDa could be detected, maybe because of the recognition of other transcription factors with similar HTH-DNA-binding domains. Nevertheless, the possibility that Rv0386 could also suffer interdomain proteolysis *in vivo* should not be neglected.

The putative transcription factor-, ATPase and DNA-binding domains of Rv0386 could also be expressed in *E. coli* as individual proteins. The purification of reasonable amounts of the transcription factor domain was however impossible. The ATPase domain was found in its majority in the insoluble fraction and only a very low amount could be purified. That is the reason why no further experiments were made. On the contrary, the DNA-binding domain could be purified in sufficient amounts to produce specific antibodies. Through Western blot it was possible to determine the high tendency of dimerization of this domain. However, antibodies anti-DB0386 were also capable of detecting *E. coli* proteins (28 and 68 kDa, approximately), probably transcription factors with a similar conformation to the DNA-binding domain as Rv0386.

The role that could be played by Rv0386 as a transcription factor in *M. tuberculosis* remains unclear. The consequences and advantages gained by joining an adenylyl cyclase domain with a transcription factor containing an ATPase motif responsible for ATP binding and hydrolysis remain unexplained. Certainly, the vast regulation possibilities of Rv0386 as an AC and as a transcription regulator *in vivo* must be the reason why this multimeric signaling unit could evolutionarily arise.

5.4 Remaining questions

Since Rv0386 has evolved to use specific variant residues in an altered catalytic mechanism and of unsuspected regulation possibilities by joining this AC domain with a transcriptional regulator, two main questions remain open after this work: Is it possible to elucidate the structure of the AC domain for an understanding of its catalytic properties observed until now through biochemical analysis? And what is the role that Rv0386 plays *in vivo* as a transcription factor? Anyhow, the first of these questions is hopefully going to be answered soon, since the first crystallization attempts of the Rv0386 AC domain were started successfully.

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6   Summary

I report on two class III ACs of *Mycobacterium tuberculosis* which possess very different structural as well as catalytic characteristics: the mammalian-like membrane-anchored Rv1625c and the transcription-factor-attached Rv0386. As a complementary study to published data (Guo et al., 2001), additional point mutations were made which demonstrated the essential role of the six canonical amino acids for catalysis in Rv1625c. The cytosolic mutants of Rv1625c N372A, N372T and D300S were used to investigate dimerization with mammalian AC catalytic units. Rv1625c engineered to contain forskolin binding amino acids cannot be stimulated by the diterpene. The similarities in conformation and mechanisms of catalysis between ACs and GCs was confirmed through the formation of functional chimeras between Mycobacterium Rv1625c and a guanylyl cyclase of Paramecium. The versatility of the class III cyclase homology domains concerning their modular architectures and mechanisms of catalysis was demonstrated with the biochemical characterization of Rv0386. This enzyme has a substrate-defining mechanism distinctly different of that of mammalian ACs. In addition by using ATP as well as GTP as a substrate it is a unique AC isoform unknown so far. Mutational studies of the Rv0386 AC domain proved the essential role that is played by a glutamine and an asparagine instead of the canonical lysine and aspartate for recognition of ATP and GTP as substrates. Diffraction-quality crystals of this AC domain were obtained as a first step to decipher the molecular and structural particularities of its catalytic function. Sequence comparisons identified an ATPase, a HTH DNA-binding and a transcription factor domain in Rv0386. How these domains affect AC activity in a concerted regulatory mechanism remains a pressing question for future studies.

7 Zusammenfassung

Im Rahmen der vorliegenden Arbeit wurden zwei Klasse III ACn aus *Mycobacterium tuberculosis* mit unterschiedlicher strukturellen und katalytischen Eigenschaften untersucht: die Mammalia-ähnliche Rv1625c und die an einen Transkriptionsfaktor gebundene Rv0386. Als ergänzende Studien zu bereits publizierten Daten (Guo et al., 2001), wurden zur Überprüfung der wesentlichen Rolle der sechs kanonischen Aminosäuren, die bei Mammalia-ACn in der Katalyse beteiligt sind und die in Rv1625c konserviert und auch katalytisch relevant sind, zusätzliche Punktmutationen durchgeführt und getestet. Die Rv1625c Mutanten N372A, N372T und D300S, die einzeln inaktiv sind, wurden für Rekonstitutionsversuchen benutzt wobei die Bildung katalytisch aktiver Homodimere in Rv1625c nachgewiesen wurde. Punktmutationen in Rv1625c, die Forskolin-bindende Aminosäuren in Säugercyclasen betreffen, erfahren dennoch keine Stimulierbarkeit durch das Diterpen. Die Ähnlichkeiten in Struktur und katalytischem Mechanismus zwischen ACn und GCn wurden hier bestätigt, da Chimären zwischen *Mycobacterium* Rv1625c und einer Guanylatcyclase aus *Paramecium* aktive katalytische Zentren gebildet haben. Die Vielseitigkeit der Klasse-III Cyclase Homologie Domänen hinsichtlich ihres modularen Aufbaus und ihres katalytischen Mechanismus wurde hier durch die biochemische Charakterisierung von Rv0386 nachgewiesen. Dieses Enzym zeigte einen Mechanismus zur Erkennung des Substrates, der sich von dem der Mammalia-ACn deutlich unterscheidet. Dieser ermöglicht die Erkennung sowohl von ATP als auch GTP als Substrat und ist eine Eigenschaft, die bis jetzt in keiner anderen AC Isoform beobachtet wurde. Mutationen der AC Domäne beweisen die essentielle Rolle, die Glutamin und Asparagine statt dem kanonischen Lysin und Aspartat bei der Erkennung der Substrate ATP und GTP in Rv0386 spielen. Der Erhalt der ersten diffraktionsfähigen Kristalle der AC Domäne innerhalb dieser Arbeit ist ein erster Schritt zur Erkenntnis der Besonderheiten ihrer katalytischen Funktion. Die Suche nach Sequenz Ähnlichkeiten zeigte, dass Rv0386 zusätzlich zu der AC Domäne aus ATPase-, HTH DNA-bindungs- und Transkriptionsfaktor-Domänen besteht. Noch zu beantworten bleibt die Frage, wie die regulatorische Verbindung zwischen der AC Domäne und dem Transkriptionsregulator AC Aktivität und weitere unbekannte Regulationsmechanismen beeinflusst.

8   Appendix

8.1 DNA and protein sequences of Rv0386

DNA and protein sequences of Rv0386 (Sanger Institute MTV036.21 or Swiss-Prot/TrEMBL accession number O53720).

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                                                                  bp    aa
ATG AGC AAG TTG CTG CCA CGG GGC ACA GTG ACA TTG CTG TTG GCC      45
 M   S   K   L   L   P   R   G   T   V   T   L   L   L   A              15
GAC GTC GAG GGA TCC ACC TGG CTG TGG GAG ACC CAT CCA GAC GAC      90
 D   V   E   G   S   T   W   L   W   E   T   H   P   D   D              30
ATG GGT GCT GCC GTG GCG CGC CTC GAC AAA GCC GTG TCT GGT GTG      135
 M   G   A   A   V   A   R   L   D   K   A   V   S   G   V              45
ATT GCC GCC CAT GAC GGC GTA CGC CCA GTC GAG CAG GGT GAG GGT      180
 I   A   A   H   D   G   V   R   P   V   E   Q   G   E   G              60
GAT AGC TTT GTC CTC GCG TTC GCC TGC GCG TCG GAT GCC GTG GCC      225
 D   S   F   V   L   A   F   A   C   A   S   D   A   V   A              75
GCC GCG TTG GAC TTG CAG CGA GCG CGG CTC GCA CCG ATC CGG TTG      270
 A   A   L   D   L   Q   R   A   R   L   A   P   I   R   L              90
CGC ATA GGC GTG CAC ACC GGG GAG GTC GCG CTC CGC GAC GAA GGC      315
 R   I   G   V   H   T   G   E   V   A   L   R   D   E   G             105
AAC TAT GCC GGT CCG ACC ATC AAC CGG ACC GCG CGC CTG CGT GAC      360
 N   Y   A   G   P   T   I   N   R   T   A   R   L   R   D             120
TTG GCG CAT GGG GGC CAG ACG GTG CTC TCG GGC GTG ACC GAA AGC      405
 L   A   H   G   G   Q   T   V   L   S   G   V   T   E   S             135
CTG GTC ATC GAT CGC CTC CCG GAC AAA GCA TGG CTG GTT GAC CTG      450
 L   V   I   D   R   L   P   D   K   A   W   L   V   D   L             150
GGG ACG CAC GCG CTG CGG GAT CTG TCG CGT CCG GAG CGG GTA ATG      495
 G   T   H   A   L   R   D   L   S   R   P   E   R   V   M             165
CAG CTG TGT CAT CCC GAA TTG CGT ATC GAT TTC CCG CCG CTG CGG      540
 Q   L   C   H   P   E   L   R   I   D   F   P   P   L   R             180
GTG GCC AAT GAC GAT GTG GCC CAT GGT CTT CCG GTG CAC CTG ACG      585
 V   A   N   D   D   V   A   H   G   L   P   V   H   L   T             195
CGT TTT GTG GGG CGC GGC GCG CAG ATC ACC GAG GTG CAC CGG TTG      630
 R   F   V   G   R   G   A   Q   I   T   E   V   H   R   L             210
GTG ACC GAT AAC CGG TTG GTG ACC CTG ACC GGC GCC GGC GGC GTG      675
 V   T   D   N   R   L   V   T   L   T   G   A   G   G   V             225
GGC AAG ACA CGG CTG GCG GCG CAG CTC GCG GCG CAG ATC GCC GGT      720
 G   K   T   R   L   A   A   Q   L   A   A   Q   I   A   G             240
GAG TTC GGT CGC GCG TGG TTC GTG GAT CTG GCG CCG ATC ACG GAC      765
 E   F   G   R   A   W   F   V   D   L   A   P   I   T   D             255
CCC GAC TTG GTG CCG GTC ACG GTG GCG GGC GCG CTG GGA CTG CAC      810
 P   D   L   V   P   V   T   V   A   G   A   L   G   L   H             270
GAC CAG CCG GGC CGC TCC ACG ACG GAC ACC GTG CTG CGC TTT CTT      855
 D   Q   P   G   R   S   T   T   D   T   V   L   R   F   L             285
GGC GGG CGT CCA GCC CTG GTG GTG CTG GAT AAC TGC GAG CAC CTG      900
 G   G   R   P   A   L   V   V   L   D   N   C   E   H   L             300
CTG GAT GCG ACG GCG GCC TTG GTG TTA GCG CTG GTG AAA GCG TGC      945
 L   D   A   T   A   A   L   V   L   A   L   V   K   A   C             315
CGG GGG GTG AGG TTG CTG GCA ACT TGT CGT GAG CCG CTC CGG GTC      990
 R   G   V   R   L   L   A   T   C   R   E   P   L   R   V             330
GAG GGT GAG GTG AGC TAC CGG GTG CCG TCG CTG TCA CTG AGC GAT      1035
 E   G   E   V   S   Y   R   V   P   S   L   S   L   S   D             345
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GAA GCC GTT GAG ATG TTT TGC TAC CGG GCT CAG CGA GTC CGG CCG    1080
 E   A   V   E   M   F   C   Y   R   A   Q   R   V   R   P           360
GAC TTT CGC CTC ACC GAC GAC AAC TCC GCC GCA GTG ACC GAG ATC    1125
 D   F   R   L   T   D   D   N   S   A   A   V   T   E   I           375
TGC AAA CGG CTG GAC GGT TTG CCG CTG GCG ATC GAG CTG GCG GCT    1170
 C   K   R   L   D   G   L   P   L   A   I   E   L   A   A           390
GCG CGG CTG CGG TCG ATG ACG CTT GAC GAG ATC ATC GAT GGC TTG    1215
 A   R   L   R   S   M   T   L   D   E   I   I   D   G   L           405
CGT GAC CGG TTC GCG CTG TTG ACC GGC GGT GCG CGC ACG GCC GCG    1260
 R   D   R   F   A   L   L   T   G   G   A   R   T   A   A           420
CAC CGG CAG CAG ACG CTG TGG GCC TCG GTG GAT TGG TCG TAC ACG    1305
 H   R   Q   Q   T   L   W   A   S   V   D   W   S   Y   T           435
CTA TTG ACC GAG CCG GAA CGT ACC TTG TTT CGC CGG CTT GCG GTG    1350
 L   L   T   E   P   E   R   T   L   F   R   R   L   A   V           450
TTT GTG GGT TGC TTT TTT GTC GAC GAC GCA CAG GCG GTT GCC TGC    1395
 F   V   G   C   F   F   V   D   D   A   Q   A   V   A   C           465
AGC GGC GAT GTG CAG CGC TAC CAG GTC CTT GAC GAG ATC ACC CTG    1440
 S   G   D   V   Q   R   Y   Q   V   L   D   E   I   T   L           480
CTG GTC GAC AAG TCA CTG GTG ATG GCC GAC GAC AAC AGC GGC CGG    1485
 L   V   D   K   S   L   V   M   A   D   D   N   S   G   R           495
ACG TGC TAT CGG TTA TGC GAG ACG ATG CGC CAC TAC GCG TTG GAA    1530
 T   C   Y   R   L   C   E   T   M   R   H   Y   A   L   E           510
AAA CTC TCC GAG GCT GGC GAG GTG GAC GCC GTG TTT GCG CGG CAC    1575
 K   L   S   E   A   G   E   V   D   A   V   F   A   R   H           525
CGT GAC TAC TAC ACG GCG CTG GCT GCC AGG GTC GAC AAT CCC GGA    1620
 R   D   Y   Y   T   A   L   A   A   R   V   D   N   P   G           540
CCC TCC GAT TAT TCG CAC TGC CTC GAC CAA GCC GAA ACC GAG ATC    1665
 P   S   D   Y   S   H   C   L   D   Q   A   E   T   E   I           555
GAC AAC CTA CGT GCC GCC TTT GTG TGG AAC CGG GAA AAT TCC GAC    1710
 D   N   L   R   A   A   F   V   W   N   R   E   N   S   D           570
ACC GAG GGC GCC TTG GCG CTG GCG TCC TCC CTG TTG CGG GTA TGG    1755
 T   E   G   A   L   A   L   A   S   S   L   L   R   V   W           585
ATG ACG CGG GGG CGC ATC CAG GAG GGG CGC GCC TGG TTT GAC AGC    1800
 M   T   R   G   R   I   Q   E   G   R   A   W   F   D   S           600
ATT CTT GCC GAC GAG AAT GCG CGT CAT CTC GAG GTG GCG GCC GCG    1845
 I   L   A   D   E   N   A   R   H   L   E   V   A   A   A           615
GTG CGC GCC CGG GCA TTG GCC GAC AAG GCC CTG CTC GAC ATC TTC    1890
 V   R   A   R   A   L   A   D   K   A   L   L   D   I   F           630
GTC GAC GCC GCC GCC GGT ATG GAG CAG GCC CAA CAG GCT TTG GTG    1935
 V   D   A   A   A   G   M   E   Q   A   Q   Q   A   L   V           645
ATC GCG CGC GAG GTC GAT GAA CCG GCG CTG CTG TCC CGG GCG CTC    1980
 I   A   R   E   V   D   E   P   A   L   L   S   R   A   L           660
ACG GCC TGC GGC TTG ATC GCG GTA GCG GTA GCT CGC GCC GAT GCG    2025
 T   A   C   G   L   I   A   V   A   V   A   R   A   D   A           675
GCC GCG TCT TAT TTC GCC GAG GCG ATC GAC CTG GCA CGA GCG GTA    2070
 A   A   S   Y   F   A   E   A   I   D   L   A   R   A   V           690
GAC GAC CGG TGG AGG CTG GCC CAG ATC CTT ACC TTT CAG GCG GTC    2115
 D   D   R   W   R   L   A   Q   I   L   T   F   Q   A   V           705
GAT GCG GTC GTG GCG GGT GAC CCG GTC GCG GCA CGC CCG GCC GCC    2160
 D   A   V   V   A   G   D   P   V   A   A   R   P   A   A           720
CAA GAG GCA CGC GAG CTG GCT GCC GCG ATC GGT GAC CAC TCC AAT    2205
 Q   E   A   R   E   L   A   A   A   I   G   D   H   S   N           735
GCG CTG TGG TGC CGC TGG TGT CTC GGC TAC GCC CAG CTG ATG CGG    2250
 A   L   W   C   R   W   C   L   G   Y   A   Q   L   M   R           750
GGG GAG CTG GCC GCG GCC GCC GCC CAA TTC GGC GAG GTG GTG GAC    2295
 G   E   L   A   A   A   A   A   Q   F   G   E   V   V   D           765
GAG GCC GAG GCG TCT CAG GAA GTG CTG CAC AAG GCC AAC AGC CTG    2340
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    E   A   E   A   S   Q   E   V   L   H   K   A   N   S   L            780
CAG GGC CTG GCC TTC GCG CTC GCC TAC CAG GGT GAA TTG AGT GCG       2385
    Q   G   L   A   F   A   L   A   Y   Q   G   E   L   S   A            795
GCT AGG GCG GCG GCC GAC GCC GCT CTC GAG GCC GCC GAG CTG GGC       2430
    A   R   A   A   A   D   A   A   L   E   A   A   E   L   G            810
GAG TAC TTC GCG GGT ATG GGC TAC TCG GCG TTG ACC ACG GCC GCG       2475
    E   Y   F   A   G   M   G   Y   S   A   L   T   T   A   A            825
TTG GCC GCC GGC GAC GTG CAG ACG GCT CAA CAT GCC AGC GAG GCG       2520
    L   A   A   G   D   V   Q   T   A   Q   H   A   S   E   A            840
GCC TGG CGG AAC TTG AGT TTG GCG CTG CCC CTC TCG GCA GCG GTG       2565
    A   W   R   N   L   S   L   A   L   P   L   S   A   A   V            855
CAG CGC GCG TTC AAT GCC CAG GCT GCA CTG GCT GGT GGT GAC CTT       2610
    Q   R   A   F   N   A   Q   A   A   L   A   G   G   D   L            870
AGC GCA GCG CGT CGT TGG TGT GAC GAT GCC GTG CAG TCA ATG ACC       2655
    S   A   A   R   R   W   C   D   D   A   V   Q   S   M   T            885
GGC CAT CAT CTG GCG ATG GCG CTG GCG ACT CGC GCC AGG ATC GCG       2700
    G   H   H   L   A   M   A   L   A   T   R   A   R   I   A            900
GTC GCC GAG GGC AAG CGG GAA GAA GCC GAA CGC GAC GCG CAT AAG       2745
    V   A   E   G   K   R   E   E   A   E   R   D   A   H   K            915
GCG CTC GCG TGC GCG GCC GAG AGC GGG GCA CAC CTG GAT CTC CCC       2790
    A   L   A   C   A   A   E   S   G   A   H   L   D   L   P            930
GAC GTG CTC GAA TGC CTT GCC GGC CTG GCC AGC GAC GCC GGC ACC       2835
    D   V   L   E   C   L   A   G   L   A   S   D   A   G   T            945
CAC CAT GCG GCG GCA CGA CTC TTC GGC GCC GCC GAG GCT ATC CGA       2880
    H   H   A   A   A   R   L   F   G   A   A   E   A   I   R            960
CAG CAG ATC GGC TCG GTC CGC TTC GCG ATT TAC CGT TCG GAC TAT       2925
    Q   Q   I   G   S   V   R   F   A   I   Y   R   S   D   Y            975
GTG CAG TCG GTG ACG GCT CTG CGA GAT GCG ATG GGG GAG AAA GAC       2970
    V   Q   S   V   T   A   L   R   D   A   M   G   E   K   D            990
TTC GAC GCT GCA TGG GCC GAA GGT GCC GCG TTG TCG ATC AAG GAG       3015
    F   D   A   A   W   A   E   G   A   A   L   S   I   K   E           1005
ACG ATC GCC TAT GCG CAA CGT GGC CAC TCC TGG CGC AAA CGA CCG       3060
    T   I   A   Y   A   Q   R   G   H   S   W   R   K   R   P           1020
GCC ACC GGT TGG GAA TCG CTT ACT CCG ACC GAG ATT GAC GTC GTG       3105
    A   T   G   W   E   S   L   T   P   T   E   I   D   V   V           1035
CGA CTG GTT GGC GAG GGA CTG GCC AAC AAG GAC ATC GCG ACG CGG       3150
    R   L   V   G   E   G   L   A   N   K   D   I   A   T   R           1050
CTT TTC GTC TCA CCG CGA ACA GTG CAA ACG CAC CTG ACG CAC GTC       3195
    L   F   V   S   P   R   T   V   Q   T   H   L   T   H   V           1065
TAC ACC AAA CTC GGC TTC ACC TCG CGA CTG CAA CTC GCT CAA GCG       3240
    Y   T   K   L   G   F   T   S   R   L   Q   L   A   Q   A           1080
GCC GCC CGC CGT ACC TGA                                            3258
    A   A   R   R   T   .                                             1085
```

8.2 Results of the Protein-Protein BLAST Search

At NCBI a Protein-Protein BLAST search (blastp) with the full length Rv0386 and its AC, ATPase, Transcription factor and DNA-binding domains was made (Nov-26-2003; http://www.ncbi.nlm.nih.gov/BLAST/; Altschul et al., 1997). The 5 best hits are here reported.

Database: All non-redundant GenBank CDS translations+PDB+SwissProt+PIR+PRF

8.2.1 Blastp of the full length Rv0386

```
                                                                Score     E
Sequences producing significant alignments:                    (bits) Value
gi|15609625|ref|NP_217004.1|  hypothetical protein Rv2488c [...   890   0.0
gi|31792554|ref|NP_855047.1|  PROBABLE TRANSCRIPTIONAL REGUL...   759   0.0
gi|15840308|ref|NP_335345.1|  transcriptional regulator, Lux...   688   0.0
gi|15840309|ref|NP_335346.1|  hypothetical protein [Mycobact...   223   2e-56
gi|29832482|ref|NP_827116.1|  putative multi-domain regulato...   219   2e-55
```

8.2.2 Blastp of the adenylyl cyclase domain

```
                                                                Score     E
Sequences producing significant alignments:                    (bits) Value
gi|31791563|ref|NP_854056.1|  PROBABLE TRANSCRIPTIONAL REGUL...   300   9e-81
gi|15607527|ref|NP_214900.1|  hypothetical protein Rv0386 [M...   300   1e-80
gi|15839770|ref|NP_334807.1|  transcriptional regulator, Lux...   299   1e-80
gi|15840309|ref|NP_335346.1|  hypothetical protein [Mycobact...   191   5e-48
gi|15842015|ref|NP_337052.1|  transcriptional regulator, Lux...   178   4e-44
```

8.2.3 Blastp of the ATPase domain

```
                                                                Score     E
Sequences producing significant alignments:                    (bits) Value

gi|15609625|ref|NP_217004.1|  hypothetical protein Rv2488c [...   228   8e-59
gi|31792554|ref|NP_855047.1|  PROBABLE TRANSCRIPTIONAL REGUL...   219   3e-56
gi|15840814|ref|NP_335851.1|  transcriptional regulator, Lux...   217   1e-55
gi|15608030|ref|NP_215405.1|  hypothetical protein Rv0890c [...   197   1e-49
gi|15840308|ref|NP_335345.1|  transcriptional regulator, Lux...   197   1e-49
```

8.2.4 Blastp of the transcription factor domain

```
                                                                Score   E
Sequences producing significant alignments:                    (bits) Value

gi|15839770|ref|NP_334807.1|  transcriptional regulator, Lux...  1428   0.0
gi|15607527|ref|NP_214900.1|  hypothetical protein Rv0386 [M...  1428   0.0
gi|31791563|ref|NP_854056.1|  PROBABLE TRANSCRIPTIONAL REGUL...  1411   0.0
gi|15842015|ref|NP_337052.1|  transcriptional regulator, Lux...   728   0.0
gi|15609625|ref|NP_217004.1|  hypothetical protein Rv2488c [...   727   0.0
```

8.2.5 Blastp of the DNA-binding domain

```
                                                                Score     E
Sequences producing significant alignments:                    (bits) Value

gi|15839770|ref|NP_334807.1|  transcriptional regulator, Lux...   162   1e-39
gi|15607527|ref|NP_214900.1|  hypothetical protein Rv0386 [M...   162   1e-39
gi|31791563|ref|NP_854056.1|  PROBABLE TRANSCRIPTIONAL REGUL...   148   2e-35
gi|15840308|ref|NP_335345.1|  transcriptional regulator, Lux...   127   4e-29
gi|15608030|ref|NP_215405.1|  hypothetical protein Rv0890c [...   127   5e-29
```

8.3 Sequence alignments of Rv0386

The sequence of the AC domain of Rv0386 was aligned with mammalian AC's, mycobacterial Rv1625c and *Paramecium* GC sequences for identification of variations in amino acids that are critical for AC and GC catalysis and for presence or absence of other features like the "arm region". The sequences of the other individual domains of Rv0386 were aligned with those proteins who showed the highest similarity after BLAST Search.

```
Rv1625c      202 :-------LRDTA--RAEAVMEAEHDRSEALLANMLPASIAERLKEPE---
AC2_C2_RAT   825 :YYCRLDFLWKNKFKKEREEIETMENLNRVLLENVLPAHVAEHFLARSLKN
ParaGC_C1   1589 :LEV---FLGRINMNKENQIMSD-------ILSILLPQFIRDRINKAG---
Rv0386         1 :-----------------------------MSKLLPR-------------

                                          Me
                                          ⇩
Rv1625c      240 :RNIIADKYDEASVLFADIVGF----TERASSTAPAD--LVRFLDRLYSAF
AC2_C2_RAT   875 :EELYHQSYDCVCVMFASIPDFKEFYTESDVNKEGLE--CLRLLNEIIADF
ParaGC_C1   1626 :QYDIQEDQGMVAVLFCDIIDFDQL-----IKNEQSN--VVDILDKLFRRF
Rv0386         8 :--------GTVTLLLADVEGS----TWLWETHPDDMGAAVARLDKAVSGV

                                    Pu   Me
                                    ⇩    ⇩
Rv1625c      284 :DELVDQ---HGLEKIKVSGDSYMVVSGVPRPRPD-----------HTQAL
AC2_C2_RAT   923 :DDLLSKPKFSGVEKIKTIGSTYMAATGLSAIPSQEHAQEPERQYMHIGTM
ParaGC_C1   1669 :D-LLCQQ--HEVQKIETVGKTYMAAAGLKIHVSQKSNP-------VNKV
Rv0386        46 :IAAHDG---V-RPVEQGEGDSFVLAF----------------------

                                           Pγ                           Pu
                                           ⬇                            ⇩
Rv1625c      320 :ADFALDMTNVAAQLKDPRGN-P-VPLRVGIATGPVVAGVVGSRRFFYDVW
AC2_C2_RAT   973 :VEFAYALVGKLDAINKHSFN-D-FKLRVGINHGPVIAGVIGAQKPQYDIW
ParaGC_C1   1708 :ISLALDMKRYVMSNE------T-FQIKIGIHYGNVIAGVIGHHKPQFSLI
Rv0386        68 :--ACASDAVAAALDLQRARL-APIRLRIGVHTGEVALRDEG------NYA

                      Ts    Ts
                      ⬇     ⬇
Rv1625c      368 :GDAVNVASRMESTDSVGQIQVPDEVYERLKD-DFVLRERGH----INVKG
AC2_C2_RAT  1021 :GNTVNVASRMDSTGVLDKIQVIEETSLILQTLGYTCTCRGI----INVKG
ParaGC_C1   1751 :GDTINTASRICSTAESWDVAISEQAYRQTNKYELVYVQRDV----VA-KG
Rv0386       109 :GPTINRTARLRDLAHGGQTVLSGVTESLVID-RLPDKAWLVDLGTHALRD
```

Fig. 8.1: Complete alignment of the AC domain of Rv0386 with the mycobacterial Rv1625c AC domain, the mammalian C2 cytosolic domain (rat) and the C1 region of the *Paramecium* GC. Shown are the purine-binding sites (Pu), the transition state-stabilizing sites (Ts), the γ-phosphate-binding site (Pγ), the metal binding sites (Me) and the "arm or dimerization region" (represented with a top bar).

```
Rv0386  179 : -LRVANDDVAHGLPVHLTRFVGRGAQITEVHRL---VTDNR----LVTLT
AfsR    258 : PGRAPSDGRKGNIRPRLTTFVGREPELDALRSE---LPGAR----LVTLT
Apaf-1  109 : --YVRTVLCEGGVPQRPVVFVTRKKLVNAIQQKLSKLKGEP---GWVTIH
I2C-1   153 : ---KQETRTPSTSLVDDSGIFGRKNEIENLVGRLLSMDTKRKNLAVVPIV
L6tr    220 : --DIWSHISKENLILETDELVGIDDHITAVLEK-LSLDSEN--VTMVGLY
RPM1    156 : ---WVNNISESSLFFSENSLVGIDAPKGKLIGRLLSPEPQR---IVVAVV


Rv0386  221 : GACGVGKTRLAAQLAAQ--IAGEFGRAWFVDLAPITDPDLVPVTVAGALG
AfsR    301 : GPGGSGKTRLAEEAAAG--LD----QAWLVELAPLDRPEAVPGAIVNALG
Apaf-1  154 : GMAGCGKSVLAAEAVRDHSLLEGCFPGGVHWVSVGKQDKSGLLMKLQNIC
I2C-1   200 : GMGGMGKTTLAKAVYNDERVQKHFGLTAWFCVSEAYDAFRITKGLLQEIG
L6tr    265 : GMGGIGKTTTAKAVYN--KISSCFDCCCFIDNIRETQEKDGVVVLQKKLV
RPM1    200 : GMGGSGKTTLSANIFKSQSVRRHFESYAWVTISKSYVIEDVFRTMIKEF-
              ▲        ▲▲▲


Rv0386  269 : ---LHD-----------QPGR---STTDTVLRFLGGRPALVVLDNCEHLL
AfsR    345 : ---LRETV----LLTGDRPAGQ-DDPVALLVEYCAPRSQLLVLDNCEHVI
Apaf-1  204 : TRLDQDESFSQRLPLNIEEAKD----RLRILMLRKHPRSLLILDDVWDSW
I2C-1   250 : STDLKADDNLNQLQVKLKADDNLNQLQVKLKEKLNGKRFLVVLDDVWNDN
L6tr    313 : SEILRIDS--GSVGFNNDSGG-----RKTIKERVSRFKILVVLDDVDEKF
RPM1    249 : ---YKEAD--TQIPAELYSLG-YRELVEKLVEYLQSKRYIVVLDDVWTTG


Rv0386  302 : D-ATAALVLALVKACRGVRLLATCREPLRVEGE------VSYRVPSLSLS
AfsR    387 : G-AAARLVETLLTRCPGLTVLATSREPLGVPGE------SVRPVEPLTQ-
Apaf-1  250 : ---------VLKAFDSQCQILLTTRD-KSVTDSVMGPKYVVPVESSLGK-
I2C-1   300 : YPEWDDLRNLFLQGDIGSKIIVTTRK-ESVALM---MDSGAIYMGILSS-
L6tr    356 : --KFEDMLGSPKDFISQSRFIITSRS-MRVLGTLNENQCKLYEVGSMSK-
RPM1    293 : --LWREISIALPDGIYGSRVMMTTRD-MNVASFPYGIGSTKHEIELLKE-


Rv0386  345 : DEAVEMFCYRAQRVRPDFRLTDDNSAAVTEICKRLDGLPLAIELAAARLR
AfsR    429 : EQAQRLFTARAGAVRPDADAVLRDEEAVAEICRRLDGLPLAIELAAARLR
Apaf-1  289 : EKGLEILSLFVNMKK------ADLPEQAHSIIKECKGSPLVVSLIGALL-
I2C-1   345 : EDSWALFKRHSLEHK-DPKEHPEFEEVGKQIADKCKGLPLALKALAGMLR
L6tr    402 : PRSLELFSKHAFKKN---TPPSYYETLANDVVDTTAGLPLTLKVIGSLL-
RPM1    339 : DEAWVLFSNKAFPASLEQCRTQNLEPIARKLVERCQGLPLAIASLGSMMS
```

Fig. 8.2 : Alignment of the ATPase domain of Rv0386 with representative proteins containing also an ATP/GTP-binding motif A (P-loop or Walker A box with a consensus sequence [AG]-x(4)-G-K-[ST]; represented here through black triangles) and disease resistance genes signatures (represented with top bars). Aligned were: AfsR (P25941), Apaf-1 (O14727), I2C-1 (O24015), L6tr (Q40254) and RPM1 (Q39214). The Swiss-Prot/TrEMBL accession numbers are in parentheses.

a

```
BVGA_BORPER    149 :LSNRELTVIQLLAQGMSNKDIADSMFLSNKTVSTYKTRLLQKLNATSLVE
FIMZ_ECOLI     150 :LSNREVTILRYLVSGLSNKEIADKLLLSNKTVSAHKSNIYGKIGLHSIVE
FIMZ_SALTY     150 :LSNREVTVLRYLANGMSNKEIAEQLLLSNKTISAHKANIFSKIGLHSIVE
NARP_HAEIN     149 :LTDREMGVLRQIATGLSNKQIAAQLFISEETVKVHIRNLLRKLNVHSRVA
YXJL_BACSU     157 :FTKREIEVIQQMAYGLRNEDIAEKLFVSESTVKTHVHRILQKCNAQDRTQ
SCO3008        189 :LTDRELEVLKLVATGMNNRDIAKELFISENTVKNHVRNILEKLQLHSRME
Rv0890c        821 :LTPTERDVVRLVSEGLSNKDIAKRLFVSPRTVQTHLTHVYAKLGLPSRVQ
Rv0386        1027 :LTPTEIDVVRLVGEGLANKDIATRLFVSPRTVQTHLTHVYTKLGFTSRLQ
                           ▲    ▲   ▲▲   ▲  ▲    ▲▲       ▲▲   ▲
```

b

```
narL      1 :SNQEPATILLIDDHPMLRTGVKQLISMAPDITVVGEASNGE-------QG
uhpA      1 :----MITVALIDDHLIVRSGFAQLLGLEPDLQVVAEFCSGR-------EA
Rv0386  867 :GGDLSAARRWCDDAVQSMTGHHLAMALATRARIAVAEGKREEAERDAHKA
```

```
narL     44 :IELAESLDPDLILLDLNMPGMNGLETLDKIREKSLSGRIVVFSVSNHEED
uhpA     40 :LAGLPGRGVQVCICDISMPDISGLELLSQIP-KGMA--TIVLSVHDSPAL
Rv0386  917 :LACAAESGAHLDLPDV-LECLAGLASDAGTHHAAARLFGAAEAIRQQIGS
```

```
narL     94 :VVTALKRGADGYLLKDMEPEDLLKALHQAAAGEMVLSEALTPVLAASLRA
uhpA     87 :VEQALNAGARGFLSKRCSPDELIAAVHTVATGGCYL----TPDIAVKLAA
Rv0386  966 :VRFAIYRSDYVQSVTALRDAMGEKDFDAAWAEGAALSIKETIAYAQRGHS
```

```
narL    144 :NRATTERDVNQLTPRERDILKLIAQGLPNKMIARRLDITESTVKVHVKHM
uhpA    133 :GRQ--D----PLTKRERQVAEKLAQGMAVKEIAAELGLSPKTVHVHRANL
Rv0386 1016 :WRKRPATGWESLTPTEIDVVRLVGEGLANKDIATRLFVSPRTVQTHLTHV
```

```
narL    194 :LKKMKLKSRVEAAVWVHQERIF
uhpA    177 :LEKLGVSNDVELAHRMFDGW--
Rv0386 1066 :YTKLGFTSRLQLAQAAARRT--
```

Fig. 8.3: a) Alignment of the DNA-binding domain of Rv0386 with representative proteins containing a Helix-Turn-Helix DNA-binding signature (top bar). The consensus sequence of this signature is represented here with black triangles. Aligned were: BVGA (P16574), FIMZ (P26319), NarP from *Haemophilus influenza*, YXJL from *Bacillus subtilis*, SCO3008 from *Streptomyces coelicolor* and Rv0890c from *M. tuberculosis*. **b**) Detailed alignment with narL (P10957) and uhpA (P27667) showing the DNA-binding motif and other conserved regions. Swiss-Prot/TrEMBL accession numbers are in parentheses.
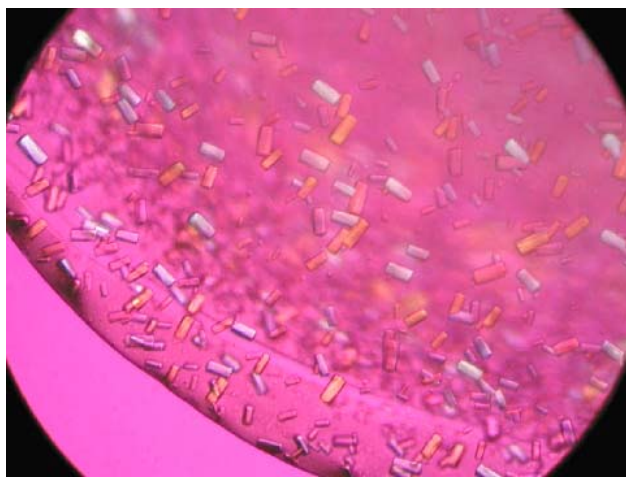
8.4 Crystal pictures

Representative pictures of some of the crystals obtained with the catalytic domain of Rv0386 within this work. First diffraction-data obtained is also shown.

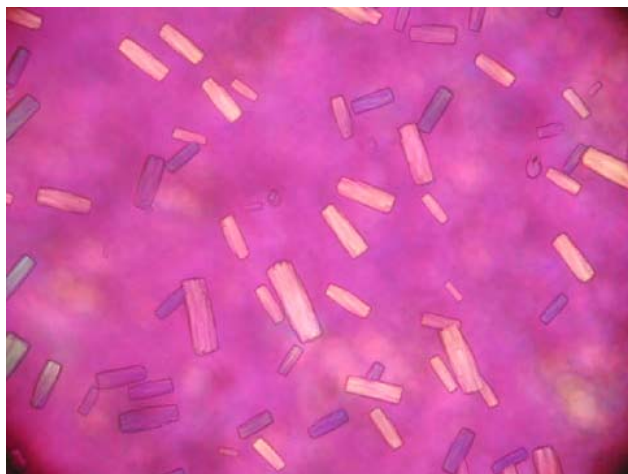### 8.4.1 Crystals of the AC domain of Rv0386 with N-terminal His-tag

a

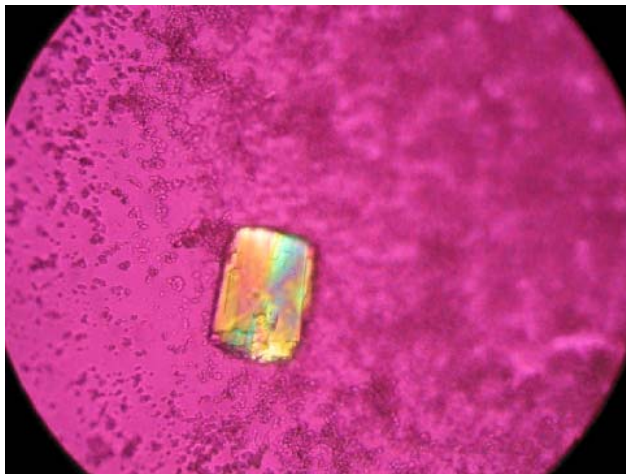b

c

d

e

f
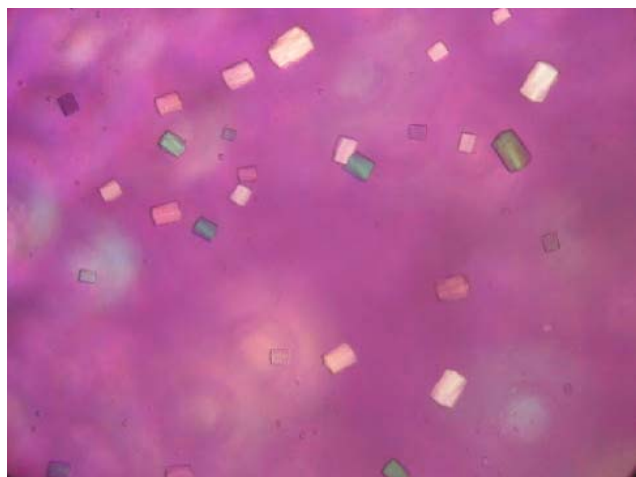
Fig. 8.4: Crystals obtained with the N-terminal His-tagged AC domain dialyzed after Ni-NTA-purification in 50 mM Tris-HCl pH 8.5, 10 mM NaCl, 2 mM β-mercaptoethanol and 10% glycerol. Precipitant solution was 10 % PEG 6000 + 2 M NaCl (a,b,e and f); 12 % PEG 6000 + 2 M NaCl (c) and 10 % PEG 4000 + 2 M NaCl (d). Incubation temperatures were: 16 °C (a and d) and 12 °C (b,c,e and f). Protein concentrations used were: a) 16 µg/µl; b) 25 µg/µl; c) 20 µg/µl ; d) 12 µg/µl ; e and f) 22 µg/µl. Approximate size of the crystals: a) 15x17 µm and 50x50 µm; b) 50x20 µm; c) 25x75 µm and 90x30 µm; d) 175x125 µm; e) 150x75 µm and f) 150x50 µm.

8.4.2 Crystals of the AC domain of Rv0386 with C-terminal His-tag
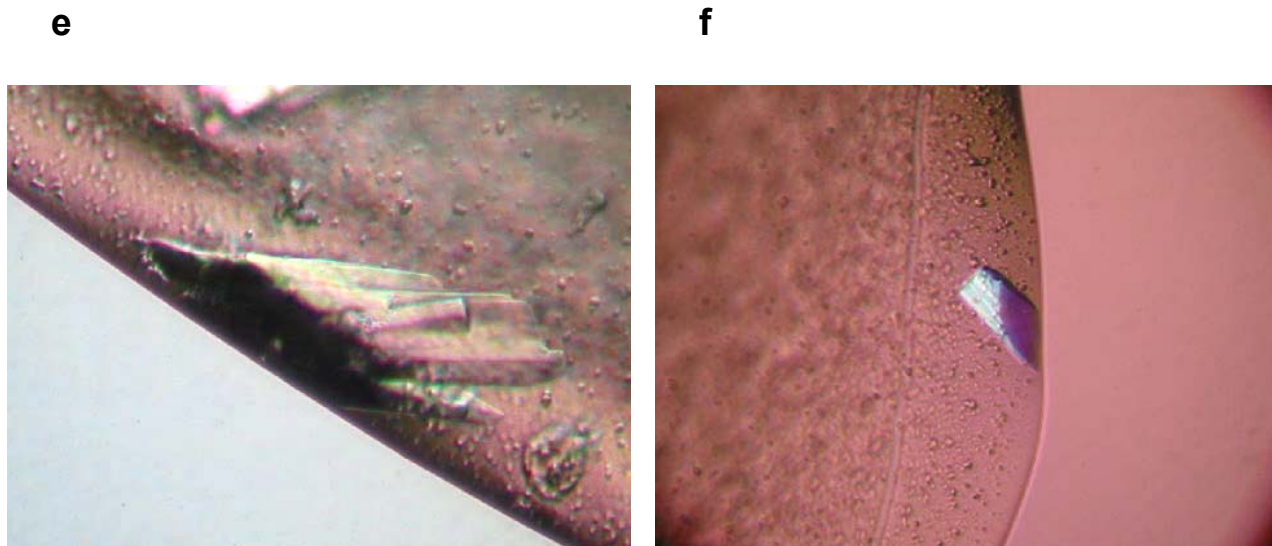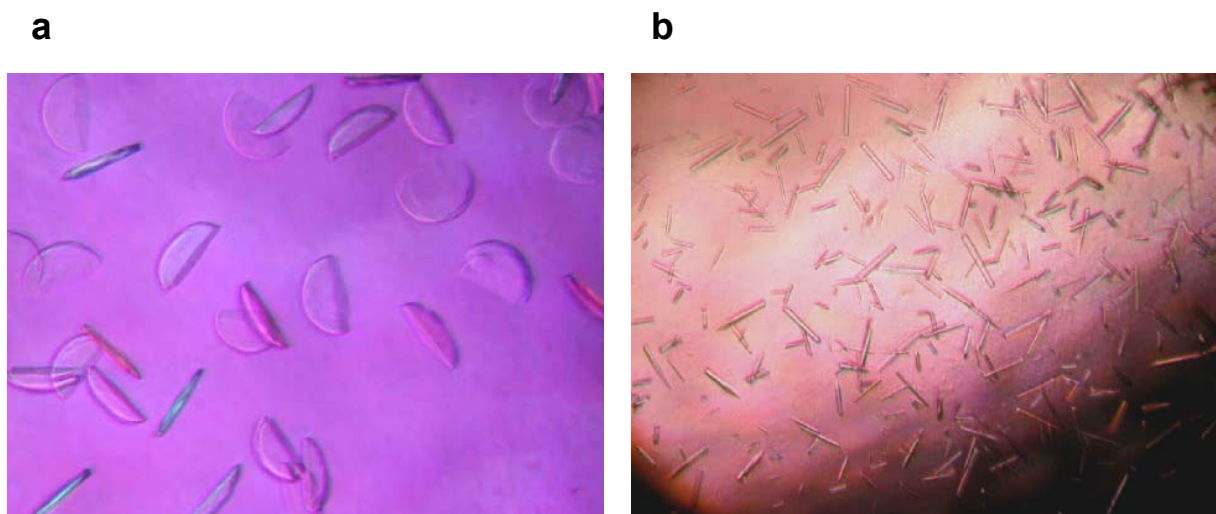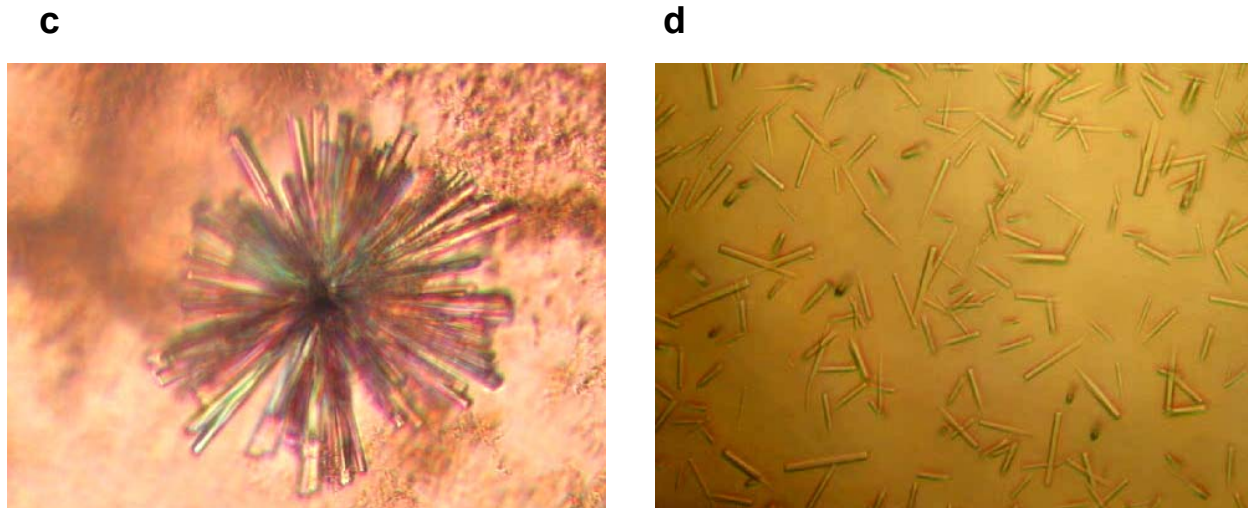
a

b

c

d

Fig. 8.5: Crystals obtained with the C-terminal His-tagged AC domain dialyzed after Ni-NTA-purification in 50 mM Tris-HCl pH 8.5, 10 mM NaCl, 2 mM β-mercaptoethanol and 5% glycerol. Protein concentration was 9.8 µg/µl. Protein was 14 h incubated with 1 mM ATP at 0 °C. Incubation temperature was 16 °C. Precipitant solutions were: a) 15 % PEG 4000 + 0.1 M Tris HCl pH 8.5 + 0.2 M sodium acetate; b) 10 % PEG 6000 + 0.1 M Hepes pH 7.5 + 5 % MPD; c) 30 % PEG 4000 + 0.1 M Tris HCl pH 8.5 + 0.2 M magnesium chloride; d) 0.5 M sodium acetate + 0.1 M imidazole pH 6.5. First diffraction data could be obtained with the crystals of picture d (see data below).

```
Shell Lower Upper Average      Average     Norm. Linear Square
   limit    Angstrom       I   error   stat. Chi**2  R-fac  R-fac
        25.00  15.81   37.3    6.6    3.1  1.000  0.105  0.078
        15.81  13.13   38.2    7.6    3.9  1.000  0.138  0.122
        13.13  11.66   53.9   11.0    6.7  1.000  0.117  0.106
        11.66  10.69   79.9   11.6    8.9  0.999  0.096  0.087
        10.69   9.97   63.4    9.9    7.1  1.000  0.109  0.110
         9.97   9.42   58.7    8.7    6.0  1.000  0.120  0.106
         9.42   8.97   49.3   13.8    8.7  1.001  0.160  0.148
         8.97   8.59   50.3   15.1   10.2  1.000  0.197  0.196
         8.59   8.28   32.3   11.3    6.9  1.000  0.226  0.174
         8.28   8.00   22.5   16.8   10.3  1.001  0.382  0.335
    All reflections    49.0   11.3    7.2  1.000  0.146  0.128
 finishing up after  8 cycles - overall R 14.6 percent - crystal q2-1
 calculating ...
 ===========================================================
  dataset: q2-1.tru   logfile: q2-1.truncate-log
 ===========================================================
  unit cell:    82.941 82.941 277.636 90.000 90.000 90.000
  space group                           =           75
  starting resolution                   =        24.77
  finishing resolution                  =         8.00
  number of amino acids in AU           =         1600
  fraction of unit cell occupied by atoms =    53.1%
  solvent content                       =        46.9%
 ===========================================================
```

Fig. 8.6: Preliminar diffraction data of the crystals of figure 8.5 d collected on the ID13 Micro Focus Beam Line (ESRF-Grenoble, France). Dr. Ivo Tews and Dr. Felix Findeisen from Biochemie Zentrum Heidelberg made the measurements.

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Meine akademischen Lehrer neben Herrn Prof. Dr. J. E. Schultz waren die Damen und Herren:

Robinson, V.A.	Pharmakologie, Biopharmazie, Toxikologie, Diplomarbeit; Puebla, México.
Anaya, C.	Organische Chemie; Puebla, México.
Garcés, J.	Biochemie; Puebla, México.
Parra, L.	Biologie und Biophysikalische Chemie; Puebla, México.
Piquer, A.	Pharmakologie und Medikamentenanalyse; Puebla, México.
Quiroz, M.A.	Physikalische Chemie und Instrumentalanalyse; Puebla, México.
Reyna, S.	Biochemie, Mikrobiologie und Immunologie; Puebla, México.
Roth, H.	Pharmazeutische Chemie; Puebla, México.
Sánchez, E.	Chemie der natürlichen Produkte; Puebla, México.

Lebenslauf

Persönliche Daten

Name: Lucila Isabel Castro Pastrana

Geburtstag: 22.06.1975

Geburtsort: Puebla, Puebla, México.

Ausbildung

1981-1991   Grundschule und Gymnasium, Puebla, México.

1991-1994   Abitur, Puebla, México.

1994-1999   Lizenziatstudium in Pharmabiochemie (Schwerpunkt Pharmazie) an der Universität de las Américas-Puebla (UDLA-P), México.

1999-2000   Laborpraktikum Betreuung bei der Chemie und Biologie Abteilung der Universität de las Américas-Puebla, México.

2000-2004   CONACYT-DAAD Stipendiatin zur Promotion an der Fakultät für Chemie und Pharmazie der Universität Tübingen. Der experimentelle Teil dieser Arbeit wurde im Zeitraum von März 2001 bis November 2003 unter der Anleitung von Prof. Dr. J. E. Schultz durchgeführt.

Curriculum vitae

Lucila Isabel Castro Pastrana

Born on the 22nd of June, 1975, in Puebla, Puebla, México.

School:	1981-1991	in Puebla (México)
High School:	1991-1994	in Puebla (México)
University:	1994-1999	Under-graduate studies at the Department of Chemistry and Biology at the University de las Américas-Puebla (UDLA-P), México; studies in chemistry, biology and pharmacy.
	1999-2000	Laboratory assistant at the Department of Chemistry and Biology at the UDLA-P, México
	2000-2004	PhD Thesis in pharmaceutical biochemistry (CONACYT-DAAD grantee) at the University of Tübingen, Faculty of Chemistry and Pharmacy.