Adenylyl Cyclase Rv1264 from Mycobacterium tuberculosis Has an Autoinhibitory N-terminal Domain∗

Jürgen U. Linder†, Anita Schultz, and Joachim E. Schultz

From the Abteilung Pharmazeutische Biochemie Fakultät für Chemie und Pharmazie Universität Tübingen Morgenstelle 8, 72076 Tübingen, Germany

Mycobacterium tuberculosis contains 15 class III adenylyl cyclase genes. The gene Rv1264 is predicted to be composed of two distinct protein modules. The C terminus seems to code for a catalytic domain belonging to a subfamily of adenylyl cyclase isozymes mostly found in Gram-positive bacteria. The expressed protein was shown to function as a homodimeric adenylyl cyclase (1 μmol of cAMP·mg⁻¹·min⁻¹). In analogy to the structure of the mammalian adenylyl cyclase catalyst, six amino acids were targeted by point mutations and found to be essential for catalysis. The N-terminal domain represents a novel protein domain, the occurrence of which is restricted to several adenylyl cyclases present in Gram-positive bacteria. The purified full-length enzyme was 300-fold less active than the catalytic domain alone. Thus, the N-terminal domain appeared to be autoinhibitory. The N-terminal domain contains three prominent polar amino acid residues (Asp¹⁰⁷, Arg¹³², and Arg¹⁹¹) that are invariant in all seven sequences of this domain currently available. Mutation of Asp¹⁰⁷ to Ala relaxed the inhibition and resulted in a 6-fold increase in activity of the Rv1264 holoenzyme, thus supporting the role of this domain as a potential novel regulator of adenylyl cyclase activity.

CAMP serves as a second messenger in virtually all organisms; yet at least three independent classes of adenylyl cyclases (AC) exist. Class I ACs are present in bacteria such as Escherichia coli or Yersinia. Class II ACs are toxins, e.g. from Bacillus anthracis or Bordetella pertussis. Class III ACs are present in all phyla (1). The catalytic domain of the huge number of class III ACs has been termed cyclase homology domain (CHD). Based on distinct amino acid motifs, class III ACs have been subclassified (2), and in lower organisms, particularly in bacteria, class III CHDs seem to be linked with different protein domains that most likely impart peculiar regulatory features. However, so far only a few studies addressed bacterial class III ACs.

In the completed genome of Mycobacterium tuberculosis, 15 open reading frames were identified that contain a CHD (2). The availability of this information enables us to study each mycobacterial AC isoform individually in vitro with the perspective to determine its contribution to the cAMP regulatory system during tuberculosis disease development in vivo. Two of the 15 AC open reading frames (Rv1625c and Rv2435c) belong to the mammalian-type ACs, and recent work concentrated on the membrane-bound mammalian-type AC present in Mycobacterium, Rv1625c (3,4). Four predicted mycobacterial ACs (Rv1318c, Rv1319c, Rv1320c, and Rv3645) contain CHDs that are part of a subclass consisting of ACs from, among others, Anaabaena, Stigmatella, Rhizobium, and Treponema (2). The remaining nine mycobacterial CHDs (Rv1264, Rv1647, Rv2212, Rv0386, Rv1358, Rv1359, Rv2488c, Rv0891c, and LipJ) are most similar to CHDs detected in other Gram-positive bacteria.

Here we investigated the gene product of Rv1264. Earlier, AC genes of this subtype were identified by complementation cloning in Breibacterium liquefaciens and Streptomyces (5,6). Those genes code for modular proteins with the CHD located C-terminally. The dismal expression of these ACs in E. coli precluded detailed biochemical studies (5,6). Thus, Rv1264 was used in an attempt to characterize this AC subtype. In addition, the biochemical characterization of the Rv1264 catalyst might constitute a starting point for future studies on the remaining eight related AC genes present in M. tuberculosis.

We were able to express reasonable amounts of the Rv1264 AC catalytic domain in E. coli with high AC activity. The catalytic site is the result of homodimerization, and catalysis depends on the same amino acids previously identified as crucial in mammalian ACs. The holoenzyme was much less active than the catalytic domain alone, suggesting an autoinhibitory function of this unique N-terminal domain, which contains no similarity to any other known protein module and so far is identified only in altogether seven ACs, i.e. four in Mycobacteria, two in Streptomyces, and one in Breibacterium.

EXPERIMENTAL PROCEDURES

Materials—Genomic DNA from M. tuberculosis was provided by Dr. Boettger (University of Zürich Medical School). Radiochemicals were from ICN or Hartmann Analytik. All enzymes were purchased from Roche Diagnostics or New England Biolabs. pQE30, nickel-ni- trioltriacetic acid-agarose, and anti-RGS-His antibody were from Qiagen. Fine chemicals were from Merck KGaA, Roche Diagnostics, Roth, and Sigma.

Construction of Expression Plasmids—The open reading frame of Rv1264 (GenBank accession number Z77137) including 26 bp downstream of the stop codon was amplified from M. tuberculosis genomic DNA by PCR. A BamHI site was added to the 5’ end, and a PstI site 10 bp 3’ of the stop codon was used as second restriction site. The cassette Rv1264,1–397, was cloned into the BamHI and XmaCI sites of pQE30, thus adding an MRGSH6GS tag N-terminally. Similarly, Rv1264,211–397, plus 26 bp at the 3’ end were amplified, fitted with BamHI sites at both ends, and cloned into the BamHI site of pQE30. Point mutations were introduced by PCR using the expression cassettes as templates. Nearby unique restriction sites were used for assembly of the fragments. The following additional restriction sites were introduced by silent mutations: a StuI site at base position 411 for construc-

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† To whom correspondence should be addressed. Tel.: 49-7071-295952; E-mail: juergen.linder@uni-tuebingen.de

‡ The abbreviations used are: AC, adenylyl cyclase; CHD, cyclase homology domain.

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tion of Rv1264, a PinAI site at nucleotide 766 for Rv1264, D211–397, D261A and K265A mutants, and a Nhel site at base 962 for mutants D312A, N319A, and R323A. All inserts in pQE30 were checked for correctness by DNA sequencing.

The expression plasmid for the N-terminal domain was generated from pQE30 by optimization of Rv1264, a N-terminal His$_6$ tag and C-terminal SSP tripeptide extension. Primer sequences will be provided on request.

Expression and Purification of Proteins—Expression plasmids were transformed into E. coli BL21(DE3)/pREP4. The cells were induced with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside and harvested after expression for 6 h at room temperature. The bacteria were washed with buffer (50 mM Tris-HCl, pH 8, 2 mM 3-thioglycerol), sonicated for 30 s, and treated with 0.2 mg/ml lysozyme for 30 min on ice. The incubation was continued for a further 30 min at 0 °C after addition of 0.5 mM MgCl$_2$ and 50% glycerol at −20 °C. Aliquots (25 μl of cell suspension) were transferred into a column, washed with 10 ml buffer containing 50 mM sodium phosphate, pH 7, 20% ethylene glycol, and 0.5 mM MnCl$_2$, and subsequently washed with 5 ml of buffer B (lysis buffer containing 37 mM A, and subsequently washed with 5 ml of buffer B (lysis buffer containing 15 mM imidazole, 5 mM MgCl$_2$). The protein was eluted with 0.4 μl of buffer C (37.5 mM Tris-HCl, pH 8, 250 mM imidazole, 2 mM MgCl$_2$, 1.5 mM 3-thioglycerol). Purified proteins could be stored without loss of activity in the presence of 50% glycerol at −20 °C.

Adenylyl Cyclase Assay—Activity was measured for 10 min at 37 °C in a volume of 0.1 ml. The standard reaction contained 22% glycerol, 50 mM Tris-HCl, pH 8.0, 3 mM MnCl$_2$, 0.5 mM [γ-32P]ATP (25 kBq), and 2 mM [2,8-3H]cAMP (150 Bq) to determine yield during the reaction. All data are the means of two to six points and are denoted with their standard deviations.

Cross-linking by Glutaraldehyde—Rv1264(211–397), dialyzed against cross-linking buffer (50 mM sodium phosphate, pH 7, 20% glycerol, 5 mM MgCl$_2$). After removal of precipitates, 310 mM protein were incubated with 7 mM glutaraldehyde (final concentration) for 60 min at room temperature. The reaction was quenched by the addition of 0.25 volumes of SDS-PAGE sample buffer, and 15 μl were separated on a 15% SDS-PAGE gel. The proteins were blotted onto a polyvinylidene difluoride membrane and visualized with an anti-RGS-His$_4$ antibody (3).

RESULTS

Sequence Features of the M. tuberculosis AC Rv1264—Rv1264 was analyzed by BLAST (8) and Smith-Waterman (9) searches. The N terminus was similar to the respective N-terminal AC domains detected in another six firmicute-type species, with the exception of the presence of an N-terminal extension. Primer sequences will be provided on request.

Expression and purification of the AC Rv1264(211–397) was dialyzed against cross-linking buffer (50 mM sodium phosphate, pH 7, 20% glycerol, 5 mM MgCl$_2$). After removal of precipitates, 310 mM protein were incubated with 7 mM glutaraldehyde (final concentration) for 60 min at room temperature. The reaction was quenched by the addition of 0.25 volumes of SDS-PAGE sample buffer, and 15 μl were separated on a 15% SDS-PAGE gel. The proteins were blotted onto a polyvinylidene difluoride membrane and visualized with an anti-RGS-His$_4$ antibody (3).

The specific activity tripled with increasing protein concentration as expected for an equilibrium between the catalytic AC fold. This gap is immediately ahead of a conserved region comprising 12 amino acids (DXNXR) that includes a second substrate-binding Asp and an Asn and an Arg that stabilize the transition state. The crystal structure of mammalian ACs indicates that this sequence of invariable length constitutes a prominent dimerization region (10, 13). Therefore, we examined whether the catalytic domain of Rv1264 functions as a homodimer in the canonical sense that two domains associate in a head-to-tail fashion using the pinpointed six catalytic amino acid residues. This would indicate significant differences to the known AC structure and suggest a considerable flexibility with respect to the protein scaffold that provides the backbone for the catalytic AC fold.

Biochemical Characterization of the Catalytic Domain of Rv1264—The CHD Rv1264(211–397), expressed in E. coli mostly in inclusion bodies. The minor soluble fraction of the expression product (10%) was purified to homogeneity (Fig. 2). Rv1264(211–397) displayed high AC activity with Mn$^{2+}$ as a cofactor (1.2 ± 0.1 μmol of cAMP·mg$^{-1}$·min$^{-1}$), whereas Mg$^{2+}$-mediated catalysis was rather poor (6 ± 2 nmol of cAMP·mg$^{-1}$·min$^{-1}$). ATP substrate specificity was stringent, GTP was not accepted irrespective of the metal cation employed. The temperature optimum was at 37 °C, the activation energy of 53 kJ/mol was within the usual range, and the pH optimum was around 7.3 (tested range, 5.6–8.5). The V$_{max}$ of 1.3 μmol of cAMP·mg$^{-1}$·min$^{-1}$ was derived from a Michaelis-Menten plot. The specific activity is thus comparable with that of the catalytic domain of AC Rv1625c (3) (Table I). The Hill coefficient of 1.6 indicated a pronounced cooperativity for ATP, consistent with the presence of two catalytic sites. This was supported by the nonlinear protein dependence of the cyclase reaction (Fig. 3A). The specific activity tripled with increasing protein concentration as expected for an equilibrium between monomers and oligomers. An apparent association constant of 50 ± 15 nm was calculated from a reciprocal plot of the dependence of the specific activity on the enzyme concentration, indicating a high affinity of the Rv1264(211–397), monomers for each other. The formation of dimers was substantiated by glutaraldehyde cross-linking experiments. At 310 nm Rv1264(211–397), which was used in standard assays, a band of a dimer-sized protein was detected by a Western blot (Fig. 3B). The limited extent of cross-linking is rationalized by the fact that Rv1264(211–397), has only two Lys residues, which are the preferred targets for cross-linking by glutaraldehyde.

Site-directed Mutagenesis of Rv1264(211–397)—Because of the peculiar gap in a conserved stretch of protein mentioned above (Fig. 1B), it was necessary to ascertain whether the same
FIG. 1. Alignment of the two domains of AC Rv1264. Sequences of the proteins encoded by *M. tuberculosis* gene Rv1264 (GenBank™ accession number Z77137), gene Rv2212 (GenBank™ accession number Z70283), their homologs from *M. smegmatis* as analyzed by BLAST from preliminary genome data, *B. liquefaciens* AC (GenBank™ accession number X57541), *Streptomyces coelicolor* AC (GenBank™ accession number AL512667), and *S. griseus* AC (GenBank™ accession number AB018557) were aligned. Identities and similarities (I/L/M/V, D/E, N/Q, R/K, S/T, and Y/F) are shown inversed.

**A**. Alignment of the N-terminal domains. Three invariant polar amino acids are marked, the two targeted by point mutations by arrows, and the third by a triangle.

**B**. Alignment of the cyclase homology domains including *M. tuberculosis* gene Rv1625c (GenBank™ accession number Z95554). A significant gap in firmicute-type ACs is highlighted by a bar. The six functional amino acids targeted by point mutations are marked by arrows and a γ-phosphate binding (Pγ) Arg is indicated with a vertical line. Ad, adenine ring binding; Me, metal-cofactor binding; Tr, transition state stabilizing.
residues crucial for catalysis in mammalian-type CHDs were used (10–12). Six critical amino acids were individually mutated to alanine in Rv1264(211–397). The proteins were expressed in E. coli and purified to homogeneity via their N-terminal His6 tag (Fig. 2). In all mutants, activity was below 1% of the maximal specific activity. Rv1264(211–397) was tested with 0.18 μM protein, Rv1264 holoenzyme was tested with 3.7 μM, and Rv1264D107A was tested with 2.1 μM enzyme as described under “Experimental Procedures.”

Table I

| V \text{max} (nmol mg^{-1} min^{-1}) | SC_{50} (nmol mg^{-1}) | Hill coefficient |
|-------------------------------------|------------------------|------------------|
| Rv1264(211–397)                     | 1250 ± 150             | 0.3 ± 0.1        | 1.6 ± 0.2 |
| Rv1264                             | 34 ± 4                 | 1.5 ± 0.1        | 1.9 ± 0.1 |
| Rv1264D107A                        | 109 ± 16               | 1.2 ± 0.2        | 1.7 ± 0.1 |

A molar excess of inactive Rv1264(211–397)D222A or Rv1264(211–397)D265A activated Rv1264(211–397)R323A 3-fold to about 200 nmol mg^{-1} min^{-1}. This was further evidence of a dimeric catalyst and for correctly folded mutant proteins. The lower than wild-type levels of cyclase activity could indicate that the mutations caused a slight and local conformational deterioration or were to some extent involved in dimerization (see below). 

Characterization of Rv1264 Holoenzyme—Next the Rv1264 holoenzyme was expressed in E. coli and purified (Fig. 5A). The specific activity was 2.5 ± 0.2 nmol of cAMP mg^{-1} min^{-1}, i.e. more than 300-fold less than that of Rv1264(211–397). Activity of the holoenzyme was only detectable with Mn^{2+} as a metal cofactor; Mg^{2+} did not support activity. A kinetic characterization showed a calculated V \text{max} of 3% compared with Rv1264(211–397), and the apparent affinity for ATP was reduced about 6-fold (Table 1). This suggested that the N-terminal domain is an intrinsic inhibitor of AC Rv1264. The Hill coefficient of 1.9 indicated a strong cooperativity, i.e. the dimerization was a prominent feature of the holoenzyme.

The similarity of AC Rv1264 to the cloned B. liquefaciens AC was taken as a hint to look for small metabolites as effectors because recombinant B. liquefaciens AC was reported to be stimulated by pyruvate (6). This was in agreement with an unspecific activation of a purified AC from B. liquefaciens by compounds such as α-ketocarboxylic acids, glycine, alanine, and lactate (16). The Rv1264 holoenzyme was not activated by 10 mM D,L-lactate, 1 mM pyruvate, α-ketobutyrate, D-alanine, L-
alanine, phosphoenolpyruvate, and 19 other compounds from the glycolysis, citric acid cycle, and amino acid-related biochemical pathways.

**Inhibition by the N-terminal Domain**—The N terminus might inhibit the AC activity of Rv1264 autonomously or only if fused to the catalytic domain. Therefore, we expressed and purified the N terminus (amino acids 1–207) (Fig. 5A). Even at 15 μg/ml the N-terminal domain reduced the activity of Rv1264(211–397) by only 30% (data not shown). Thus, the inhibition of the catalytic domain appeared to require a linked configuration.

We hypothesized that the invariably conserved Asp and Arg residues in the N terminus might be important for inhibition and that mutation of these residues to Ala might attenuate inhibition. Therefore, we generated Rv1264D107A and Rv1264R132A mutant holoenzymes. The recombinant proteins were purified from E. coli (Fig. 5B). The Rv1264D107A mutant had the same low activity as the wild-type protein (Fig. 5C). However, the removal of a carboxyl group in the Rv1264D107A mutant resulted in a 7-fold more active AC (Fig. 5C). A kinetic analysis of Rv1264D107A revealed a 3-fold increase in $V_{\text{max}}$ and a slightly enhanced affinity for ATP (Table I). This established this unique N-terminal domain of Rv1264 as a likely autoinhibitory module.

**DISCUSSION**

The occurrence of 15 genes coding for class III ACs in *M. tuberculosis* (2) implies that cAMP-mediated signal transduction may be a central and versatile tool that this pathogen can employ to process multiple environmental challenges. Nine of these genes code for CHDs that are typical for ACs in Gram-positive bacteria. Thus, the understanding of the structure-function relationships in this type of CHDs appears important for an understanding of cAMP signaling in *M. tuberculosis* and other bacteria. AC Rv1264 was chosen for this study because it has the same modular composition as the single AC identified in *Streptomyces* and an AC present in *B. liquefaciens* (5, 6).

We addressed three major questions: Is AC Rv1264 operating as a homodimer like mammalian-type AC Rv1625c despite a major gap in a conserved region implicated in dimerization? Are the same amino acids that are known to be critical for catalysis in mammalian ACs also involved in catalysis in Rv1264? What is the potential function of the novel N-terminal domain?

The former two aspects were investigated by use of the recombinant CHD, i.e. Rv1264(211–397). A sequence comparison...
of Rv1264 with mammalian-type ACs demonstrated that all critical amino acids can be properly aligned. This argues for a canonical homodimerization. However, a prominent seven-amino acid gap will result in the almost complete elimination of a distinct loop between an antiparallel β-sheet that is formed by the β3 and β3′ strands in the mammalian AC heterodimer (10, 13). This loop has been shown to be important for dimerization, and its absence in Rv1264 raised doubts about its catalytic mechanism. Four experimental approaches were used to demonstrate a functional homodimerization of Rv1264.

First, the ATP dependence of Rv1264(211–397) was positively cooperative, consistent with more than one catalytic site in a dimer. Second, the specific activity increased with the protein concentration as expected for an assembly of monomers in catalyst formation. Third, glutaraldehyde cross-linking demonstrated the physical presence of Rv1264(211–397) dimers. Fourth, two almost inactive mutants of Rv1264(211–397) N319A and R323A, which functionally correspond to mammalian AC C1 domains, were partially complemented by addition of inactive mutant proteins such as Rv1264(211–397)D222A and D265A, which were the functional equivalents of mammalian AC C2 domains. In the latter experiments the reconstitution of a single wild-type catalytic center was predicted (see Fig. 4A for a pictogram). However, the observed specific AC activity was much less than Rv1264(211–397). The lack of a full complementation of these Rv1264 C1/C2 equivalents may be the consequence of a profound difference of dimerization epitopes compared with mammalian ACs, as suggested by the gap in the dimerization arm (marked by a bar in Fig. 1B). In fact, it cannot be excluded that amino acids that were targeted by the point mutations also affect the orientation of the monomers and thus play an additional role in catalyst formation that obviously could not be substituted in a mixture of two otherwise complementatory mutants.

The individual mutation of all six amino acids that are implicated in catalysis (10–12, 14) was detrimental. The protein folding of these mutants appeared to be largely intact because there was either measurable residual activity in mutants targeting the stabilization of the transition state (N319A and R323A) and the adenine binding site (R261A and D312A) or a complementation with inactive mutants in the metal-binding sites (D222A and D265A). These features are highly similar to results obtained by alanine mutagenesis of the mammalian-type AC Rv1625c of M. tuberculosis (3). Finally, of the nine mycobacterial ACs that belong to this subtype, only three (Rv1264, Rv1647, and Rv2212) have the unaltered set of six amino acids assumed to be critical for catalysis. It will be interesting to investigate whether the amino acid changes in the other six ACs that comprise two to four of the six catalytic amino acids in each isozyme will be compatible with AC activity. Preliminary data indicate that the conservation of the catalytic center is considerably relaxed in those ACs. Obviously, this raises novel questions concerning the structural details of the catalytic cleft in different AC subtypes.

The AC activity of the Rv1264 holoenzyme was almost completely masked by its N-terminal domain. This was in agreement with and explained the tiny activity of Rv1264(1–397) that has been reported recently (4). Because the recombinant and purified N-terminal domain alone (Rv1264(1–207)) did not inhibit the CHD Rv1264(211–397), when added separately, the physical linkage of both protein modules seems to be required and sufficient for the autoinhibitory effect. One prerequisite for a domain to be a regulator is the flow of information from the regulator to the effector, and the key property of an inhibitor is that a perturbation of its function leads to the relief of inhibition, i.e. an activation. Both conditions are met by the N-terminal domain of Rv1264 because the mutation of a conserved Asp107 to Ala led to a highly significant increase of cyclase activity. A comparison of kinetic parameters of Rv1264(1–397), Rv1264(211–397), and Rv1264(1–397)D107A indicated that the inhibitory module decreased the catalytic rate as well as the apparent affinity for ATP. Thus, it may put a conformational constraint on the catalytic domain via the linker region.

A similar modularized AC from B. liquefaciens was reported to be activated by pyruvate, other α-ketocarboxylic acids, lactate, and some amino acids (5, 16). Similar tests with the mycobacterial Rv1264 AC failed to relieve the inhibition. Therefore, the physiological signal that may alleviate the inhibition of the catalytic region remains enigmatic at present.

The inhibitory domain of AC Rv1264 is novel and was not identified previously in other proteins. Therefore, the attempt of a mutational analysis had to rely fully on the analysis of similarity patterns present in six other ACs of this subtype. Because Rv1264 holoenzyme can be purified in large quantities, an elucidation of its structure will be possible and probably allow a more detailed insight into the relationship between the autoinhibitory and catalytic domains.

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