Eukaryotic-like Adenylyl Cyclases in Mycobacterium tuberculosis H37Rv

CLONING AND CHARACTERIZATION*

Sathyavelu K. Reddy‡, Madhavi Kamireddi, Kiran Dhanireddy, Lynn Young, Andrew Davis, and Prasad T. Reddy§

From the DNA Technologies Group, Biotechnology Division, Chemical Science and Technology Laboratory, National Institute of Standards and Technology, Gaithersburg, Maryland 20899

Screening the Mycobacterium tuberculosis H37Rv genomic library for complementation of catabolic defect for cAMP-dependent expression of maltose operon produced the adenylyl cyclase gene (Mtb cya, GenBank™ accession no. AF017731 (1997)) annotated later as Rv1625c (Cole, S. T., Brosch, R., Parkhill, J., Garnier, T., Churcher, C., Harris, D., Gordon, S. V., Eiglmeier, K., Gas, S., Barry, C. E., III, et al. (1998) Nature 393, 537–544). The deduced amino acid (aa) sequence (443 aa) encoded by Mtb cya contains a single hydrophobic domain of six transmembrane helices (152 aa) in the amino-terminal half of the protein. Flanking this domain are an arginine-rich (17%) amino-terminal cytoplasmic tail (46 aa) and a carboxyl-terminal cytoplasmic domain (245 aa) with extensive homology to the catalytic core of eukaryotic adenylyl cyclases. Site-directed mutagenesis of Arg43 and Arg44 to alanine/glycine showed a loss of adenylyl cyclase activity, whereas mutagenesis to lysine restored the activity. Hence it is proposed that the formation of the catalytic site in Mtb adenylyl cyclase requires an interaction between Arg43 and Arg44 residues in the distal cytoplasmic tail and the carboxyl-terminal cytoplasmic domain. Mtb adenylyl cyclase activity at the physiological concentration of ATP (1 mM) was 475 nmol of cAMP/min/mg of membrane protein in the presence of Mn²⁺ but only 10 nmol of cAMP/min/mg of membrane protein in the presence of Mg²⁺. The physiological significance of the activation of Mtb adenylyl cyclase by Mn²⁺ is discussed in view of the presence of manganese transporter protein in mycobacteria and macrophages wherein mycobacteria reside.

* Certain commercial equipment, instruments, and materials are identified in this paper to specify the experimental procedure as completely as possible. In no case does such identification imply a recommendation or endorsement by the National Institute of Standards and Technology nor does it imply that the material, instrument, or equipment identified is necessarily the best available for the purpose. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

This paper is available on line at http://www.jbc.org

EXPERIMENTAL PROCEDURES

Enzymes and Chemicals—Restriction endonucleases, T4 DNA ligase, and T4 polynucleotide kinase were purchased from New England Biolabs, Beverly, MA. Calf intestinal alkaline phosphatase was from Roche Molecular Biochemicals. Acrylamide, bisacrylamide, and phenol were obtained from Life Technologies Inc. Deoxynucleoside triphosphates were purchased from Amersham Pharmacia Biotech. SeaKem GTG agarose and NuSieve GTG agarose were purchased from FMC BioProducts, Rockland, ME. Dye-conjugated Dideoxyterminator DNA sequencing kits were from PerkinElmer Life Sciences. PCR products and DNA from agarose gels were purified using kits from Qiagen, Valencia, CA. Mutagenesis kit was from Bio-Rad. [α-³²P]ATP (30 Ci/mmol) and

1 The abbreviations used are: AC, adenylyl cyclase; PCR, polymerase chain reaction; kbp, kilobase pair(s); aa, amino acid(s); Mtb, Mycobacterium tuberculosis; ORF, open reading frame.


**Adenylyl Cyclases from M. tuberculosis H37Rv**

**Molecular Cloning of cya Gene**—Twenty micrograms of *M. tuberculosis* H37Rv chromosomal DNA (generously provided by Drs. John Belisle and Patrick Brennan, Colorado State University) was partially digested with 1 unit of Sau3A I restriction endonuclease at 37 °C for 15 min. DNA fragments, size-selected for 2-6 kb, were excised from a 1% agarose gel (0.8% NuSieve GTG-agarose, 0.2% SeaKem GTG-agarose) and purified by phenol extraction and ethanol precipitation. DNA fragments were ligated into the BamHI site of pBR322. The cya deletion strain TP610 was electrophorographed (Life Technologies, Inc.) with the ligation products, and the cells were spread on MacConkey/maltose plates containing ampicillin (100 μg/ml). Eight red colonies indicative of maltose fermentation, which requires cAMP production, were purified from the Mtb deletion strain, was obtained from Dr. Antoine Danis-Perez, Pasteur Institute, Iogenic Engineering Laboratory, Paris, and CA8445 (cya/crp deletion) were obtained from Dr. Alan Peterkofsky, National Institutes of Health (13).

**Construction of Full-length Mtb cya Gene in pRE Expression Vector**—M. tuberculosis AC was purified from the 3.4-kbp insert obtained in the first step for the construction of a full-length Mtb cya gene containing the BamHI site of the Mtb cya gene coding for protein expression.

**Expression of Adenylyl Cyclase(s)**—The pRE1-Mtb cya recombinant plasmid was digested with NdeI and BalIII to remove the DNA fragment coding for the amino-terminal 57 amino acids. A DNA cassette was constructed containing the pRE1-Mtb cya recombinant plasmid that was ligated into the pRE1-Mtb cya deletion strain. The sequence of the oligonucleotides, with bold letters indicating the mutation, was as follows: Oligo 1, 5'-dTGACGACACAGTCTGGACACGCG-3'; Oligo 2, 5'-dGACGACGTCTGGACACGCG-3' (see Table I, Oligo 2, for R31G); 5'-dGACGACGTCTGGACACGCG-3' (see Table I, Oligo 2, for R4G); 5'-dGACGACGTCTGGACACGCG-3' (see Table I, Oligo 2, for R44G); 5'-dGACGACGTCTGGACACGCG-3' (see Table I, Oligo 2, for R46A); 5'-dGACGACGTCTGGACACGCG-3' (see Table I, Oligo 2, for R43A); 5'-dGACGACGTCTGGACACGCG-3' (see Table I, Oligo 2, for R43K); 5'-dGACGACGTCTGGACACGCG-3' (see Table I, Oligo 2, for R44K); 5'-dGACGACGTCTGGACACGCG-3' (see Table I, Oligo 2, for R46K); and 5'-dGACGACGTCTGGACACGCG-3' (see Table I, Oligo 2, for R43K/R44K).

**Mutations were confirmed by DNA sequencing using dye-conjugated Dideoxynucleotidetoprimers, digested with NdeI and BalIII, purified from low melting agarose gel, and cloned into the pRE1-Mtb cya recombinant plasmid for the construction of a full-length Mtb cya deletion strain.**

**Expression of Adenylyl Cyclase(s)**—The pRE1-Mtb cya recombinant plasmid was digested with NdeI and BalIII to remove the DNA fragment coding for the amino-terminal 57 amino acids. A DNA cassette was constructed containing the pRE1-Mtb cya recombinant plasmid that was ligated into the pRE1-Mtb cya deletion strain. The sequence of the oligonucleotides, with bold letters indicating the mutation, was as follows: Oligo 1, 5'-dTGACGACACAGTCTGGACACGCG-3'; Oligo 2, 5'-dGACGACGTCTGGACACGCG-3' (see Table I, Oligo 2, for R31G); 5'-dGACGACGTCTGGACACGCG-3' (see Table I, Oligo 2, for R4G); 5'-dGACGACGTCTGGACACGCG-3' (see Table I, Oligo 2, for R44G); 5'-dGACGACGTCTGGACACGCG-3' (see Table I, Oligo 2, for R46A); 5'-dGACGACGTCTGGACACGCG-3' (see Table I, Oligo 2, for R43A); 5'-dGACGACGTCTGGACACGCG-3' (see Table I, Oligo 2, for R43K); 5'-dGACGACGTCTGGACACGCG-3' (see Table I, Oligo 2, for R44K); 5'-dGACGACGTCTGGACACGCG-3' (see Table I, Oligo 2, for R46K); and 5'-dGACGACGTCTGGACACGCG-3' (see Table I, Oligo 2, for R43K/R44K).

**Mutations were confirmed by DNA sequencing using dye-conjugated Dideoxynucleotidetoprimers, digested with NdeI and BalIII, purified from low melting agarose gel, and cloned into the pRE1-Mtb cya recombinant plasmid for the construction of a full-length Mtb cya deletion strain.**

**Expression of Adenylyl Cyclase(s)**—The pRE1-Mtb cya recombinant plasmid was digested with NdeI and BalIII to remove the DNA fragment coding for the amino-terminal 57 amino acids. A DNA cassette was constructed containing the pRE1-Mtb cya recombinant plasmid that was ligated into the pRE1-Mtb cya deletion strain. The sequence of the oligonucleotides, with bold letters indicating the mutation, was as follows: Oligo 1, 5'-dTGACGACACAGTCTGGACACGCG-3'; Oligo 2, 5'-dGACGACGTCTGGACACGCG-3' (see Table I, Oligo 2, for R31G); 5'-dGACGACGTCTGGACACGCG-3' (see Table I, Oligo 2, for R4G); 5'-dGACGACGTCTGGACACGCG-3' (see Table I, Oligo 2, for R44G); 5'-dGACGACGTCTGGACACGCG-3' (see Table I, Oligo 2, for R46A); 5'-dGACGACGTCTGGACACGCG-3' (see Table I, Oligo 2, for R43A); 5'-dGACGACGTCTGGACACGCG-3' (see Table I, Oligo 2, for R43K); 5'-dGACGACGTCTGGACACGCG-3' (see Table I, Oligo 2, for R44K); 5'-dGACGACGTCTGGACACGCG-3' (see Table I, Oligo 2, for R46K); and 5'-dGACGACGTCTGGACACGCG-3' (see Table I, Oligo 2, for R43K/R44K).

**Mutations were confirmed by DNA sequencing using dye-conjugated Dideoxynucleotidetoprimers, digested with NdeI and BalIII, purified from low melting agarose gel, and cloned into the pRE1-Mtb cya recombinant plasmid for the construction of a full-length Mtb cya deletion strain.**

**Expression of Adenylyl Cyclase(s)**—The pRE1-Mtb cya recombinant plasmid was digested with NdeI and BalIII to remove the DNA fragment coding for the amino-terminal 57 amino acids. A DNA cassette was constructed containing the pRE1-Mtb cya recombinant plasmid that was ligated into the pRE1-Mtb cya deletion strain. The sequence of the oligonucleotides, with bold letters indicating the mutation, was as follows: Oligo 1, 5'-dTGACGACACAGTCTGGACACGCG-3'; Oligo 2, 5'-dGACGACGTCTGGACACGCG-3' (see Table I, Oligo 2, for R31G); 5'-dGACGACGTCTGGACACGCG-3' (see Table I, Oligo 2, for R4G); 5'-dGACGACGTCTGGACACGCG-3' (see Table I, Oligo 2, for R44G); 5'-dGACGACGTCTGGACACGCG-3' (see Table I, Oligo 2, for R46A); 5'-dGACGACGTCTGGACACGCG-3' (see Table I, Oligo 2, for R43A); 5'-dGACGACGTCTGGACACGCG-3' (see Table I, Oligo 2, for R43K); 5'-dGACGACGTCTGGACACGCG-3' (see Table I, Oligo 2, for R44K); 5'-dGACGACGTCTGGACACGCG-3' (see Table I, Oligo 2, for R46K); and 5'-dGACGACGTCTGGACACGCG-3' (see Table I, Oligo 2, for R43K/R44K).

**Mutations were confirmed by DNA sequencing using dye-conjugated Dideoxynucleotidetoprimers, digested with NdeI and BalIII, purified from low melting agarose gel, and cloned into the pRE1-Mtb cya recombinant plasmid for the construction of a full-length Mtb cya deletion strain.**

**Cloning of Putative cya Genes Revealed from M. tuberculosis Genome Sequence**—M. tuberculosis genes annotated as RV1264 and RV1320c (7) were amplified by PCR using pJN DNA polymerase, genomic DNA, 5′ end and 3′ end primers containing restriction recognition sequences for NdeI and XbaI, respectively. The sequences of the primers were: RV1264-5′ end primer, 5′-dGGAATTCCTATAGACGAGGAGCGGCGCGC-3' (NdeI primer) and 5′-dTTACGAGACAGGTCTTACGAGGCCGCG-3′ (XbaI primer). The amplified fragment was purified from unreacted primers, digested with NdeI and XbaI, purified from low melting agarase gel, and cloned into a similarly digested pRE1 expression vector. Recombinants were isolated from E. coli C600 λ lysogen and introduced into E. coli MZ1 for protein expression.

**Expression of Adenylyl Cyclase(s)**—The pRE1-Mtb cya recombinant plasmid was digested into E. coli strain MZ1, which carries the temper-
Adenylyl Cyclases from M. tuberculosis H37Rv

Table I

| phenotypic properties of cya genes from M. tuberculosis |
|--------------------------------------------------------|
|安全感 | 段落 | 处理 | T. coli AP850 (cya deletion) | E. coli CA8445 (cycA deletion) |
|模式 | 模式 | 模式 | 模式 | 模式 |
|Lactose | Maltose | Lactose | Maltose |
|--- | --- | --- | --- |
|Rv1625c (Mtb cya) in pREL | + | + | + |
|Rv1264 in pREL | + | + | + |
|Rv1320c in pREL | + | + | + |
|Mtb cya clones 1–8 (in pBR322) | + | + | + |
|Mtb cya clones 1–8 (in pRE1) | + | + | + |

RESULTS

Molecular Cloning of Mtb cya Gene—The full-length Mtb cya gene was isolated in two steps. First, the gene corresponding to the carboxyl-terminal 365 amino acids was cloned by complementation of the catabolic defect for maltose fermentation in the cya deletion strain, T. coli TP610, which lacks adenylyl cyclase activity. Eight red colonies, indicative of putative positive transformations, were identified on MacConkey/Maltose plates. Cyclic AMP synthesis, measured by the in vitro adenylyl cyclase assay in extracts of T. coli TP610 harboring one or the other of the eight clones, was barely above the detection level (level of detection is 1 pmol of cAMP/min/mg of protein; data not shown) but was apparently enough to “turn on” the maltose operon in vivo. Restoration of the catabolic defect with all of these clones was dependent on the cAMP receptor protein, since a cya/crp deletion strain transformed with these plasmids failed to ferment lactose and maltose (Table I). A GenBank search for the presence of a common ORF in all eight clones revealed that the ORF had extensive homology (see below) to the catalytic core of eukaryotic adenylyl and guanylyl cyclases.

It is noteworthy that the orientation of all eight of the inserts was identical and the DNA sequence of all the clones had a start point within about 100 base pairs of each other, but the end points were significantly different, ranging from about 1 to 3.4 kbp. Because there is no apparent translation initiation signal corresponding to the ORF, we reasoned that these clones encoded a fusion protein of the 97 amino acids of the tetracycline resistance protein fused to the BamHI cloning site in pBR322 and an adenylyl cyclase catalytic core capable of synthesizing cAMP. Indeed, sequence analysis at the fusion revealed that Trp97 of the tetracycline resistance protein was fused to Ile79 in cya clones 1, 3, and 7; Ile109 in cya clones 2, 6, and 8; and Ile113 in cya clones 4 and 5. The absence of the translation initiation signal corresponding to this ORF in these eight clones also led us to believe that the full-length cya gene could not be obtained either under the transcriptional control of the constitutive tet promoter in pBR322 or its own transcriptional unit in a multicopy plasmid. This observation is in accord with our previous finding that uncontrolled expression of adenylyl cyclase with concomitant overproduction of cAMP is lethal to E. coli cells, which led to the development of the pRE expression vector for controlled lethal gene expression (10). Consequently, Southern hybridization was used to obtain that portion of the cya gene coding for the amino-terminal terminus of Mtb AC in the pRE vector as described under “Experimental Procedures.”

The complete coding sequence of the Mtb cya gene was assembled from the overlapping sequences of the clones obtained in pBR322 and pRE1 vectors. The nucleotide sequence and the deduced amino acid sequence of the Mtb cya gene are presented in Fig. 1 and have been deposited in GenBank (accession no. AF017731). A putative open reading frame of 443 amino acids, beginning with GTG as an initiation codon at the nucleotide triplet 841–843 and terminating with the nucleotide triplet TGA at 2170–2172, was identified. From the M. tuberculosis genome sequence published later (7), the putative start codon for the Mtb cya gene was suggested to be the GTG triplet at 916–918 in the same reading frame as above. The ribosome binding sequences for both of the GTG initiators are poor matches to the consensus sequence. However, visual examination of the consensus sequence suggested that the GTG initiation codon at nucleotide 841–843 has a relatively better ribosome binding sequence (nucleotides 826, 829, 830, and 831, underlined in Fig. 1). Hence we have expressed adenylyl cyclase in the E. coli MZ1/pRE1 expression vector system beginning at nucleotide 841. The Mtb cya gene that we cloned earliest (GenBank accession no. AF017731 (1997)) and a gene with annotation Rv1625c from M. tuberculosis genome sequence (7) published later are identical except for the difference in the position of initiation codon.

Putative Secondary Structure of Mtb AC—A hydropathy plot (not shown) of the Mtb adenylyl cyclase protein sequence suggests that the amino-terminal domain (aa 47–199) of the enzyme contains six transmembrane helices (Fig. 2), which is consistent with the observation that the Mtb adenylyl cyclase expressed in E. coli is membrane-bound (see below). With the exception of one negatively charged amino acid in the fourth...
(Asp) and fifth (Glu) helices, the other helical residues are all hydrophobic. The amino-terminal cytoplasmic tail is rich in arginine residues (17%), and their importance in adenylyl cyclase activity has been elucidated (see below).

Expression and Activity Analysis of Adenylyl Cyclase—The expression profile (Fig. 3) and activity (Table II) of Mtb adenylyl cyclase from M. tuberculosis H37Rv. The open reading frame corresponding to adenylyl cyclase has two putative GTG initiation codons, one beginning at nucleotide 841 and the other at nucleotide 916. Because the GTG initiator at nucleotide 841 has a relatively better consensus ribosome binding sequence (underlined), we have expressed adenylyl cyclase in pRE1 expression vector (10) beginning at nucleotide 841. The TGA stop codon is identified by an asterisk.

The nucleotide and deduced amino acid sequences of the cya gene from M. tuberculosis H37Rv. Fig. 1. A schematic topological model of Mtb AC. The putative six-membrane helices are boxed. The cytoplasmic carboxyl-terminal domain (26 kDa) is represented by an oval shape. A putative interaction between the Arg$^{43}$ and Arg$^{44}$ residues with ATP and the carboxyl-terminal domain is depicted. Single-letter amino acid abbreviations are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
transmembrane protein could all be incorporated into the membrane space, we investigated the localization and the associated activity of the enzyme in the membrane and cytosolic fractions (Table II). About 65% of adenyl cyclase activity was detected in the cytoplasmic membrane, whereas essentially all of the expressed protein with low activity was localized in the cytosolic fraction. This suggests that the portion of adenyl cyclase incorporated into the membrane has an ordered structure and is a high activity form but the soluble enzyme is misfolded.

**Mn-ATP Is a Better Substrate for Mtb AC Than Mg-ATP**—We investigated the divalent metal ion requirement for adenyl cyclase activity. Cytoplasmic membranes enriched with the Mtb AC were isolated from *E. coli* MZ1 as described under “Experimental Procedures.” Kinetic studies using membranes containing Mtb AC revealed that the Michaelis-Menten constant ($K_m$) for adenosine triphosphate in the presence of 20 mM Mn$^{2+}$ was $3.5 \mu M$ with a $V_{max}$ of $210 \text{ nmol of cAMP/min/mg}$ of protein, whereas the $K_m$ for adenosine triphosphate in the presence of 2 mM Mn$^{2+}$ was $50 \mu M$ with a $V_{max}$ of $475 \text{ nmol of cAMP/min/mg}$ protein (Table III). This amounts to a 70-fold lower $K_m$ for Mn-ATP$^2+$ compared with Mg-ATP$^2+$. The rate of the reaction with 1 mM Mg-ATP$^2+$ and 1 mM Mn-ATP$^2+$, at the physiological concentration of ATP, was 10 and 475 nmol of cAMP formed/min/mg of membrane protein, respectively, which amounts to a 47-fold activation by Mn$^{2+}$.

**Site-directed Mutagenesis of Amino-terminal Arginine Residues**—Based on a putative secondary structure (Fig. 2), the arginine-rich amino-terminal cytoplasmic tail contributes to the net positive charge of this region. Although these arginine residues satisfy the “positive inside” rule in the membrane protein topology (20), we investigated whether these arginine residues have any role in AC activity in view of the ability of arginine residues to bind ATP, the substrate for adenyl cyclase. A mutant version of Mtb AC was constructed wherein all arginine residues to the level of expression were changed to alanine and glycine residues. This mutant Mtb AC was the same as for the wild type adenyl cyclase (Fig. 3, lane 3, respectively), and the mutant had no adenyl cyclase activity over and above the uninduced level with Mg-ATP$^2+$ or Mn-ATP$^2+$ as the substrate (Table IV). This result suggests an important role for the amino-terminal cytoplasmic tail in the catalysis of ATP to cAMP by the cytoplasmic domain. However, we cannot discount the possibility that topological perturbations might have occurred in the disposition of membrane helices upon a drastic change of...
Adenylyl cyclase activity in the site-directed mutants

French press extracts were prepared from 3-h heat-induced cells as described under “Experimental Procedures.” Eighty micrograms of crude extract protein was loaded in each lane of the 10% SDS-polyacrylamide gel. Lane 1, molecular weight markers; lane 2, E. coli MZ1/pRE1 negative control; lane 3, wild type Rv1625c; lane 4, R43A mutant; lane 5, R44G mutant; lane 6, R43A/R44G mutant; lane 7, R43A/R44G mutant; lane 8, R43K mutant; lane 9, R44K mutant; lane 10, R46K mutant; lane 11, R43K/R44K mutant; lane 12, 8R-G/A mutant; lane 13, a positive control (overproduces E. coli sugar transport protein IIAG) (23). The protein band corresponding to Mtb adenylyl cyclase Rv1625c is marked with an arrow.

![Image](http://www.jbc.org/)

TABLE IV

| Ac source | % wild type AC activity |
|-----------|------------------------|
|           | Mg-ATP<sup>a</sup> | Mn-ATP<sup>b</sup> |
| Wild type | 100                    | 100                    |
| 8R→4G/4A | 77 ± 0.1               | 77 ± 0.1               |
| R4G      | 82 ± 1                 | 82 ± 1                 |
| R18A     | 82 ± 1                 | 82 ± 1                 |
| R18A/R19G | 72 ± 6              | 72 ± 6                 |
| R27A     | 82 ± 3                 | 78 ± 3                 |
| R31G     | 115 ± 6                | 118 ± 2                |
| R43A     | 67 ± 0.2               | 61 ± 1                 |
| R44G     | 83 ± 0.0               | 41 ± 0.4               |
| R46A     | 83 ± 0.3               | 85 ± 4                 |
| R43A/R44G | 0.2 ± 0.1            | 1.3 ± 0.1              |
| R43K     | 63 ± 0.1               | 77 ± 0.1               |
| R44K     | 80 ± 0.2               | 116 ± 0.5              |
| R46K     | 92 ± 0.2               | 135 ± 0.17             |
| R43K/R44K | 7 ± 0.2              | 39 ± 3                 |

<sup>a</sup> 100% activity for wild type adenylyl cyclase with Mg-ATP as the substrate equals 5.8 ± 0.5 nmol of cAMP/min/mg of protein.

<sup>b</sup> 100% activity for wild type adenylyl cyclase with Mn-ATP as the substrate equals 70 ± 16 nmol of cAMP/min/mg of protein.

All the arginine residues to glycine and alanine residues such that adenylyl cyclase was rendered inactive. To examine which of the arginine residue(s), if any, may play a critical role in the activity, site-directed mutagenesis of each arginine residue was performed.

An adenylyl cyclase activity profile of the wild type and site-directed mutants is presented in Table IV. Adenylyl cyclase mutants R4G, R18A, R18G, R19G, R27A, R31G, and R46A are all nearly as active (70–120%) as the wild type enzyme with either Mg-ATP<sup>2-</sup> or Mn-ATP<sup>2-</sup> as the substrate. It is clear from these data that Arg<sup>43</sup> and Arg<sup>44</sup> have a significant role in the activity of the enzyme. The R43A and R44G mutants are only about 35% as active as the wild type enzyme with Mg-ATP<sup>2-</sup> whereas the double mutant R43A/R44G is nearly inactive having only about 0.2% activity of the wild type enzyme. With Mn-ATP<sup>2-</sup> as the substrate, the R43A mutant has 60% activity and the R44G mutant has 40% activity, reflecting the higher affinity of adenylyl cyclase for this substrate (Table III). The double mutant R43A/R44G, however, has only 1% activity even with Mn-ATP<sup>2-</sup> as the substrate. This interesting observation suggests that Arg<sup>43</sup> and Arg<sup>44</sup> vicinal residues have critical role in the catalysis of ATP to cAMP by the cytoplasmic domain. We tested whether a conserved substitution like lysine for arginine would restore the activity. We found that lysine restored the activity that was lost by alanine/glycine substitutions at Arg<sup>43</sup> and Arg<sup>44</sup>. The double lysine mutant R43K/R44K resulted in an ~30-fold activation of adenylyl cyclase activity compared with the R43A/R44G mutant (0.2 → 7% with Mg-ATP<sup>2-</sup> and 1.3 → 39% with Mn-ATP<sup>2-</sup> as substrate). These results suggest that the formation of the catalytic site requires an interaction between the arginine-rich distal amino-terminal cytoplasmic tail and the carboxyl-terminal cytoplasmic domain.

Protein expression for the wild type (Fig. 4, lane 3) and the site-directed mutants R43A, R44G, R46A, R43A/R44G, R43K, R44K, R46K, and R43K/R44K (lanes 4–11, respectively) of adenylyl cyclase showed that mutant protein(s) is expressed just as well as the wild type enzyme. This observation rules out the possibility that the differences in the activity of various mutants of adenylyl cyclase is not a reflection of the level of the enzyme expression but is due to the mutation itself.

Expression and Activity Analysis of Other Putative Adenylyl Cyclases—The M. tuberculosis H37Rv genome sequence revealed five genes (Rv1264, Rv1318c, Rv1319c, Rv1320c, and Rv1625c) annotated as putative adenylyl cyclases (7). The Mtb cya gene we cloned is identical to Rv1625c. Genes Rv1318c, Rv1319c, and Rv1320c have about 70% identity among themselves, whereas Rv1264 and Rv1625c have about 20% identity to this group of genes and to each other. We cloned Rv1320c and Rv1264 to represent all putative cya genes, expressed in E. coli (Fig. 3) and tested for adenylyl cyclase activity (Table V). The Rv1320c gene product (62 kDa) represented about 5% of the E. coli protein (lane 3) but had no adenylyl cyclase activity under the standard assay conditions. Although the Rv1264 gene product (42 kDa) was expressed to about 10% of E. coli protein (lane 2), it was only 0.5% as efficient as Rv1625c (Mtb cya) in synthesizing cAMP. Phenotypic analysis of Rv1264 to ferment lactose/maltose was positive, and that of Rv1320c was negative (Table I), consistent with adenylyl cyclase activity profile (Table V).

Calmodulin, Forskolin, and Mammalian G<sub>α</sub> Have Marginal Effects on Mtb AC—It is well established that cAMP synthesis by the chimer of the cytoplasmic catalytic domains of eukaryotic adenylyl cyclase is activated by G<sub>α</sub>-GTP<sub>γ</sub>S (50-fold), forskolin (150-fold) and the combination of G<sub>α</sub>-GTP<sub>γ</sub>S and forsk-
Adenylyl Cyclases from M. tuberculosis H37Rv

Kolin with a synergistic effect (600-fold) (21, 22). Because the Mtb AC shows extensive homology with the catalytic core in the cytoplasmic domains of eukaryotic adenylyl cyclase (Fig. 5), we tested the effect of Gsα-GTPγS, forskolin, and calmodulin on the membranous Mtb AC. Only a modest stimulation (25–50%) of the Mtb AC by these effectors alone or in combination was observed (data not shown). However, it is interesting to note that the basal activity of Mtb AC expressed in E. coli (2.0 nmol of cAMP/min/mg) (21). The crystal structure of the eukaryotic adenylyl cyclase in complex with forskolin and Gsα-GTPγS identified the essential amino acid residues involved in the activation of the enzyme by these effectors (23, 24). Based on this structural information and sequence alignment (Fig. 5), it is clear that two critical residues for activation by forskolin, Thr426 and Ser927 in the eukaryotic type I adenylyl cyclase CI and CII domains, respectively, are replaced by Asn372 and Asp300 in the Mtb AC. Although the other predicted contacts, Phe254, Trp367, and Val371, are conserved in Mtb AC, the absence of Thr at position 372 and Ser at position 300 in the Mtb AC might explain the diminished activation of this enzyme by forskolin.

DISCUSSION

Analysis of Putative cya Genes—We report here the cloning of adenylyl cyclase gene(s) (cya) from M. tuberculosis H37Rv and biochemical characterization of the enzyme. Cole et al. (7) deciphered the nucleotide sequence of the M. tuberculosis genome and suggested five gene products as putative adenylyl cyclases based on 30–40% homology within 200 amino acids of the catalytic core of eukaryotic adenylyl cyclase. These putative cya genes are annotated as Rv1625c, Rv1264, Rv1318c, Rv1319c, and Rv1320c. Gene products of these five, viz. Rv1318c, Rv1319c, and Rv1320c, are a highly homologous group with 70% identical amino acids. Gene products of Rv1625c and Rv1264 have only about 20% identity among themselves and with the gene cluster of Rv1318c, Rv1319c, and Rv1320c. In our attempt to clone the Mtb cya gene by complementation of the catalytic defect in the E. coli cya deletion strain, in work done before the genome sequence was published (25), we obtained eight clones of the same gene, Rv1625c (Mtb cya), but none of the other (GenBankTM accession no. AF017731). Hence, we cloned Rv1264 and Rv1320c (to represent Rv1318c and Rv1319c) by PCR to evaluate the function of the encoded proteins. The protein encoded by Rv1265c is undoubtedly adenylyl cyclase as observed here. The Rv1264 gene product has about 0.5% activity compared with Rv1625c, although the protein encoded by Rv1264 was expressed better than Rv1625c (Fig. 3). The protein encoded by Rv1320c did not function as adenylyl cyclase under the conditions tested. It is not surprising then that cloning by complementation assay produced only Rv1625c from the chromosomal library but none of the other genes. From this analysis, we can corroborate the annotation of Rv1264 as adenylyl cyclase but not Rv1320c or its homologues Rv1318c and Rv1319c. However, we cannot discount the possibility that Rv1318c, Rv1319c, and Rv1320c gene products may require some unidentified cofactor and function as adenylyl cyclases in their native environment.

Homology with Eukaryotic Adenylyl Cyclases—The lack of similarity of Mtb AC to other prokaryotic adenylyl cyclases but its conserved nature with respect to eukaryotic adenylyl cyclases (see below) is noteworthy. The Mtb AC contains an aminoterminal cytoplasmic tail (46 aa) followed by a single six helical transmembrane domain (152 aa) and a cytoplasmic catalytic domain (245 aa) and hence belongs to a new class of adenylyl cyclases; such an organization was found in Stigmatella aurantiaca, a Gram-negative myxobacterium (26). All of the higher eukaryotic adenylyl cyclases thus far characterized, with the one exception being soluble adenylyl cyclase (27), contain the same secondary structural organization but duplicated. Consequently, the monomeric molecular weight of the Mtb AC is about half that of eukaryotic adenylyl cyclases. The duplication of this class of Mtb AC sequence in the higher eukaryotic adenylyl cyclases is intriguing from the evolutionary point of view. In this context, we have cloned the cya gene from Mycobacterium smegmatis, a nonpathogenic species of mycobacte-

| TABLE V | Expression and activity analysis of adenylyl cyclases in E. coli |
|------------------|------------------|
| AC source       | AC activity      |
|                 | Mg-ATP (nmol cAMP/min/mg) | Mn-ATP (nmol cAMP/min/mg) |
| Rv1625c         | 2.50 ± 0.3       | 55.50 ± 5.0     |
| Rv1264          | 0                | 0.28 ± 0.02     |
| Rv1320c         | 0                | 0               |

Fig. 5. Alignment of the amino acid sequence of the cytoplasmic domain of Mtb adenylyl cyclase with the CI and CII domains of eukaryotic (bovine brain) adenylyl cyclase. Identical amino acids are shaded dark and similar amino acids are shaded light.
ria. The deduced amino acid sequence of *M. smegmatis* adenylyl cyclase differs from *Mtbc* adenylyl cyclase and is similar to a prokaryotic (anemana) adenylyl cyclase. Because *M. smegmatis* does not appear to have adenylyl cyclase similar to *Mtbc* AC H37Rv adenylyl cyclase, it is tempting to speculate that *M. tuberculosis* H37Rv might have acquired the streamlined eukaryotic *cyA* gene during its association with mammals.

Alignment of the carboxyl-terminal half of *Mtbc* adenylyl cyclase (aa 200–427) with the catalytic core in cytoplasmic domains CI and CII of bovine adenylyl cyclase (8) revealed matches as high as 32% identical amino acids within a stretch of 229 aa of the CI domain (aa 246–474) and 40% identical amino acids within a stretch of 247 aa of the CII domain (aa 817–1063) (Fig. 5). The similarity with conserved substitutions was 43% in the CI domain and the 54% in CII domain.

Putative Roles of Arg43 and Arg44 in Adenylyl Cyclase Activity—The amino-terminal cytoplasmic tail (46 aa) of *Mtbc* AC contains eight arginine residues that contribute to the net positive charge of this region of the protein and satisfy the positive inside rule in the membrane protein topology (20). Because it is also known that arginine residues bind ATP, in this case the substrate for AC, site-directed mutants of these arginine residues were created to determine whether they have any role in AC activity. Site-directed mutagenesis to alanine or glycine clearly showed an important role for Arg43 and Arg44 in the activity of AC, whereas Arg6, Arg18, Arg30, Arg37, Arg113, and Arg119 had no apparent effect. Mutagenesis of Arg43 and Arg44 to conserved substitution to lysine restored the enzyme activity although to a level lower than the wild type enzyme activity, which perhaps reflects the importance of the longer side chain of arginine. Further support for the role of Arg43 and Arg44 in the activity of AC comes from the observation that all of the eight of the carboxyl-terminal Mtbc *cyA* clones in pBR322, obtained by complementation of the catalytic defect, with start points in the second and third helices (Fig. 2; Ile79, Ile80, or Ile1–13 fused to Trp97 of tetracycline resistance protein) but devoid of the arginine-rich amino-terminal cytoplasmic tail, also had no appreciable activity. We propose that Arg43 and Arg44 vicinal residues bind ATP and deliver the substrate to the 26-kDa cytoplasmic domain for efficient catalysis. In higher eukaryotic adenylyl cyclases, the catalytic CII domain exhibits low adenylyl cyclase activity, but the interaction of the CII domain with the CI domain enhances the activity of the enzyme (21, 22). A similar analogy may be drawn with the *Mtbc* AC wherein the arginine-rich cytoplasmic tail may contribute to overall catalysis by the 26-kDa cytoplasmic domain.

We considered an alternative explanation of why Arg43 and Arg44 residues are important for activity. Although Arg46 would be the residue that contributes to the “snorkel effect” (28), the role of Arg43 and Arg44 is elusive in membrane topology. The putative location of Arg43 and Arg44 near the membrane interface is intriguing in that these residues may serve as the topological determinants (20) to anchor AC to the membrane and contribute to the structural topology and integrity of the enzyme and thereby to full activity. Mutagenesis of these basic amino acid residues to hydrophobic residues like alanine and glycine might perturb the anchoring process, resulting in a low activity form of the enzyme. Whether or not R43A, R44G, and R43A/R44G mutations distort the membrane helical topology, the mutations clearly affected the enzyme activity. We have shown in Table I that 35% of the activity of the wild type AC is in the 100,000 × g soluble fraction, and 65% of the activity is associated with the membranes. Contrary to this distribution of enzyme activity, greater than 99% of AC protein is found in the soluble fraction as judged by SDS-polyacrylamide gel electrophoresis Coomassie stain, whereas membranous AC protein could not be detected by this technique (data not shown). In this context it should be noted that the protein expression profiles for the wild type (Fig. 4, lane 2) and all of the mutants of AC (R43A, lane 4; R44G, lane 5; R46A, lane 6; R43A/R44G, lane 7; R43K, lane 8; R44K, lane 9; R46K, lane 10; R43K/R44K, lane 11; and 8R → G/A, lane 12) are nearly identical. Given these data, even if a mutant AC failed to integrate into the membrane with proper topology, the mutant AC(s) should have had all of the activity associated with the soluble fraction if the mutation had no effect on the enzyme activity. The double mutant R43A/R44G and the mutant wherein all of the eight arginine residues are mutated (8R → G/A) are nearly inactive (Table V). Point mutation effects of R43A and R44G are smaller, and the double mutation effect is more than additive. Hence, it appears that Arg43 and Arg44 are involved in facilitating the activity of Mtbc AC; a hypothetical model representing an interaction between these arginine residues and the carboxyl terminus is presented in Fig. 2.

Role of Adenylyl Cyclase in Bacterial Pathogenesis—Adenylyl cyclase has been shown to be an important virulent factor in bacterial pathogens like *B. pertussis* and *B. anthracis* (2). The soluble secreted form of adenylyl cyclase from these two species invades host cells and is activated by the host calmodulin, thereby depleting ATP pool and elevating cAMP concentration in the host cell. This unregulated conversion of ATP to cAMP compromises the bactericidal activity of the host immune system (5, 6). We have suggested that Mtbc AC is a six helical transmembrane protein and have shown that the active form of the enzyme is membrane-bound. Hence, the enzyme cannot be secreted into the host cell to alter the physiological concentration of ATP/cAMP. In any event, calmodulin has no effect on Mtbc AC. There may exist alternative mechanisms for creating the ATP/cAMP imbalance in macrophages where mycobacteria reside. The physiological significance of the activation of Mtbc AC by Mn2+ cannot be ignored. The genomic sequence indicates that a homologue of the macrophage N ramp Mn2+ transporter protein (29, 30) is present in *M. tuberculosis* H37Rv (Rv9924c) (7). It is known that macrophages can import Mn2+ and thereby provide a rich Mn2+ environment for the potential activation of resident Mtbc AC. The elevated cAMP thus obtained can be secreted into the macrophages, which would inhibit several phagocyte-associated processes including lysosomal fusion (31), and mycobacteria can propagate within the host macrophages.

Padh and Venkitasubramanian (32, 33) have documented the presence of cAMP in mycobacteria. However, intracellular cAMP-mediated functions via cAMP receptor protein were not observed (34). The *M. tuberculosis* H37Rv genome sequence revealed eukaryotic-like protein kinases (7, 35), which may be involved in the intracellular cAMP-mediated signaling pathways. An important extracellular function for cAMP from *M. tuberculosis* H37Rv may be the prevention of fusion between phagosomes and lysosomes. This working model needs to be tested experimentally to assign a role for adenylyl cyclase in the pathogenesis of *M. tuberculosis*.

Acknowledgments—We thank Dr. Alan Peterkofsky (National Institutes of Health) and Dr. Malcolm Byrnes (National Institute of Standards and Technology) for their valuable comments on the manuscript.

REFERENCES
1. Dolin, P. J., Raviglione, M. C., and Kochi, A. (1994) Bull. W. H. O. 72, 213–220
2. Masure, H. R., Shattuck, R. L., and Storm, D. R. (1987) Microbiol. Rev. 51, 60–65
3. Wolff, J., Cook, G. H., Goldhammer, A. R., and Berkowitz, S. A. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 3840–3844

---

*2 M. Kamireddi and P. Reddy, unpublished results.*
Eukaryotic-like Adenylyl Cyclases in *Mycobacterium tuberculosis* H37Rv: CLONING AND CHARACTERIZATION

Sathyavelu K. Reddy, Madhavi Kamireddi, Kiran Dhanireddy, Lynn Young, Andrew Davis and Prasad T. Reddy

*J. Biol. Chem.* 2001, 276:35141-35149.
doi: 10.1074/jbc.M104108200 originally published online June 28, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M104108200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 34 references, 15 of which can be accessed free at http://www.jbc.org/content/276/37/35141.full.html#ref-list-1