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Purified Recombinant Hypothetical Protein Coded by Open Reading Frame Rv1885c of *Mycobacterium tuberculosis* Exhibits a Monofunctional AroQ Class of Periplasmic Chorismate Mutase Activity*

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Naturally occurring variants of the enzyme chorismate mutase are known to exist that exhibit diversity in enzyme structure, regulatory properties, and association with other proteins. Chorismate mutase was not annotated in the initial genome sequence of *Mycobacterium tuberculosis* (*Mtb*) because of low sequence similarity between known chorismate mutases. Recombinant protein coded by open reading frame Rv1885c of *Mtb* exhibited chorismate mutase activity *in vitro*. Biochemical and biophysical characterization of the recombinant protein suggests its resemblance to the AroQ class of chorismate mutases, prototype examples of which include the *Escherichia coli* and yeast chorismate mutases. We also demonstrate that unlike the corresponding proteins of *E. coli*, *Mtb* chorismate mutase does not have any associated prephenate dehydratase or dehydrogenase activity, indicating its monofunctional nature. The Rv1885c-encoded chorismate mutase showed allosteric regulation by pathway-specific as well as cross-pathway-specific ligands, as evident from proteolytic cleavage protection and enzyme assays. The predicted N-terminal signal sequence of *Mtb* chorismate mutase was capable of functioning as one in *E. coli*, suggesting that *Mtb* chorismate mutase belongs to the AroQ class of chorismate mutases. It was evident that Rv1885c may not be the only enzyme with chorismate mutase enzyme function within *Mtb*, based on our observation of the presence of chorismate mutase activity displayed by another hypothetical protein coded by open reading frame Rv0948c, a novel instance of the existence of two monofunctional chorismate mutases ever reported in any pathogenic bacterium.

Mycobacterium tuberculosis (*Mtb*)[1] has developed ingenious mechanisms to survive inside the hostile environment presented by the host and to acquire essential nutrients from this adverse environment (1–3). The emergence of drug-resistant strains and synergy with the AIDS virus has further aggravated the disease scenario (4–6). For the development of new therapeutic intervention strategies, there is a need for identification of novel targets that are not only unique to *Mtb* but blocking of which would either prove lethal to the bacterium or render it extremely susceptible to the host immune response. In this context, understanding the mechanism of action of the aromatic amino acid pathway enzymes of *Mtb* assumes the utmost importance because most of the corresponding genes have been proven essential for the bacterium and have no human or mammalian counterpart (7, 8). Moreover, amino acid auxotrophs of *Mtb* do not survive or multiply in macrophages (9, 10), suggesting that these amino acids are not available within the compartment of the macrophage in which the bacteria reside.

Chorismic acid is the last common precursor in the aromatic amino acid biosynthesis pathway and is a substrate for multiple enzymes (11, 12). Hence it has always been of interest to explore how a microbe partitions chorismate into diverse metabolic pathways. Various enzymes that utilize chorismate as a substrate include chorismate mutase (CM), anthranilate synthase, isochorismate synthase, and *p*-amino benzoate synthase (13). Chorismate mutase is a key regulatory enzyme of the shikimate pathway that catalyzes the Claisen rearrangement of chorismate to prephenate (Fig. 1), a committed step in L-Phe and L-Tyr biosynthesis. Besides being a rare example of an enzyme that catalyzes a pericyclic rearrangement reaction, chorismate mutase has also gathered attention due to the presence of completely different protein folds and ligand binding pockets in different organisms. Members of the AroQ class of chorismate mutases are all helix-bundle proteins, whereas members of the AroH class of chorismate mutases possess a trimeric pseudo-α/β-barrel structure (14). The chorismate mutases of yeast and *E. coli* belong to the AroQ class of chorismate mutases (15–21) and are allosterically regulated by aromatic amino acids. The monofunctional chorismate mutase of *Bacillus subtilis* falls under the AroH class and is a feedback insensitive enzyme (22, 23).

The low similarity at the amino acid sequence level among known chorismate mutases makes the enzyme one of the finest examples of convergent evolution of enzyme reaction mechanisms (24). However, this low sequence similarity also accounts

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[1] The abbreviations used are: *Mtb*, *Mycobacterium tuberculosis*; ORF, open reading frame; ss, signal sequence; aa, amino acid(s); XP, 5-bromo-4-chloro-3-indolyl phosphate; CPB, citrate phosphate buffer; MES, 4-morpholineethanesulfonic acid; CM, chorismate mutase; rRv1885c, recombinant Rv1885c; rRv0948c, recombinant Rv0948c; PDT, prephenate dehydratase; PDH, prephenate dehydrogenase; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

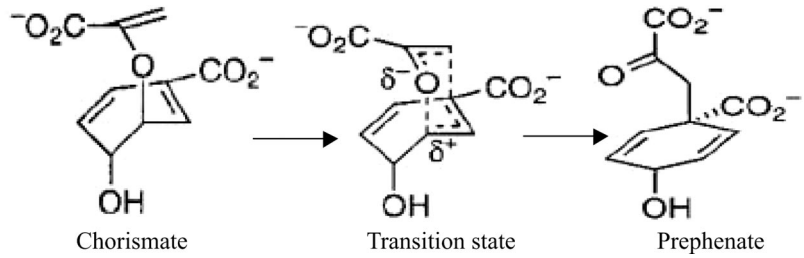

FIG. 1. **Conversion of chorismate to prephenate proceeds via a chair-like transition state.** This reaction is a rare example of an enzyme-catalyzed pericyclic reaction.

for the lack of confidence in the annotation of the enzyme in genome annotation projects. On the other hand, the uniqueness of the enzyme also makes it an attractive target for herbicides and antibacterial compounds (25).

Chorismate mutase was not annotated in the initial genome sequence *of Mtb*. However, the recent Cluster of Orthologous groups (NCBI) annotation of the *Mtb* genome suggests that chorismate mutase activity can be attributed to two ORFs, Rv1885c and Rv0948c (www.ncbi.nlm.nih.gov/COG/). The ORF Rv1885c has been annotated in the TubercuList web server (genolist.pasteur.fr/TubercuList/), Institute Pasteur, as a conserved hypothetical protein with some similarity to the monofunctional chorismate mutases of *Erwinia herbicola* (28.6% identity in a 133-aa overlap). Rv0948c has also been annotated as a conserved hypothetical protein, equivalent to a conserved hypothetical protein (105 aa) from *M. leprae* (NP_301237.1 NC_002677) that is also similar to the N terminus of some chorismate mutase/prephenate dehydratase enzymes.

We intended to study whether these two hypothetical proteins indeed show *in vitro* chorismate mutase activity and, if they do, how they are related to the two known classes of chorismate mutases (AroQ and AroH). Our approach involved expressing the *Mtb* ORFs Rv1885c and Rv0948c in *E. coli* and determining the biochemical and biophysical properties of the encoded proteins. Whereas more extensive studies were carried out with the protein coded by ORF Rv1885c, we were also able to demonstrate that Rv0948 also possesses chorismate mutase activity, although with a reduced turnover. Kinetic and regulatory studies of rRv1885c indicate several unique properties of the enzyme that include feedback regulation by pathway-specific as well as cross-pathway-specific ligands in the same manner. We have also used a gene fusion approach for functional characterization of the predicted N-terminal signal sequence of *Mtb* chorismate mutase. Our study provides sufficient evidence to conclusively place the protein coded by ORF Rv1885c of *Mtb* in the AroQ class of periplasmic chorismate mutases.

EXPERIMENTAL PROCEDURES

Bacterial Strains/Plasmids—All bacterial strains used in this study are described in Table I. The plasmid vectors (with their sources) and the recombinant plasmids constructed are also described in the same table. Integrity of all the plasmid clones was confirmed by DNA sequencing.

Cloning, Overexpression, and Purification of Recombinant Proteins in E. coli—The *Mtb* ORFs Rv1885c and Rv0948c were amplified from *Mtb* H37Rv genomic DNA using primer pairs with specific restriction enzyme sites (Rv1885c, ATCATATGTTGCTTACCCGTCCACGTGA (forward) and ATCTCGAGGGCCGGCGGTAGG (reverse); Rv0948c, AATCATATGAGACCAGAACCCCCACATCACGA (forward) and ATAAAGCTTGTGACCGAGGCGGCCCCT (reverse)). The PCR products were cloned into the corresponding sites of pET23a expression vector (Novagen). In this way, plasmids pETCM1 and pETCM2 capable of encoding C-terminal His_6-tagged versions of the two putative *Mtb* chorismate mutases were generated (Table I).

E. coli BL21(DE3) cells transformed with pETCM1 and pETCM2 were grown in 1 liter of LB medium supplemented with 100 μg/ml ampicillin and 10% glycerol. Isopropyl 1-thio-β-D-galactopyranoside was added to the mid-log phase culture at a final concentration of 0.1 mM. The cells were kept in an incubator shaker for another 5 h at 27 °C/150 rpm to allow protein expression. A low temperature was used to allow the protein to enter the soluble phase. After induction, the cells were harvested by centrifugation and resuspended in 20 ml of lysis buffer (10 mM Tris-HCl, 100 mM NaCl, and 10% glycerol, pH 7.5) with 0.1 mM phenylmethylsulfonyl fluoride and disrupted using a sonicator. After another round of centrifugation for 10 min at 10,000 $\times$ *g*, the supernatant was applied to a Talon cobalt affinity resin (Clontech).

Affinity Chromatography—The supernatant was allowed to bind to Talon resin (Clontech) packed in a polypropylene column. The recombinant protein was purified to near homogeneity after washing the column with 5 bed volumes of lysis buffer containing 30 mM imidazole and elution with 250 mM imidazole. The eluates were analyzed by SDS-PAGE and dialyzed against Tris buffer to remove salts and imidazole. The purity of the enzyme was checked by SDS-PAGE followed by Coomassie Blue staining.

Enzyme Assays and Kinetic Studies—Chorismate mutase activity assays were carried out as described by Davidson and Hudson (26), with a few modifications. This assay is based on enzymatic conversion of chorismate to prephenate, which is terminated by the addition of HCl. Prephenate can be further converted to phenylpyruvate under acidic conditions that can be measured spectrophotometrically at an alkaline pH (27). Reaction volumes of 100 μl contained 10 mM Tris-HCl, pH 7.5, chorismic acid (0.5–5 mM), and, optionally, tyrosine, phenylalanine, or tryptophan (50 μM to 3 mM). The pH of the reaction was maintained by varying the ionic strength of the buffer. The reaction was started by adding 10–200 pmol of the purified protein to the pre-warmed chorismate solution (at 30 °C), and it was stopped by the addition of 10 μl of 1.6 N HCl. After a second incubation of the mixture at 37 °C for 15 min, 890 μl of 1.58 N NaOH was added, and the absorbance was recorded at 320 nm. To exclude the absorbance caused by the uncatalyzed arrangement of chorismate, blanks of increasing chorismate concentrations without enzyme were prepared, and their absorbance was also recorded. These readings were then subtracted from the absorbance measured for enzyme activities at different concentrations of the substrate.

For determination of the optimum enzyme and substrate concentrations, 10–800 pmol of enzyme and 0.5–3 mM chorismic acid were used. Buffers of different pH values (CPB, pH 4; CPB, pH 4.5; MES, pH 6; HEPES, pH 7; Tris-HCl, pH 7.5; Tris-HCl, pH 8) were used to determine the optimum pH for enzyme activity. Chorismate mutase activity was also assessed at different temperatures (15 °C to 80 °C). One unit of enzyme was defined as the amount of enzyme required to form 1 μmol product/min at 37 °C.

Prephenate dehydratase and prephenate dehydrogenase assays were carried out as previously described (26, 28).

Phe, Tyr, and Trp Feedback Inhibition Assays—Allosteric regulation of chorismate mutase activity by L-Phe, L-Tyr, and L-Trp was measured at 100–800 μM concentrations of the effectors.

Western Blot—Western blot with the cytosolic and periplasmic fractions of *E. coli* BL21 cells expressing *Mtb* chorismate mutase (Rv1885c) was carried out with monoclonal anti-His antibody using the manufacturer's instructions (Santa Cruz Biotechnology). The *E. coli* periplasmic fraction was isolated using a modified osmotic shock procedure described by Qiagen Inc.

Limited Proteolysis—To study the effects of various ligands on enzyme stability, integrity, and accessibility of active sites, limited proteolysis of *Mtb* chorismate mutase was carried out. Trypsin was taken as the protease of choice. Ten μg of recombinant protein (1 μg/μl concentration) was taken, to which different concentrations of the ligand (tyrosine, phenylalanine, tryptophan, salicylate) were added. The reaction buffer contained 50 mM Tris, pH 7.5, and 100 mM NaCl. Trypsin was added at a 1:1000 ratio, and the reaction was incubated at 25 °C for 30 min, following which trypsin was inactivated by the addition of 0.5 mM phenylmethylsulfonyl fluoride. The reaction was mixed with an equal volume of 2× Tris-Tricine loading dye and loaded on a 10% Tris-Tricine gel.

CD Spectrometry—The CD spectrum of the recombinant protein

TABLE I
Bacterial strains and plasmids

Strains/plasmids	Description	Source/ref.
E. coli strains		
BL21 DE3	*F* $^-$ *ompT hsdS*$_B$ (r_B- m_B-) *gal lon dcm* (ΔDE3)	Studier and Moffatt (54)
DH5α	Δ(*argF-lac*)*U169 supE33 hsdR17 recA1 endA1 gyrA96 thi-1 relA1*(ϕ80 *lacZ* Δ*M15*)	Hanahan (53)
AD494	*F′ lac pro lacI*^{q} Δ *(ara- leu)7697 araD 139* Δ*(lac) X74 galE galK rpsLphoR* Δ*(phoA) PvuII* Δ*(malF)3 thi trxB*::*kan*	Derman *et al*. (41)
GJ1902	*zbh*-900::Tn*10*dKan(Ts)*1 lacZ*::Tn*10*dKan *proC259*	Dr. J. Gowrishankar, lab stock
MG1655	Wild-type *E. coli* K-12	
MGPH1	MG1655 Δ *phoA lacZ*:: Tn*10*dKan	This work
Plasmids		
pET23a	Expression vector	Novagen
pETCM1	pET23a derivative with *Mtb* CM1 (Rv1885c) cloned in NdeI/XhoI sites	This work
pETCM2	pET23a derivative with *Mtb* CM2 (Rv0948c) cloned in NdeI/XhoI sites	This work
pGEMTEasy	Cloning vector	Promega
pCDF1	pGEMT Easy derivative with ss-less *E. coli pho*A cloned in vector MCS	This work
pCDF2	pET23aCM1 derivative with ss-less *E. coli pho*A cloned in SalI/XhoI sites downstream of *Mtb* CM1 signal sequence	This work
pBAD 18	Expression vector	Guzman *et al.* (30)
pCDF3	pBAD18 derivative with the putative *Mtb* CM1 signal sequence appended *E. coli pho*A cloned in XbaI/HindIII sites	This work
pCDF4	pBAD18 derivative with ss-less *E. coli* phoA containing a 16-aa N-terminal extension cloned in XbaI/HindIII sites	This work

(Rv1885c) was recorded in a wavelength range of 190–250 nm in steps of 1 nm, with four accumulations at each step. The spectral baseline was corrected by subtracting the respective blanks. Ellipticity, represented in millidegrees, was plotted as a function of wavelength (in nm). The percentage of helicity for secondary structure determination was calculated using the K2D software available on-line (www.embl-heidelberg.de/~andrade/k2d/)

Determination of the Quaternary Structure of Mtb Chorismate Mutase—The oligomeric state of recombinant proteins was determined using analytical size exclusion chromatography using a Superdex 200 (HP 10/30) fast protein liquid chromatography column from Amersham Biosciences. Chromatography was performed at room temperature with 10 mM Tris, 100 mM NaCl, and 1 mM dithiothoreitol as the running buffer, the same buffer in which the recombinant protein was eluted. A standard curve was prepared according to the instruction manual using the Low Molecular Weight Gel Filtration calibration kit from Amersham Biosciences. The void volume was determined using blue dextran 2000. The elution parameter K_{av} was calculated as follows: $K_{av} = V_e - V_o/V_t - V_o$, where V_e = elution volume for the protein, V_o = column void volume, and V_t = total bed volume. K_{av} was plotted against log Molecular Weight. The protein sample was chromatographed on the gel filtration column at a concentration of 4 mg/ml in the presence of dithiothreitol, and the elution volume was recorded.

Construction of an E. coli Strain Lacking Endogenous Alkaline Phosphatase Activity—Strain MGPH1 lacking endogenous alkaline phosphatase (PhoA) activity was constructed via bacteriophage P1-mediated transduction in a two-step process. In the first step, a lysate prepared on strain GJ1902 was used to transduce wild-type *E. coli* strain MG1655 to *lacZ*::*Kan*, and the transductants were screened for inheritance of the linked *proC* mutation conferring proline auxotrophy. This transductant was used as a recipient in the second transduction involving strain AD494 as a donor, and selection was employed for proline protrophy. Co-inheritance of the linked *phoA* deletion was assessed by screening for transductants lacking PhoA activity on low phosphate plates containing the PhoA chromogenic substrate XP (29).

Characterization of the Putative N-terminal Signal Sequence of Mtb Chorismate Mutase in the Heterologous Host E. coli—To validate the prediction that the N terminus of *Mtb* chorismate mutase comprising the first 33 amino acids was capable of functioning as a signal sequence, we appended this sequence to PhoA, a heterologous protein, and studied its export in *E. coli*. Using standard methodologies, a chimeric protein was generated in which the first 33 amino acids of *Mtb* chorismate mutase were fused to the *E. coli* PhoA lacking its natural signal sequence of the first 21 amino acids (ΔssPhoA). Briefly, Δ*ssphoA* was PCR-amplified from *E. coli* genomic DNA using primer pairs ATGTCGACACACCAGAAATGCCTGTTCTG and ATCTCGAGAAGCTTTTATTTTCAGCCCCAGAGC and cloned into the SalI and XhoI sites of the pGEMTEasy vector (Promega), generating plasmid pCDF1. The cloned PCR product was excised using the enzymes SalI and XhoI and cloned into the corresponding sites of the pETCM1 plasmid abutting the first 33 amino acids of chorimate mutase to the signal sequence-less PhoA. This construct was designated as pCDF2. In the next step, pCDF2 was digested with XbaI and HindIII and cloned into the corresponding sites of plasmid pBAD18 (30), and the resulting construct was designated as pCDF3. In this configuration, the expression of the gene encoding the chimeric protein (CM1ssPhoA) is induced by arabinose, and translation initiating from the initiation codon of CM would terminate at the stop codon of Δ*ssphoA*. A chimeric protein is thereby produced with the first 33 amino acids of *Mtb* chorismate mutase at its N terminus, and the remainder is alkaline phosphatase lacking its native signal sequence. Using similar methods, we constructed plasmid pCDF4, expressing from the arabinose-inducible promoter a ΔssPhoA with a 16-aa N-terminal extension not expected to function as a signal sequence. An *E. coli* strain designated MGPH1, bearing a deletion of the gene for alkaline phosphatase, was constructed in two steps using bacteriophage P1-mediated transduction. Plasmids pCDF3 and pCDF4 were transformed into MGPH1. *E. coli* PhoA is known to be active only after export to the periplasmic space (31). Hence, blue-colored transformants on LB plates supplemented with the PhoA chromogenic substrate XP (40 μg/ml) and the inducer L-arabinose (0.2%) were expected only if the putative *Mtb* chorismate mutase 1 signal sequence mediated the export of the appended PhoA variant to the periplasm.

RESULTS

The Hypothetical ORF Rv1885c of Mtb Encodes a Functional Chorismate Mutase Enzyme—The gene corresponding to the ORF Rv1885c of *Mtb* was PCR-amplified, cloned, and expressed in *E. coli* BL21 cells, and recombinant protein was purified to near homogeneity (Fig. 2*A*). The kinetic properties of the purified protein were studied using chorismate mutase activity assays. The substrate saturation curves were hyperbolic for the enzyme, *i.e.* the enzyme followed Michaelis-Menten kinetics. There was no indication of positive homotropic cooperativity of *Mtb* chorismate mutase toward chorismate. K_m for the enzyme was calculated as 1.2 mM, and V_{max} was calculated as 74 μmol min^{-1} mg^{-1} (Fig. 2*B*). The molar catalytic activity (k_{cat}) was 26 s^{-1}. The effects of temperature and pH on enzyme kinetics were also studied. Because spontaneous arrangement of chorismate to prephenate is strongly dependent on temperature, blanks of the same reaction without the enzyme were also kept at different temperatures, and the readings were subtracted from those obtained in the presence of the enzyme. *Mtb* chorismate mutase was found to be maximally active in the temperature range of 37 °C to 50 °C. An increase in temperature did increase enzyme activity (as expected), and complete denaturation of the enzyme occurred at a temperature close to 60 °C.

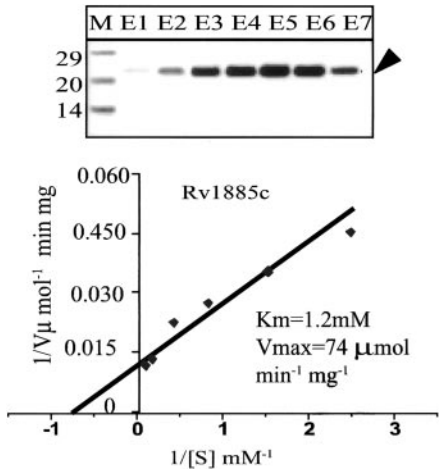

FIG. 2. ***Mtb* chorismate mutase encoded by ORF Rv1885c follows Michaelis-Menten kinetics.** *A*, purification of the chorismate mutase enzyme (Rv1885c) of *Mtb* as a recombinant protein in *E. coli*. The ORF corresponding to Rv1885c was cloned in the NdeI and XhoI sites of pET23a vector with a C-terminal His tag and expressed in *E. coli* BL21 cells. Affinity purification of recombinant protein was carried out using Talon resin (Clontech). The purified protein was resolved on 10% Tris-tricine gel, and the gel was stained with Coomassie Brilliant Blue dye. *M* represents the protein molecular size marker (Broad range, Genei, India). *E1–E7* show the successive eluted fractions of the recombinant protein. *Arrowhead* indicates the position of the 22-kDa protein, which is the predicted mass of *Mtb* chorismate mutase. *B*, the purified recombinant protein corresponding to ORF Rv1885c was assayed for chorismate mutase activity in the absence of any effector molecule. The data were fitted to functions describing Michaelis-Menten-type saturation to calculate K_m and V_{max}.

The pH value also has a significant role in determining the activity of chorismate mutase. *Mtb* chorismate mutase was found to be active between pH 6 and pH 8.5, with a pH optimum of 7.5. The pH optimum is similar to that of *E. coli* chorismate mutase, for which maximum activity is at pH 7.3. Chorismate mutase activity of rRv1885 was greatly inhibited at acidic pH. Whereas fungal chorismate mutases are reported to be more active at acidic pH, bacterial chorismate mutases (*E. coli* P protein and *Salmonella typhimurium* CM) are reported to be active at alkaline pH (14, 32). *Bacillus* chorismate mutase has maximum activity in the range of pH 5 to pH 9 (33). Hence, the pH range at which *Mtb* chorismate mutase works efficiently is similar to that of *B. subtilis*, with the optimum pH being closer to that of *E. coli* chorismate mutase.

Mtb Chorismate Mutase (Rv1885c) Does Not Display Prephenate Dehydratase (PDT) or Prephenate Dehydrogenase (PDH) Activity—Although PDT or PDH domains have not been predicted to be present in the ORF Rv1885c, on account of several examples of divergent evolution of enzymes involved in aromatic amino acid biosynthesis (12), we decided to test whether *Mtb* chorismate mutase displays PDT or PDH activities. PDT and PDH assays were accordingly carried out with the recombinant protein. Our results show that the PDT/PDH activity of rRv1885c is only 0.01% of its CM activity, which suggests that the protein does not possess any intrinsic PDT or PDH activity (data not shown). This is unlike the chorismate mutases of many other enteric bacteria, in which chorismate mutase and PDT/PDH are present as fusion proteins (14).

Mtb Chorismate Mutase (Rv1885c) Shows Allosteric Regulation by Tyrosine, Phenylalanine, and Tryptophan—Having shown that the hypothetical protein coded by ORF Rv1885c encodes a functionally active chorismate mutase enzyme with no associated PDT or PDH activity, we tested L-phenylalanine, L-tyrosine, and L-tryptophan as potential modifiers of the chorismate mutase activity of rRv1885c. A very unusual property of the enzyme could be seen. Whereas these ligands moderately enhance chorismate mutase activity at low concentrations, they inhibit the enzyme at higher concentrations. The Eadie-Scatchard plots of the three ligands show a noncompetitive-type enzyme inhibition (Fig. 3). It is interesting to note that all known forms of chorismate mutase that show allosteric regulation are usually large fusion proteins (14), whereas the *Mtb* chorismate mutase is a small protein of 22 kDa. The monofunctional chorismate mutase of *B. subtilis* is entirely resistant to feedback inhibition by aromatic amino acids (33). *E. coli* chorismate mutase exists as a fusion protein and shows allosteric inhibition only by pathway-specific inhibitors (14). Allosteric regulation of *Mtb* chorismate mutase is also unlike that of yeast chorismate mutase, in which tryptophan causes only activation, whereas tyrosine and phenylalanine cause inhibition of enzyme activity (34).

Fluorometric assays were carried out to determine whether phenylalanine binding causes a conformational change in *Mtb* chorismate mutase. A blue shift in the spectra and the quenching of net fluorescence indicate that tryptophan residues of the enzyme are buried inside as a consequence of phenylalanine binding (Fig. 4). *Mtb* chorismate mutase possesses four tryptophan residues, and therefore the quenching phenomenon is expected to be primarily due to a change in the conformation of the protein upon ligand binding (35). Fluorescence quenching is also a reflection of an alteration in the function of the enzyme. This could be seen in the inhibition of enzyme activity in the presence of aromatic amino acids.

Pathway-specific and Cross-pathway-specific Ligands Protect Mtb Chorismate Mutase from Proteolytic Cleavage—Having demonstrated that tyrosine, phenylalanine, and tryptophan are allosteric inhibitors of *Mtb* chorismate mutase activity, we proceeded to determine whether binding of these ligands causes a conformational change in the enzyme that leads to inaccessibility of the active site. We employed limited proteolysis as a structural probe to ascertain enzyme conformational change. Trypsin was taken as the protease of choice, and limited proteolysis was carried out to study the amenability of the enzyme to proteolytic cleavage in the presence of various ligands. We observed that in the presence of high concentrations (≥3 mM) of effectors (Tyr, Phe, and Trp), the enzyme was completely protected from proteolytic degradation (Fig. 5, *A–C*). Salicylic acid, another cross-pathway secondary metabolite derived from chorismate, did not confer any protection on the enzyme (Fig. 5*D*).

Mtb Chorismate Mutase Is a Dimeric Protein with a Predominantly α-Helical Structure—Whereas catalytic activity and regulatory activity of *Mtb* chorismate mutase point toward some novel properties of the enzyme, the study was continued to determine the biophysical parameters of the enzyme to define the actual class to which it belongs. Size exclusion chromatography was performed to determine the oligomeric state of the protein. The output was a single peak corresponding to the dimeric state of the recombinant protein (data not shown). In this context, *Mtb* chorismate mutase is similar to the *E. coli* or yeast chorismate mutases, which are also dimers of identical subunits (24, 36). To determine the secondary structure of *Mtb* chorismate mutase, the CD spectrum was recorded on a JASON spectropolarimeter (Fig. 6). The data were analyzed using the K2D software available on-line. The results suggest a predominantly α-helical structure for the enzyme. This is reminiscent of the AroQ class of enzymes from yeast and *E. coli* (24, 37) that are also helical proteins. Members of the AroQ class of chorismate mutases consist of unregulated and regulated (AroQr) enzymes and are unusually divergent among closely related organisms (38). This structure showed 71% helices with essentially no β-sheets.

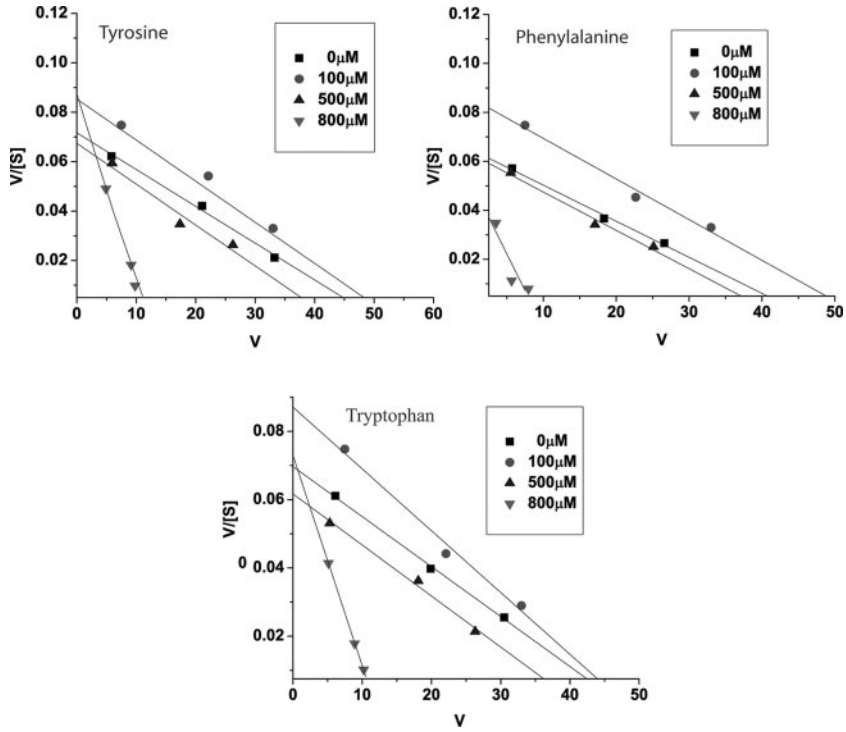

FIG. 3. **Eadie-Scatchard plots showing allosteric regulation of *Mtb* CM (Rv1885c) by aromatic amino acids.** The CM activty of Rv1885c was tested in the presence of various allosteric effectors (tyrosine, phenylalanine, and tryptophan). Low concentrations (up to 100 μM) of all three aromatic amino acids led to a modest increase in enzyme activity. However, at higher concentrations of the effectors ($\geq$500 μM), CM activity was greatly inhibited.

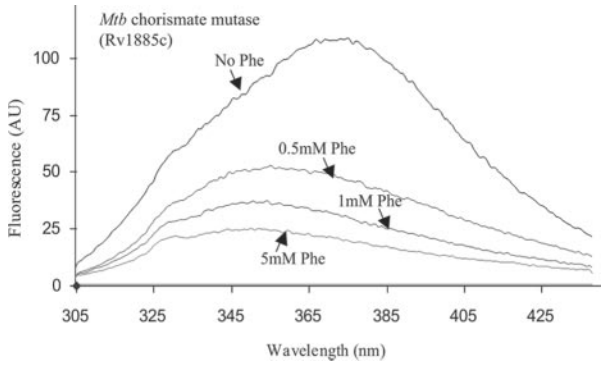

FIG. 4. **Phenylalanine binding leads to a change in the fluorescence spectrum of *Mtb* CM.** Fluorescence emission spectra were recorded by exciting the protein at 280 nm and recording the emission spectra in the range of 305–440 nm. A blue shift was observed upon incubation of rRv1885c with phenylalanine, indicating that the tryptophan residues are buried inside as a consequence of phenylalanine binding.

The N-terminal Signal Sequence of Mtb Chorismate Mutase Can Mediate the Export of Signal Sequence-less E. coli Alkaline Phosphatase to the Periplasmic Space—Whereas our experimental evidence in support of a dimeric α-helical structure for *Mtb* chorismate mutase classified the enzyme as an AroQ-class chorismate mutase, the prediction of a signal peptide in the N terminus of the protein further led us to determine whether *Mtb* chorismate mutase belongs to the periplasmic subclass of chorismate mutases. The predicted signal sequence showed a high similarity score to that of Gram-positive as well as Gram-negative bacterial signal sequences. The chorismate mutases of *E. herbicola*, *Pseudomonas aeruginosa*, and *S. typhimurium* also possess an N-terminal signal sequence and differ in this respect from other members of the AroQ class of chorismate mutases (39). A study was therefore initiated to assess the function of the N-terminal signal sequence (ss) of *Mtb* chorismate mutase in the heterologous host *E. coli*. Our approach involved tagging the putative *Mtb* chorismate mutase signal sequence to the *E. coli* alkaline phosphatase (PhoA) lacking its native signal sequence. The recombinant DNA molecule encoding such a fusion was cloned in pBAD18 vector (30), wherein its expression is driven from an arabinose-inducible promoter. The resultant construct pCDF3 was used to transform an *E. coli* strain lacking PhoA. PhoA is known to be inactive in the cytosolic space and active only after export into the periplasm (31). On the other hand, PhoA lacking its ss is retained in the cytoplasm, does not possess export competence, and is inactive (40–42). PhoA activity results in the ability to cleave the cognate chromogenic substrate XP, leading to the appearance of blue-colored colonies. We observed that pCDF3 transformants of the *E. coli* strain lacking endogenous PhoA activity gave rise to blue-colored colonies, whereas those bearing vector alone remained white, on LB plates supplemented with L-arabinose and XP (Fig. 7*A*). In addition, transformants of MGPH1 expressing from *ara*pBAD promoter a ss-less version of PhoA with a 16-aa N-terminal tag encoded by plasmid pCDF4 gave rise to white colonies on XP indicator plates. The said 16-aa extension is not expected to encode a ss. Consequently, the PhoA activity was localized to the cytoplasm, in which it is inactive. We confirmed that the PhoA activity expressed from pCDF4 was cytoplasmic because it could be reactivated in strains lacking thioredoxin reductase activity (data not shown), a diagnostic feature of cytoplasmically retained PhoA (41). This indicated that only the putative *Mtb* chorismate mutase signal sequence could mediate the export of a heterologous protein to the periplasmic space. In an independent approach, we also assessed localization of chorismate mutase in the periplasmic and cytoplasmic fractions of *E. coli* BL21DE3 cells expressing rRv1885c and rRv2386c. Rv2386c is an ORF of *Mtb* that does not possess a predicted signal sequence at its N terminus. A very large proportion of the chorismate mutase activity was confined to the periplasmic space in cells expressing rRv1885c, whereas the protein product of Rv2386c partitioned exclusively in the cytoplasm (Fig. 7*B*). From these genetic and biochemical studies it could be concluded that the Rv1885c-encoded chorismate mutase is a periplasmic protein, at least in a heterologous host.

Rv1885c Is Not the Sole Chorismate Mutase Enzyme of Mtb: Rv0948c Also Shows Chorismate Mutase Activity, Although with a Reduced Turnover—The Cluster of Orthologous groups

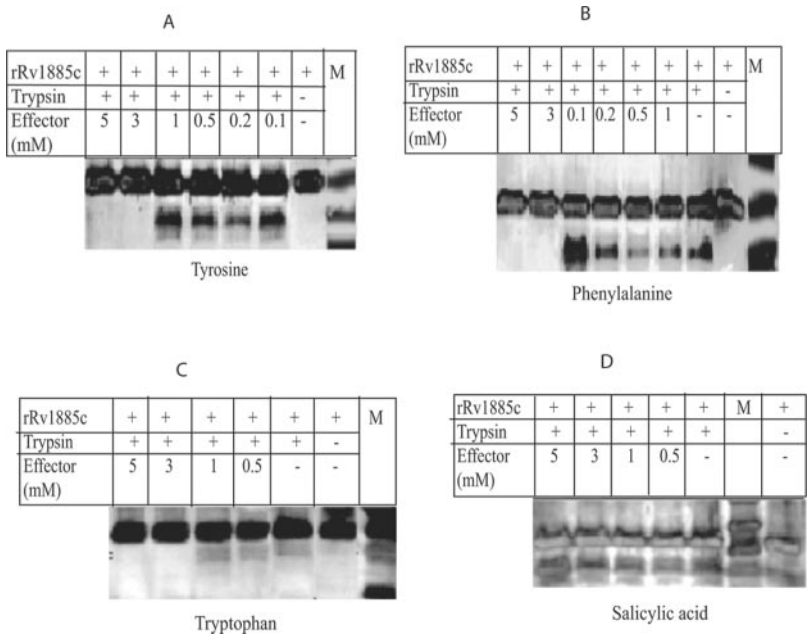

FIG. 5. **Aromatic amino acids at high concentrations provide protection to *Mtb* chorismate mutase from trypsin cleavage as evident from limited proteolysis studies.** *Mtb* chorismate mutase enzyme (Rv1885c) is protected from tryptic cleavage in the presence of high concentrations of tyrosine, phenylalanine, and tryptophan (≥3 mM). Salicylic acid, another cross-pathway effector molecule, did not protect *Mtb* CM (Rv1885c) from proteolytic cleavage. *M* represents the protein molecular size marker.

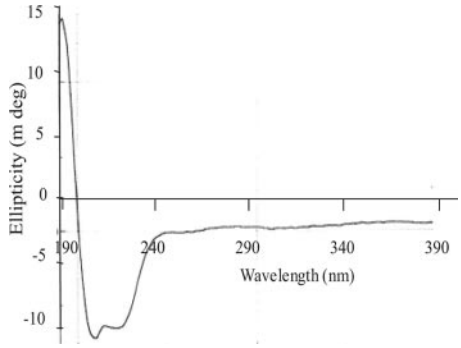

FIG. 6. **CD spectrum of *Mtb* chorismate mutase (Rv1885c) suggests a predominantly α-helical secondary structure of the protein.** The graph has been plotted as ellipticity (in millidegrees (*m deg*)) as a function of wavelength (in nm). The percentage of helicity for secondary structure determination was calculated using the K2D software available on-line (www.embl-heidelberg.de/~andrade/k2d/). The spectrum confirms a highly helical structure of the recombinant protein with signature peaks at 208 and 222 nm.

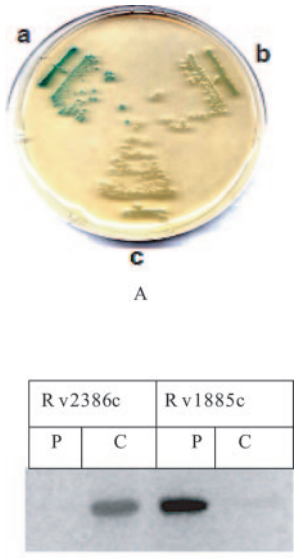

FIG. 7. **The N terminus of *Mtb* chorismate mutase (Rv1885c) encodes a functional signal sequence.** *A*, *E. coli* strain MGPH1 lacking endogenous PhoA was transformed with plasmids pBAD18, pCDF3, and pCDF4. The transformants were streaked on LB plates supplemented with 40 μg/ml XP, 0.2% L-arabinose, and the appropriate antibiotic. Alkaline phosphatase activity is observed only in cells expressing *Mtb* CM1 ss-tagged version of PhoA from plasmid pCDF3 (*a*). Cells transformed with plasmids pBAD18 (*b*) and pCDF4 (*c*) remain white. *B*, Western blot demonstrating the presence of *Mtb* chorismate mutase in the periplasmic space of *E. coli*. 5 mg of protein from the cytosolic (C) and the periplasmic (P) fractions of *E. coli* BL21 (DE3) cells expressing the His6 tagged ORFs Rv2386c and Rv1885c were transferred to nitrocellulose membrane and probed with monoclonal anti-histidine antibodies. Rv2386c is used as a control protein that does not possess an N-terminal signal sequence. As evident only *Mtb* chorismate mutase, product of the ORF Rv1885c shows a periplasmic location.

annotation of the *Mtb* genome suggests that chorismate mutase activity could be attributed to two ORFs of *Mtb*, Rv1885c and Rv0948c. Hence, we cloned and expressed Rv0948 as well in *E. coli* and assayed the recombinant protein for chorismate mutase activity. It could be seen that even rRv0948 has a chorismate mutase activity, although its molar catalytic activity is 60-fold lower than that of Rv1885c (Fig. 8). The K_m and V_{max} of rRv0948 were determined as 5 mM and 1.2 μmol min^{-1} mg^{-1}, respectively. K_m and V_{max} of Rv0948 are unaltered even when assayed in the presence of an unrelated protein, IdeR. This is a reliable indication of the *in vitro* specificity of Rv0948 toward chorismate and supports our view that Rv0948 codes for a CM enzyme, although its activity is modest.

The existence of another chorismate mutase in the *Mtb* genome is consistent with the hypothesis that a free monofunctional chorismate mutase never exists in a single copy in any genome (43). However, it is inconsistent with the hypothesis that among two copies of chorismate mutases, at least one is a fusion protein. rRv0948 also showed no PDT or PDH activity, indicating that it is also a monofunctional protein (data not shown). Rv0948 was also found to be resistant to proteolytic digestion in the presence of phenylalanine and tryptophan (data not shown).

DISCUSSION

Chorismate mutase is known to be the sole example of an enzyme that catalyzes a pericyclic rearrangement reaction and has therefore drawn extensive attention from biologists and

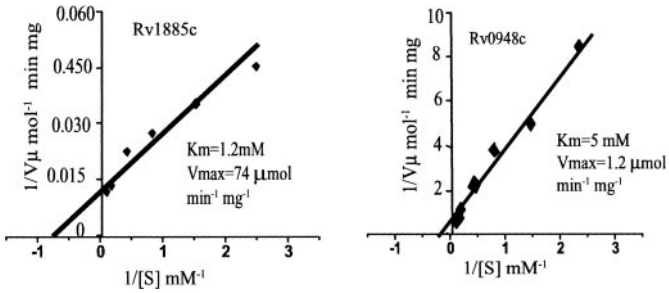

FIG. 8. **Comparative study of the chorismate mutase activity of the ORFs Rv1885c and Rv0948c.** Purified fractions of both the proteins were used for CM activity assay. V *versus* [*S*] plot of rRv1885c and rRv0948c. As evident from the graph, Rv0948c also possesses CM activity; however, the specific activity of rRv0948c was 60-fold lower than the specific activity of Rv1885c.

chemists. Earlier reports that have referred to differences in the structure and regulatory properties of the enzyme from different sources led us to initiate the study of the respective properties of the equivalent protein from *Mtb*. Our approach involved a detailed biochemical and biophysical characterization of recombinant *Mtb* chorismate mutase, encoded by ORF Rv1885, and comparison of these properties with enzymes from other sources. Our results conclusively place *Mtb* chorismate mutase, coded by ORF Rv1885c, in the AroQ class of periplasmic chorismate mutases.

In the course of this study, we also came across several unique properties of *Mtb* chorismate mutase that are important in the context of differentiating the enzyme from the bacterial and fungal counterparts. The first and foremost observation with respect to the regulation of *Mtb* chorismate mutase by aromatic amino acids revealed an unusual property of the enzyme. Whereas *Mtb* chorismate mutase was found to be allosterically regulated by pathway-specific (Tyr and Phe) as well as cross-pathway-specific (Trp) ligands in the same manner, it showed a modest increase in enzyme activity at low concentrations and complete inhibition at higher concentrations of effectors. Whereas it is difficult to explain the observation, our results suggest that the activity of *Mtb* CM is a reflection of a complex interplay between ligand concentration and enzyme activity. We also propose that this unusual property of *Mtb* chorismate mutase must be coordinately regulated with other enzymes of the aromatic amino acid pathway. It would be worthwhile to mention that a recent study from our laboratory indicates that *Mtb* PheA (prephenate dehydratase), the enzyme that catalyzes the subsequent step in the biosynthesis of phenylalanine, is regulated in exactly the opposite manner, *i.e.* it is inhibited by low concentrations and activated by higher concentrations of aromatic amino acids (44). This suggests in that in *Mtb* sequential enzymes of the aromatic amino acid biosynthesis pathway are regulated in an opposite manner to bring about a balance of intracellular concentrations of these amino acids.

Because binding of ligands causes inhibition of enzyme activity, we investigated whether ligand binding would also protect the enzyme from proteolytic cleavage. This experiment was expected to generate another line of evidence that ligand binding induces a conformational change in the enzyme. Limited proteolysis studies suggest that ligand binding imparts a much more rigid structure to the enzyme, leading to enhanced stability and inaccessibility of the active site and protease cleaving sites. A cross-pathway-specific secondary metabolite, salicylic acid, could not protect *Mtb* chorismate mutase from proteolytic cleavage. Salicylic acid is also derived from chorismate. This suggests that only aromatic amino acids and not secondary metabolites have a binding pocket on the enzyme.

The chorismate mutase reaction is also known to occur spontaneously, and we also observed an increase in conversion of chorismate to prephenate at higher temperatures. However, there was a distinct enhancement of the reaction rate upon addition of the enzyme. We believe that despite a spontaneous conversion of chorismate to prephenate, there is a need for enzyme-catalyzed conversion because chorismate is a substrate for multiple enzymes, and the fate of the compound is determined by the enzyme that acts upon it, which in turn is dependent on the requirement of the organism (45).

It is also interesting to note that unlike most enteric bacteria, in which chorismate mutase exists in fusion with other enzymes of the aromatic amino acid biosynthesis pathway, *Mtb* chorismate mutase is predicted to be a non-fusion protein (www.sanger.ac.uk/cgi-bin/Pfam/speciesview.pl?family=CM_2&acc=PF01817&ncbicode=83332&name=Mycobacterium%20tuberculosis). However, because of several reports of divergent evolution of aromatic amino acid biosynthesis enzymes, we tested whether the amino acid sequence of *Mtb* chorismate mutase could also contribute to PDT or PDH activity. Our results confirm that *Mtb* chorismate mutase is a monofunctional protein, *i.e.* it has no associated PDT or PDH activity. The chorismate mutases that show allosteric regulation are usually large fusion proteins. *Mtb* chorismate mutase appears to be unique in the sense that despite being a small monofunctional protein, it shows a complex pattern of allosteric regulation. Whereas *Mtb* chorismate mutase does not possess a discrete ACT domain that is required for amino acid binding, it is possible that the complex regulatory activity of the enzyme rests in the uncharacterized C terminus of the enzyme.

Among the monofunctional AroQ proteins, the existence of a cleavable signal peptide has been reported in *E. herbicola*. Later, AroQ chorismate mutases from *S. typhimurium* and *P. aeruginosa* were also shown to have a periplasmic location (46). Periplasmic chorismate mutases possess a signal peptide at the N terminus and usually have a C terminus of an unknown function. The C terminus of *Mtb* chorismate mutase is also unique, and its precise function is still unclear. Based upon its ability to export *E. coli* PhoA to the periplasmic space, we tentatively place *Mtb* chorismate mutase in the unique periplasmic subclass of AroQ proteins. However, our study also necessitates a requirement for identification of other periplasmic enzymes of the aromatic amino acid biosynthesis pathway of *Mtb*. This study would shed further light on whether there is a periplasmic pathway of aromatic amino acid biosynthesis in *Mtb*. In the absence of the existence of such a pathway, we propose that the periplasmic location of *Mtb* chorismate mutase could have a role to play in modulation of the host immune response or that the enzyme could have a chemotactic role (47). It is interesting to note that the ORF Rv1885c exists in the same operon as Antigen85A, a major secreted antigen of *Mtb*. It is also worth mentioning that chorismate mutase is a virulence factor of nematode parasites that secrete chorismate mutase from their esophageal glands to alter the shikimate pathway of the plants that they parasitize (48, 49).

Finally, our evidence for the existence of another chorismate mutase in the *Mtb* genome is in accordance with the hypothesis that a free monofunctional chorismate mutase never exists in a single copy in any genome (43). Interestingly, however, this is the first report that demonstrates the existence of two monofunctional chorismate mutases in any bacterial system. In other bacteria, in which chorismate mutase is present in two copies, at least one of them is a fusion protein (50). Evolution of these fusion proteins was brought about with the purpose of coordination of gene expression as per the requirement of the cell. Hence, the issue of co-regulation of *Mtb* chorismate mu-

tases and PDT/PDH still remains. Whereas IdeR is known to govern the expression of *Mtb* prephenate dehydratase (51), regulation of expression of *Mtb* chorismate mutase is still not known. The apparent lack of synchrony in gene expression also suggests that *Mtb* chorismate mutase could be involved in some other pathway that is independent of the involvement of PDT/PDH.

Our data support the existence of two monofunctional chorismate mutases in the *Mtb* genome, yet the question remains: why two chorismate mutases? The clue lies in our observation of a huge difference in turnover number of the two chorismate mutases, which suggests that one of these enzymes could have an additional function. Additionally, the existence of one of the chorismate mutases as a secreted protein also suggests an as yet undeciphered function of the enzyme. The second chorismate mutase of *Mtb* (Rv0948) does not possess a signal sequence, which suggests that there are possibly two pathways for aromatic amino acid biosynthesis in *Mtb*. The role of the periplasmic pathway is still not clear. Our other novel observation that the periplasmic chorismate mutase of *Mtb* shows a higher turnover number also demands elucidation of some other function of the enzyme. Because chorismate-utilizing enzymes are known to have divergently evolved, it is likely that these enzymes could have some other function as well. In this context, it would be important to mention that in *P. aeruginosa*, the isochorismate pyruvate lyase enzyme PchB has been reported to show chorismate mutase activity (52). We are currently exploring whether either of the chorismate mutase enzymes could substitute for an isochorismate pyruvate lyase enzyme.

In summary, our results show that the hypothetical proteins coded by the ORFs Rv1885c and Rv0948c of *M. tuberculosis* represent the chorismate mutase enzymes of the organism and that the kinetic properties of the enzyme are under the control of strict regulatory mechanisms. To our knowledge, this is the first detailed report that provides evidence for the existence of a monofunctional AroQ class of periplasmic chorismate mutase in the *Mtb* genome. Additionally, a low level sequence similarity of the protein with other known chorismate mutases makes this protein an attractive molecule for the design of novel therapeutic compounds for intervention against tuberculosis.

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