How pantothenol intervenes in Coenzyme-A biosynthesis of Mycobacterium tuberculosis

Parimal Kumar a, Manmohan Chhibber a, Avadhesha Surolia a,b,*

a Molecular Biophysics Unit, Indian Institute of Science, Bangalore 560012, India
b National Institute of Immunology, Aruna Asaf Ali Marg, New Delhi 110067, India

Received 26 June 2007
Available online 27 July 2007

Abstract

Coenzyme A is an indispensable cofactor for all organisms and holds a central position in a number of pathways. Prokaryotic enzymes involved in the synthesis of CoA are quite different from their mammalian counterparts; hence, they are good targets for the development of antimicrobials to treat many diseases. There are antimicrobials that act by inhibiting CoA biosynthesis. It has been suggested that pantothenol exhibits antibacterial activity by competitively inhibiting pantothenate kinase, a key regulatory enzyme for CoA synthesis. Contrary to these suggestions, in this paper, we demonstrate that pantothenol acts as a substrate for Mycobacterium tuberculosis and Escherichia coli pantothenate kinases. The product, 4'-phosphopantothenol, thus formed inhibits competitively the utilization of 4'-phosphopantothenate by CoaBC. Thus, it is the failure of CoaBC to utilize 4'-phosphopantothenol as a substrate that accounts for the bactericidal activity of pantothenol.

© 2007 Elsevier Inc. All rights reserved.

Keywords: Pantothenol; Pantothenate kinase; Coenzyme A; Isothermal titration calorimetry; Mycobacterium tuberculosis; 4'-Phosphopantothenate; Pantothenate

Coenzyme A is an essential cofactor for all organisms and holds a central position in a number of cellular processes. About 4% of all known enzymes utilize CoA as a cofactor for their activity. CoA, across organisms, is synthesized by broadly similar enzymatic reactions from pantothenate (vitamin B5) [1]. The enzymes involved in the synthesis are pantothenate kinase (CoaA), 4'-phosphopantothenoylcysteine synthase and 4'-phosphopantothenoylcysteine decarboxylase (CoaBC), dephospho CoA synthase (CoaD), and CoA synthase (CoaE) [2–4].

Abbreviations: CoA, Coenzyme A; MtCoaA, Mycobacterium tuberculosis pantothenate kinase; MtCoaBC, 4'-phosphopantothenoylcysteine synthase and 4'-phosphopantothenoylcysteine decarboxylase; EcCoaA, E. coli pantothenate kinase; ITC, isothermal titration calorimetry; PEP, phosphoenol pyruvate; N5-Pan, N-pentylpantothenamide; N7-Pan, N-heptylpantothenamide; TLC, thin layer chromatography; kb, kilobases; CTP, cytidine triphosphate.

* Corresponding author. Address National Institute of Immunology, Aruna Asaf Ali Marg, New Delhi 110067, India. Fax: +91 11 26717104.
E-mail address: surolia@nii.res.in (A. Surolia).

The potential of the analogs of CoA as antimicrobials has been compromised by their inability to cross bacterial membrane [5]. Amide derivatives of pantothenate (e.g. N-pentylpantothenamide and N-heptylpantothenamide) and analogs of pantothenic acid (e.g. pantothenol and pantoyltaurine) have been described previously as antimicrobials [6–8]. These have the ability to cross the membrane. Inside the cell, N-pentylpantothenamide and N-heptylpantothenamide are phosphorylated by pantothenate kinase, and they undergo further transformation by CoaD and CoaE to ethyldethia-CoA and butyldethia-CoA, respectively. Both ethyldethia-CoA and butyldethia-CoA competitively inhibit CoA-dependent enzymes and thereby prevent bacterial growth [9].

It has been suggested that pantothenol exhibits antibacterial activity by competitively inhibiting pantothenate kinase [10,11]. Contrary to these suggestions, results reported in this study demonstrate that pantothenol acts as a substrate for Mycobacterium tuberculosis and Escherichia coli pantothenate kinases. The product, 4'-phosphopantothenol, thus
formed inhibits competitively the utilization of 4'-phosphopantothenate by CoaBC. Thus, it is the failure of CoaBC to utilize 4'-phosphopantothenol as a substrate that accounts for the bactericidal activity of pantothenol.

**Materials and methods**

[14C]-Pantothenate and [14C]-pantothenol were from American Radiolabel Chemicals. Nickel–NTA and BL21 (DE3) were from Novagen. HPLC grade organic solvents were purchased from Qualigen. All other chemicals used were of highest purity available from Sigma–Aldrich or Fluka.

**Cloning and expression of ORF Rv1092c, Rv1391, and b3974.** Pantothenate kinase (ORF Rv1092c, Rv1391, and b3974) was cloned, expressed, and purified as in [12]. ORF Rv1391 (MtCoaBC) was PCR amplified using primers 5'-GGATTTCATATGTTGGGACCATAAAGCAGATCCC-3' and 5'-CCCAAGCTTATGCCATACGCGGAAAC-3' whereas b3974 (EcCoA) was PCR amplified using primers 5'-GGG AATTCTCAATGGATATAAAGGACGAACTTATAGCAGG-3' and 5'-CCCAAGCTTTATTTGTGATCTGACCTTCTACCACA-3'. The amplified products were cloned between NdeI and HindIII sites of pET-28a vector. Protein expressions were done in BL21 (DE3) cells (Novagen) and proteins were purified on a Ni–NTA metal-affinity column. MtCoaBC was eluted at 150 mM imidazole in buffer (50 mM Tris pH 8, 300 mM NaCl, 10% glycerol) whereas EcCoA was eluted at 100 mM imidazole in the same buffer. Enzymes thus obtained were dialyzed against buffer containing 50 mM Tris, 150 mM NaCl, and 10% glycerol and 10 mM imidazole in the same buffer. Enzymes obtained were dialyzed against buffer containing 50 mM Tris, 150 mM NaCl, and 10% glycerol and 10 mM imidazole in the same buffer.

**Kinetic parameters for ATP hydrolysis.** MtCoaA was dialyzed in buffer containing 50 mM Tris, 150 mM NaCl, 8 mM MgCl₂, and 10% glycerol for 24 h. The ligand concentration was kept constant at 1 mM and ATP concentration was varied from 10–1000 μM. Values of the change in enthalpy, Kₜ, and n of the titration were determined by non-linear least-squares fitting of the data using Origin™ 7.0 software. Thermodynamic parameters for the binding of acetyl and malonyl-CoA were also evaluated in the same manner excepting that the ITC cell contained 45 and 40 μM of MtCoaA, respectively. For each binding, the injection volume used was 8 μl. Values of the change in enthalpy, Kₜ, and n of the titration were determined by non-linear least-squares fitting of the data using Origin™ 7.0 software. Change in entropy (∆S) was obtained using the equation (∆Gₜ = ∆Hₜ – T∆S), where ∆Gₜ = –RT lnKₜ, R and T represent the gas constant and the absolute temperature (K), respectively.
addition of MtCoaBC in the reaction mixture and incubated at 37°C for 10 min. Heating at 80°C for 5 min terminated the reaction. Separation of the products of MtCoaBC was done as described above for MtCoaA reaction product separation. Separated radiolabeled components were visualized by autoradiography using Fujifilm Phosphorimager. New spots that appeared were scratched from TLC plates and dissolved in 50% acetonitrile with 0.1% tri-fluoroacetic acid, and masses of these products were determined by ESI-MS.

Inhibition of MtCoaBC by 4'-phosphopantothenol. Inhibition of MtCoaBC activity by 4'-phosphopantothenol was checked using TLC. For inhibition assay, [1-^14C] 4'-phosphopantothenate was enzymatically synthesized using ATP and [1-^14C] pantothenate. 4'-Phosphopantothenol was enzymatically synthesized using ATP and pantothenol. The 25-μl reaction mix contained 50 mM Tris, 200 μM cysteine, 200 μM CTP, 200 μM DTT, 200 μM MgCl2, and increasing amounts of 4'-phosphopantothenol. The reaction was carried out at 37°C for 2 min and was then stopped by keeping it at 80°C for 5 min. TLC separation of reaction products was done as described previously. Separated radiolabeled components were visualized by autoradiography using Fujifilm Phosphorimager. The IC50 value for 4'-phosphopantothenol was determined using densitometric analysis of spots using the program Fujifilm Scienclab.

**Results**

**Expression and purification of recombinant proteins**

MtCoaA (Rv1092c) and MtCoaBC (Rv1391) were expressed in BL21 (DE3) using pET28a vector system and purified to homogeneity on Ni–NTA resin. CoaA from *E. coli*, EcCoaA, was also expressed and purified likewise.

Proteins thus obtained, showed molecular masses consistent with those predicted from their amino acid sequences (Fig. 1A).

**Complementation**

Rv1092c was subcloned in pBAD24 vector and tested for its ability to rescue the CoaA temperature-sensitive (ts) mutant of *E. coli* (DV62 cells) [14]. DV62 cells were transformed with pBAD24 plasmid alone as well as pBAD24 plasmid containing MtCoaA, under P_BAD promoter [15]. Transformants were grown at 30°C for 8 h and then streaked on minimal media plates. Mutant cells transformed with pBAD24 containing MtCoaA were rescued at 42°C, when plated on minimal media with 2 μM arabinose, whereas mutants transformed with pBAD24 alone did not grow under these conditions (Fig. 1B). Thus, the cloned ORF is indeed that of pantothenate kinase and that *M. tuberculosis* pantothenate kinase can substitute the function of *E. coli* pantothenate kinase in vivo with which it bears 50% sequence similarity.

**Biophysical parameters of substrates and inhibitor binding to MtCoaA**

Isothermal titration calorimetry (ITC) was performed to study the interaction of MtCoaA with CoA, acetyl-CoA,
malonyl-CoA, and ATP (Fig. 1C). Panels A–C, respectively, show the titration profile of CoA, acetyl-CoA, and malonyl-CoA binding to MtCoaA at 21 °C. Least-squares fit of the ITC data in panels A–C are shown in panels D–F, respectively. The values of binding constants (Kb), number of binding site (n), change in free energy, (ΔGb), binding enthalpy (ΔHb), and entropy (ΔSb) are listed in Table 1.

The next enzyme of the pathway, MtCoaBC, was examined for its substrate specificity using TLC assay. Reaction product of CoaBC with 4′-phosphopantothenol and 4′-phosphopantothenol on TLC revealed the appearance of two new spots only in the case of 4′-phosphopantothenol (Fig. 3D). No detectable new product formation on TLC and ESI-MS was observed when 4′-phosphopantothenol was used as substrate for MtCoaBC reaction (Fig. 3E). ESI-MS of spots appearing with 4′-phosphopantothenol as a substrate just as it makes use of pantothentic acid. EcCoaA was also able to use pantothanol as a substrate.

Substrate specificity

Product formation by MtCoaA or EcCoaA with pantothenate or pantothanol was determined by TLC assay and ESI-MS. The autoradiogram of the reaction of MtCoaA and EcCoaA with pantothenate and pantothanol shows the formation of 4′-phosphopantothenate and 4′-phosphopantothenol, respectively (Fig. 3A). Also, 4′-phosphopantetheine formation increased with increasing pantothanol concentration in the reaction mixture. This was further confirmed by ESI-MS where, in the reaction mixture with MtCoaA, two new peaks appeared whose masses corresponded to that of 4′-phosphopantothenol (285.2 Da) and sodium adduct of 4′-phosphopantothenol (307.2 Da), respectively (Fig. 3B). Likewise, with pantothenate 4′-phosphopantothenolate (300 Da) peak was observed (Fig. 3C). MtCoaA thus utilizes pantothanol as a substrate just as it makes use of pantothentic acid. EcCoaA was also able to use pantothanol as a substrate.

Kinetic analysis

Kinetic parameters of MtCoaA were determined by spectrophotometric-coupled assay. The Km values obtained for ATP, pantothenate, and pantothanol were 375, 100, and 280 µM, respectively (Fig. 2A–C and Table 2). Kcat values for phosphorylation of pantothenate and pantotenol were 0.61 and 0.45 s⁻¹. Kcat value for the inhibition of MtCoaA by CoA was determined using Dixon plot. The value obtained was 2 µM (D). Since ITC directly measures the heat change associated with a reaction, it provides a more accurate method to monitor the reaction kinetics as compared to the coupled assay [17]. Hence, kinetics of MtCoaA was also studied using ITC (Fig. 2E–H and Table 2). The kinetic values obtained by ITC are close to the values obtained by the spectrophotometric-coupled assay, which in turn helps validate these studies further. Moreover, the Km and Kcat values, for pantothenate and pantothanol are similar indicating that pantothanol is akin to pantothenate as a substrate.

Inhibition of MtCoaBC activity by 4′-phosphopantothenol

Since MtCoaBC cannot utilize 4′-phosphopantothenol as substrate, the possibility of inhibition of MtCoaBC by 4′-phosphopantothenol was examined. Reaction of

---

**Table 1**

Thermodynamic parameters for the binding of MtCoaA with CoA, acetyl-CoA, malonyl-CoA, γS-ATP and titration of pantothenate-saturated MtCoA with γS-ATP

<table>
<thead>
<tr>
<th></th>
<th>T (°C)</th>
<th>n</th>
<th>Kb (M⁻¹)</th>
<th>ΔHb (M⁻¹)</th>
<th>ΔGb (kcal M⁻¹)</th>
<th>ΔS (cal M⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MtCoaA with CoA</td>
<td>21</td>
<td>0.71</td>
<td>2.0 × 10⁴</td>
<td>(−8.81)</td>
<td>(−7.1)</td>
<td>(−5.73)</td>
</tr>
<tr>
<td>MtCoaA with Acetyl CoA</td>
<td>21</td>
<td>0.80</td>
<td>1.1 × 10⁴</td>
<td>(−9.00)</td>
<td>(−6.6)</td>
<td>(−7.86)</td>
</tr>
<tr>
<td>MtCoaA with Malonyl CoA</td>
<td>21</td>
<td>0.74</td>
<td>4.88 × 10⁴</td>
<td>(−8.60)</td>
<td>(−6.1)</td>
<td>(−8.50)</td>
</tr>
<tr>
<td>MtCoaA with γS-ATP</td>
<td>21</td>
<td>0.81</td>
<td>5.83 × 10⁴</td>
<td>(−4.62)</td>
<td>(−6.2)</td>
<td>(5.82)</td>
</tr>
<tr>
<td>γS-ATP with pantothenate-saturated MtCoaA</td>
<td>21</td>
<td>1.02</td>
<td>4.02 × 10⁴</td>
<td>(−3.36)</td>
<td>(−6.0)</td>
<td>(9.60)</td>
</tr>
</tbody>
</table>
MtCoaBC with 4′-phosphopantothenate in the presence of increasing concentration of 4′-phosphopantothenol led to a progressive diminution in the formation of product 4′-phosphopentetheine (Fig. 3F). Thus 4′-phosphopantethenol, the product of MtCoaA, inhibits the reaction catalyzed by MtCoaBC. Densitometric analysis of the spots of 4′-phosphopantothenate (Fig. 3F) yielded a 50% inhibition of MtCoaBC activity at 63 μM of 4′-phosphopantothenol (Fig. 3G).

**Discussion**

It has been suggested that pantothenol competitively inhibits pantothenate kinase as it competes with pantothenate for the active site of pantothenate kinase. Our studies, however, show that pantothenate kinase can phosphorylate pantothenol. Our observation is in consonance with the ability of pantothenate kinase to phosphorylate amide derivative of pantothenate [18]. Ivey et al. have crystallized the *E. coli* pantothenate kinase complex with pantothenate and modeled N5-Pan and N7-Pan on the crystallized

---

**Table 2**

Kinetic parameters for the reaction catalyzed by MtCoaA with ATP, pantothenate, and pantothenol

<table>
<thead>
<tr>
<th>Substrates</th>
<th>$K_m$ (μM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat}/K_m$ (mM$^{-1}$ s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>a</td>
<td>375 (±20)</td>
<td>0.62</td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>50 (±10)</td>
<td>0.65</td>
</tr>
<tr>
<td>Pantothenate</td>
<td>a</td>
<td>100 (±15)</td>
<td>0.61</td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>180 (±11)</td>
<td>0.62</td>
</tr>
<tr>
<td>Pantothenol</td>
<td>a</td>
<td>280 (±20)</td>
<td>0.40</td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>250 (±22)</td>
<td>0.38</td>
</tr>
</tbody>
</table>

(a) The parameters were determined from coupled assay, (b) parameters determined using ITC.
enzyme, which led them to suggest that pantothenate kinase can accept them as substrate and phosphorylate them. Since the hydroxyl group in the N5-Pan and N7-Pan, which accepts the phosphate from ATP, occupies the same position as the hydroxyl group of pantothenate, we have modeled pantothenol binding on pantothenate kinase. We observe that it docks on the protein at the same position as pantothenate does and the hydroxyl group of pantothenol overlaps with the hydroxyl group of pantothenate (data not shown). This is not surprising, as the replacement of the COOH group (of pantothenate) by the OH group (of pantothenol) will perturb its interaction minimally [19]. Expectedly, therefore, the transfer of γ-phosphate to pantothenol from ATP is as efficient as observed for pantothenate and it is the incapacitation of MtCoaBC that blocks the synthesis of CoA right in the middle of the CoA synthesis pathway. In this respect pantothenol differs from N5-pantothenamide and N7-pantothenamide as N5-pantothenamide phosphate and N7-pantothenamide phosphate, the products of pantothenate kinase reaction, are acted upon by CoaD and CoaE to metabolize them to CoA like moieties, which then inhibit CoA-dependent enzymes.

In conclusion, studies reported here explicitly show that, contrary to the widely accepted view, pantothenol does not inhibit pantothenate kinase. Instead by virtue of its striking similarity to its natural substrate, pantothenate, it is phosphorylated at its 4β-position. 4β-Phosphopantothenol thus synthesized inhibits the activity of the downstream enzyme, CoaBC, thereby blocking CoA biosynthesis. The mechanism of inhibition elucidated here with M. tuberculosis enzymes is observed with the E. coli enzymes as well. This explains the wide spectrum antibacterial activity of pantothenol. A similar mechanism is likely to operate in inhibition of the growth of Plasmodium falciparum. Since these organisms are evolutionarily quite different, we believe that the mechanism of pantothenol that we report here has ramifications for the development of novel anti-infectives.
Acknowledgments

Authors thank the Department of Biotechnology (DBT) for support to A.S. for this work and the program support of DBT in Drug and Molecular Diversity and Design for ESI-MS.

References