Inhibitors of Pantothenate Kinase: Novel Antibiotics for Staphylococcal Infections

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Pantothenate kinase (CoA) catalyzes the first step of the coenzyme A biosynthetic pathway. Here we report the identification of the Staphylococcus aureus coA gene and characterization of the enzyme. We have also identified a series of low-molecular-weight compounds which are effective inhibitors of S. aureus CoA.

Increasing reports of antibiotic resistance involving opportunistic gram-positive pathogens, including methicillin-resistant Staphylococcus aureus, have emphasized the critical need for the development of antimicrobial compounds with novel modes of action. Coenzyme A (CoA), an essential cofactor for maintaining life, is used in a multitude of biochemical reactions. In most bacteria, CoA is synthesized from pantothenic acid (vitamin B₅) in 5 steps (5), with the first step being the phosphorylation of pantothenate by pantothenate kinase (CoA). Although this pathway also exists in eukaryotes, in most cases there is no sequence homology between the prokaryotic and eukaryotic CoA biosynthetic enzymes (7, 9, 12, 18, 24, 27). Thus, there is the potential for developing highly specific inhibitors of bacterial CoA enzymes.

Unlike the case for other biosynthetic pathways of bacteria, the genes involved in CoA biosynthesis are not organized as operons. This has delayed the identification of the enzymes responsible for CoA synthesis, even though the intermediate chemical steps have been known since the 1960s (1). With the recent identification of the Escherichia coli genes encoding the enzymes CoaBC and CoAE, the entire pathway is now known for this organism (9, 10, 13, 19, 21). Interestingly, the gene coaA, which encodes the first enzyme in the pathway, has no homolog in the complete genome sequences of the S. aureus strains Mu50 and N315 (11).

Cloning and purification of S. aureus CoA. Initially, the coaA gene sequences in S. aureus strains Mu50 and N15 (GenBank accession numbers BA000017 and BA000018, respectively) were identified through searches of the ERGO comparative genomic database (previously WIT) (http://ergo.integratedgenomics.com/ERGO/) (8). We cloned the S. aureus RN4220 coaA gene and overexpressed it using standard techniques (4, 17). S. aureus RN4220 coaA was amplified by PCR, introducing an Ndel site at the start codon and an Xhol site after the stop codon, and cloned into pSTBlue1 using the Perfectly Blunt Cloning kit. The gene was excised by digestion with Ndel and Xhol and ligated into similarly digested pET-28a. The final construct encoded the N-terminal six-His-tagged S. aureus CoA.

Tuner (DE3) cells were transformed with this construct and grown at 37°C in Luria-Bertani medium–50-µg/ml kanamycin. Protein expression was induced by 500 µM isopropyl-β-d-galactoside, and cells were harvested 3 h postinduction. The cell pellet was resuspended and sonicated, and cell debris was removed by centrifugation. The supernatant was subjected to Ni-chelating column chromatography followed by a HiTrap Q Sepharose ion exchange column. Enzyme identity was confirmed through N-terminal sequencing and matrix-assisted laser desorption ionization mass spectrometry and purity was verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The molecular mass corresponded to the predicted mass of 29,096 Da.

Characterization of S. aureus CoA. To verify that this protein catalyzed the same reaction as E. coli CoA, its activity was assayed using ATP and pantothenate as substrates. The assay monitored ADP formation by coupling it to pyruvate kinase and lactate dehydrogenase. The activity was measured as a change in absorbance at 340 nm during monitoring of depletion of NADH. This change was observed only in the presence of enzyme and all other components of the reaction.

**TABLE 1. Kinetic parameters of S. aureus CoA**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>(k_{cat} ) (min⁻¹)</th>
<th>(K_m ) (µM)</th>
<th>(k_{cat}K_m ) (µM⁻¹ min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pantothenate</td>
<td>329 ± 54</td>
<td>27 ± 7</td>
<td>13 ± 5</td>
</tr>
<tr>
<td>ATP</td>
<td>283 ± 109</td>
<td>93 ± 18</td>
<td>3 ± 1</td>
</tr>
</tbody>
</table>

* Aliquots of a reaction mixture containing 80 U of pyruvate kinase/ml, 80 U of lactate dehydrogenase/ml, 200 µM NADH, and 200 µM phosphoenolpyruvate in 50 mM HEPES (pH 7.5) containing 2 mM MgCl₂ were placed in 96-well half-area plates. The concentrations of the substrates ATP and pantothenic acid varied from 10 to 300 µM. The plates were incubated at 25°C for 5 min, and the reaction was initiated by the addition of S. aureus CoA to a final concentration of 50 nM in a final assay volume of 150 µl. The reactions were monitored at 340 nm for 15 min. The parameters were determined by nonlinear regression analysis of initial velocities using GraFit v.4.09. (Erithacus Software Inc.)
Staphylococcus epidermidis bacteria: the pathogens orthologous to S. aureus coaA (1). Orthologs also occur in eukaryotes, including mammals. Phylogenetic analysis (Fig. 2) shows that CoaA functions via a similar kinetic mechanism (20). It has been suggested that S. aureus CoaA proceeds via a Bi Bi mechanism which clearly delineate eukaryotes from the bacterial species (2, 16). Its limited distribution in bacteria and yet widespread occurrence in eukaryotes suggest that staphylococcal CoaA was horizontally transferred from eukaryotes to bacteria. The high level of sequence, and likely structural, divergence between bacterial and mammalian CoaA should permit the development of S. aureus-like CoaA-specific inhibitors.

**Evolutionary relationships of staphylococcal CoaA.** Genes orthologous to S. aureus coaA occur in other gram-positive bacteria: the pathogens *Staphylococcus epidermidis* and *Staphylococcus haemolyticus*; *Oceanobacillus iheyensis*, an alkaliphilic, halophilic bacillus living in deep-sea sediments (25); *Bacillus cereus*, an alkaliphilic bacterium living in deep-sea sediments (25); *Bacillus subtilis anther*, a soil-dwelling bacterium and the causative agent of the disease anthrax; and its closest relative, *B. anthracis* (baan), a soil-dwelling bacterium and the causative agent of the disease anthrax (26). Genes are related to eukaryotic CoaA proteins are distantly related to eukaryotic CoaA proteins. The GenBank accession numbers and phylogenetic relationships of these sequences are given in Fig. 2. The Orthologs also occur in eukaryotes, including mammals. For further analysis of the kinetic mechanism, initial velocities were determined at various concentrations of substrates. For nonlinear regression analysis of the observed rates suggest that S. aureus CoaA catalyzes the production of Pantothenic acid, with the inhibitor at various concentrations (0 to 100 M). All other assay conditions were as described in Table 1. To determine MIC, a Microlab AT Plus 2 (Hamilton Co., Reno, Nev.) serially diluted 50 μl of the stock solution into cation-adjusted Mueller-Hinton broth in a 96-well microtiter plate. A 50-μl aliquot of the test isolate, prepared at 10^6 CFU/ml using a direct suspension of an overnight culture, was added to each well. The final test concentrations ranged from 0.06 to 64 μg/ml. Inoculated plates were incubated at 35°C in ambient air for 18 to 24 h. The MIC was determined as the lowest concentration of compound that inhibited visible growth.

**TABLE 2. Potential inhibitors of S. aureus CoaA.**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>IC50 (μM)</th>
<th>S. aureus Oxford</th>
<th>S. aureus WCUH29</th>
<th>S. aureus 2</th>
<th>S. aureus 4</th>
<th>S. aureus 5</th>
<th>S. aureus 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>1b</td>
<td>1.6 ± 0.1 (0.46)</td>
<td>&gt;64</td>
<td>16</td>
<td>16</td>
<td>32</td>
<td>64</td>
<td>16</td>
</tr>
<tr>
<td>1c</td>
<td>1.1 ± 0.1 (0.35)</td>
<td>4</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>1d</td>
<td>0.4 ± 0.1 (0.14)</td>
<td>8</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>1e</td>
<td>No inhibition</td>
<td>&gt;64</td>
<td>&gt;64</td>
<td>&gt;64</td>
<td>&gt;64</td>
<td>&gt;64</td>
<td>&gt;64</td>
</tr>
<tr>
<td>2b</td>
<td>2.9 ± 0.7 (1.07)</td>
<td>&gt;64</td>
<td>&gt;64</td>
<td>&gt;64</td>
<td>&gt;64</td>
<td>&gt;64</td>
<td>&gt;64</td>
</tr>
</tbody>
</table>

* To determine IC50, the reaction mix contained 100 μM ATP and 25 μM pantothenic acid, with the inhibitor at various concentrations (0 to 100 μM). All other assay conditions were as described in Table 1. To determine MIC, a Microlab AT Plus 2 (Hamilton Co., Reno, Nev.) serially diluted 50 μl of the stock solution into cation-adjusted Mueller-Hinton broth in a 96-well microtiter plate. A 50-μl aliquot of the test isolate, prepared at 10^6 CFU/ml using a direct suspension of an overnight culture, was added to each well. The final test concentrations ranged from 0.06 to 64 μg/ml. Inoculated plates were incubated at 35°C in ambient air for 18 h.
FIG. 2. Phylogenetic tree of bacterial and eukaryotic CoaA (PanK) proteins. Species names in bold type were included in the alignment in Fig. 1. The Drosophila protein fumble and known vertebrate protein families (PanK1-4) are indicated. GenBank accession numbers are given in parentheses. (The sCoaA homolog in B. anthracis strain A2012 is annotated as a hypothetical protein [15]. Preliminary B. cereus and S. epidermidis sequence data were obtained from The Institute for Genomic Research website. [http://www.tigr.org]. The S. haemolyticus sequence is available upon request.) For human and mouse PanK1, the numbers of the identical α/H9251 and β/H9252 isoforms are both listed. PanK homologs for Takifugu rubripes and Bacillus cereus were found from separate genome searches at GenBank. Phylogenetic analyses used a multiple sequence alignment of 221 conserved amino acids (available upon request). The neighbor-joining tree shown here was constructed from pairwise distances between amino acid sequences calculated using the Dayhoff matrix (PHYLIP 3.6 package [Phylogeny Inference Package], version 3.6a2 [http://evolution.genetics.washington.edu/phylip.html]). Maximum parsimony trees (PAUP4.0b5 package [version 4; Sinauer Associates, Sunderland, Mass.]) were made from 100 random sequence additions yielding a single minimal length tree of 1,030 steps. Maximum likelihood trees (PUZZLE 4.0 software [23]) were based on the JTT substitution matrix, rate heterogeneity estimates of an eight-category gamma distribution model, and α parameter estimation from the data set. Numbers along the branches show the greater than 50% percent occurrence of nodes in 1,000 bootstrap replicates of neighbor-joining (plain text) and maximum parsimony (italicized text) analyses or 1,000 maximum likelihood quartet puzzling steps (bold text). An asterisk indicates that the node was supported more than 70% by all three methods. The scale bar represents the estimated number of amino acid substitutions per site.
anism of antibacterial action is due to the inability of the CoA derivative (ethyldehydro-CoA) to form acyl-CoA esters, resulting in disruption of essential downstream pathways (22).

Given the differences between the sequences of the E. coli and S. aureus CoaA enzymes, we attempted to determine if compound 1b also acted as a substrate for the S. aureus enzyme. Studies with compound 1b and related analogs (compounds 1c to e and 2b; Fig. 3) revealed that most of these compounds are inhibitors of the S. aureus CoaA, with the exception of the N-benzyl derivative of pantothenic acid (compound 1e; Fig. 3). Indeed, it appears that compound 1e is accommodated in the active site in a manner such that it acts as a substrate for the enzyme with a specific activity equal to that of pantothenate (A. E. Choudhry et al., unpublished data). All the other compounds (1b to d and 2b; Fig. 3) inhibited S. aureus CoaA with 50 percent inhibitory concentrations (IC50s) in the low micromolar range (Table 2), indicating their potential as effective inhibitors.

The activity of these compounds against several S. aureus strains was determined by the broth microdilution method. Encouragingly, the compounds with the best IC50s exhibited very good MICs (Table 2). 1e, which is a substrate rather than an inhibitor of CoaA, exhibited no antibacterial activity. Not surprisingly, compound 2b, the phosphorylated form of compound 1b, did not exhibit any antibacterial activity either.

Further, the two compounds exhibiting the best MICs, 1c and 1d, were assessed for their cytotoxicity potential against human HepG2 liver cells as described previously (14). The lowest concentrations causing a \( \geq 50\% \) decrease in cell viability for compounds 1c and 1d are 64 and 128 \( \mu \text{g/mL} \), respectively, indicating that neither compound strongly inhibits the growth of human hepatocytes.

Here, we have identified lead compounds for the development of staphylococcus-specific drugs against pantothenate kinase. Chemical optimization of these molecules could lead to the development of novel drugs that are not compromised by existing resistance mechanisms.

We thank Erick Strauss for synthesis of compound 1b and Gilbert Scott at the Protein Core Facility, GlaxoSmithKline Pharmaceuticals, for performing the protein analysis experiments.

REFERENCES


