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A Novel Inhibitor of *Mycobacterium tuberculosis* Pantothenate Synthetase

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Pantothenate synthetase (PS; EC 6.3.2.1), encoded by the *panC* gene, catalyzes the essential adenosine triphosphate (ATP)–dependent condensation of D-pantoate and β-alanine to form pantothenate in bacteria, yeast, and plants; pantothenate is a key precursor for the biosynthesis of coenzyme A (CoA) and acyl carrier protein (ACP). Because the enzyme is absent in mammals and both CoA and ACP are essential cofactors for bacterial growth, PS is an attractive chemotherapeutic target. An automated high-throughput screen was developed to identify drugs that inhibit *Mycobacterium tuberculosis* PS. The activity of PS was measured spectrophotometrically through an enzymatic cascade involving myokinase, pyruvate kinase, and lactate dehydrogenase. The rate of PS ATP utilization was quantitated by the reduction of absorbance due to the oxidation of NADH to NAD^+ by lactate dehydrogenase, which allowed for an internal control to detect interference from compounds that absorb at 340 nm. This coupled enzymatic reaction was used to screen 4080 compounds in a 96-well format. This discussion describes a novel inhibitor of PS that exhibits potential as an antimicrobial agent. (*Journal of Biomolecular Screening* 2007:100-105)

Key words: pantothenate synthetase, *Mycobacterium tuberculosis*, high-throughput screen, antimicrobial

INTRODUCTION

MYCOBACTERIUM TUBERCULOSIS (MTB) is a notorious pathogen whose increasing resistance to antibiotics and heightened lethality in combination with AIDS makes it a major health concern worldwide. One-third of the world's population is thought to be infected with MTB; 8 million people worldwide develop MTB annually, and nearly 2 million die.[1] MTB causes more deaths than any other infectious agent in the world. Immunocompromised individuals, particularly those infected with HIV, are at great risk for infection with MTB. The World Health Organization estimates that 11.4 million people worldwide are infected with both MTB and HIV. In addition, drug-resistant MTB, including multidrug-resistant tuberculosis (MDR-TB), has been found in all regions of the world.[2] Treatment for MDR-TB often requires the use of special drugs, all of which can produce serious side effects while only providing a 40% to 60% survival rate. Therefore, it is important to locate more potent and selective antituberculosis drugs that inhibit alternate essential biosynthetic pathways.

Pantothenate synthetase (PS) catalyzes the adenosine triphosphate (ATP)–dependent condensation of pantoate and β-alanine to form pantothenate, a B-group vitamin (vitamin B5) and key precursor for the synthesis of coenzyme A (CoA) and acyl carrier protein (ACP).[3] Unlike bacteria, humans obtain pantothenate strictly from dietary sources and do not possess the genes necessary for pantothenate biosynthesis. In MTB vaccine studies, growth and virulence in panC auxotrophs was severely compromised, supporting the theory of the necessity of this enzyme and its attractiveness as an antimicrobial target.[4]

Zheng and Blanchard[5] reported a PS assay that couples the AMP produced in the condensation of β-alanine and pantoate with the reduction of NADH to NAD^+ through myokinase, pyruvate kinase, and lactate dehydrogenase. The NAD^+ produced can be monitored spectrophotometrically at 340 nm and can be correlated with PS kinetic rates. This article describes the development and optimization of Zheng and Blanchard's PS assay to a high-throughput format and the validation of that assay using 4080 compounds. A kinetic assay was chosen to minimize interference by compounds that absorbed at 340 nm.

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MATERIALS AND METHODS

Materials

His-tagged PS was expressed and purified as previously described.[6] The enzyme prior to removal of the His-tag had the same specific activity as the enzyme from which the tag had been removed (unpublished observations, ELW). Pantoic acid was synthesized as described in Zheng and Blanchard[5] and King et al.[7] ATP, AMP, potassium phosphoenolpyruvate, NADH, rabbit muscle lactic dehydrogenase, rabbit muscle pyruvate kinase, and chicken muscle myokinase were purchased from Sigma Chemical Company (St. Louis, MO).

PS was screened against the Prestwick library of 880 compounds (Prestwick Chemicals, Inc., Washington, DC) and the MicroSource Spectrum Collection of 2000 compounds (MicroSource Discovery Systems, Inc., Gaylordsville, CT). Compounds were solubilized in 100% DMSO. Test compounds were screened in a single-dose format with the final concentration at either 10 μg/mL or 10 μM, depending on the compound library.

Methods

To each well of a 96-well plate, 60 μL of PS reagent mix was added, including NADH, pantoic acid, β-alanine, ATP, phosphoenolpyruvate, $MgCl_2$, myokinase, pyruvate kinase, and lactate dehydrogenase in buffer. Compounds were then added to plates in 1-μL volumes. The reaction was initiated with the addition of 39 μL of PS, diluted in buffer. The final concentrations in the reaction were 0.4 mM NADH, 5 mM pantoic acid, 10 mM $MgCl_2$, 5 mM β-alanine, 10 mM ATP, 1mM potassium phosphoenolpyruvate, and 18 units/mL each of chicken muscle myokinase, rabbit muscle pyruvate kinase, and rabbit muscle lactate dehydrogenase diluted in 100 mM HEPES buffer (pH 7.8), 1% DMSO, and 5 μg/mL PS in a final volume of 100 μL. The test plate was immediately transferred to a microplate reader, and absorbance was measured at 340 nm every 12 sec for 120 sec. Each plate had 16 control wells in the 2 outside columns, of which 12 contained the complete reaction mixture with DMSO carrier control (full reaction) and 4 without the addition of PS (background).

To confirm specific inhibition of PS apart from the other "coupled enzymes" in the assay, the hits were tested in an assay that was performed as above, except the final concentration of lactate dehydrogenase was 0.2 units/mL and no pantoic acid was added to the reaction mixture. The coupling enzyme assay was initiated with AMP (6.5 mM final concentration) instead of PS.

Data were analyzed using IDBS ActivityBase software. Hits in the single-dose assay were determined by comparing the rates of each compound to the full reaction after subtracting the background rate:

$$\%\ \text{Inhib} = 100 \times \left(1 - \frac{\text{compound rate} - \text{background rate}}{\text{full reaction rate} - \text{background rate}}\right)$$

Dose-response curves were analyzed using XLfit equation 205 for a 4-parameter logistic fit where the maximum and minimum values were fixed at 100% and 0%.

Crystallization was carried out as described previously.[6,8] Crystals in space group $P2_1$ were obtained from the well condition 13% PEG 3000, 5% glycerol, 2% ethanol, 20 mM $MgCl_2$, 150 mM $LiSO_4$, and 100 mM imidazole (pH 8.0) at 20 °C. Nafronyl oxalate was soaked into the crystals with a final concentration of 1 mM nafronyl oxalate in the drop. The cryoprotectant was an additional 30% glycerol added to the crystallization condition, and the crystals were flash-frozen in a cryo stream of N_2 gas at 100 K. Diffraction data were collected at 100 K on a Rigaku FRD generator with an R-AXIS IV++ detector. Data reduction and scaling were carried out with DENZO and SCALEPACK.[9] The PS crystal form has 2 PS molecules in the asymmetric unit. The initial difference electron density map was calculated with CNS[10] using a model of apo-PS (PDB code 1MOP), an apo-PS data set, and a nafronyl oxalate–soaked PS data set that revealed initial positive electron density within the active site of PS. The structural model of nafronyl oxalate–soaked PS was refined in CNS, and then nafronyl oxalate was modeled into the active site before further rounds of refinement. AUTODOCK[11] was run in an attempt to dock nafronyl oxalate into the active site of PS. The program's generic algorithm run in the vicinity of the active site of PS gave the best docking results.

RESULTS

Assay validation and optimization

The PS assay is a modification of a procedure reported by Zheng and Blanchard.[5] The assay couples the AMP produced in the condensation of β-alanine and pantoate with the reduction of NADH to NAD^+ through myokinase, a reaction that generates adenosine diphosphate (ADP); then pyruvate kinase, a reaction that transfers a phosphate from phosphoenolpyruvate to ADP and generates pyruvate; and finally lactate dehydrogenase, a reaction that converts pyruvate to lactate with the oxidation of NADH to NAD^+ (**Fig. 1**). The oxidation of NADH to NAD^+ can be monitored spectrophotometrically at 340 nm.

Enzyme concentration and the optimal reaction rate were determined in 100-μL reaction volumes in 96-well plates, with enzyme concentration being adjusted to provide a linear reaction rate at 340 nm for 120 sec. Reproducibility of the full reaction rates for PS was evaluated in quadruplicate. At the optimal enzyme concentration, reaction rates with R^2 values of 0.998 indicated good linearity during the defined assay time (**Fig. 1**).

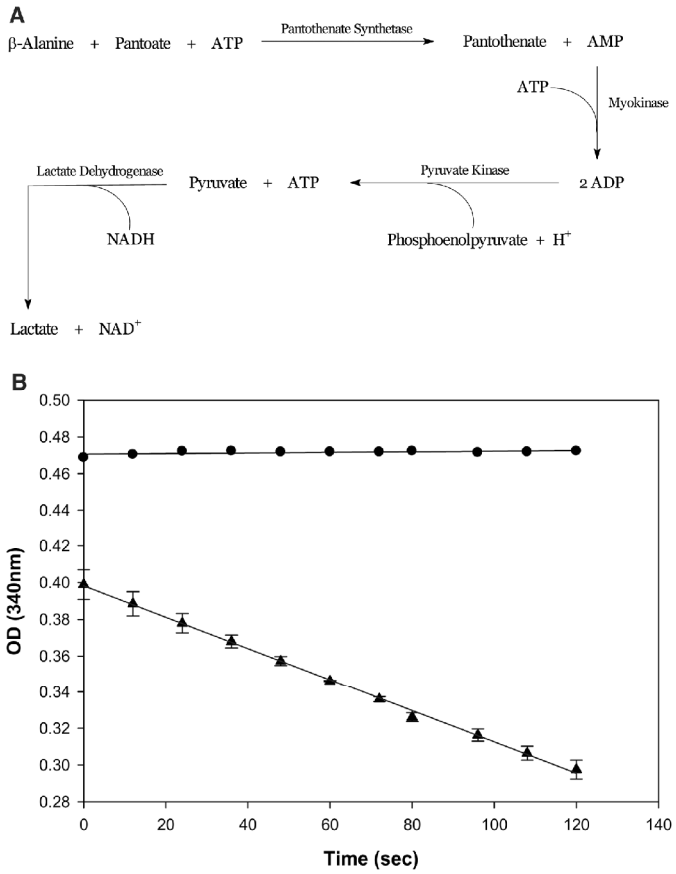

FIG. 1. Reaction scheme for pantothenate synthetase assay. The reaction scheme depicts the previously published assay,[5] which was the basis for the high-throughput screening. The time course for the optimized assay, described in detail in the Methods section, is illustrated in the graph. Pantothenate synthetase (PS) is present in the full reaction at 5 μg/mL (triangles) but absent in the background reaction (circles). Each reaction was run in quadruplicate to determine the average reaction rate for the full reaction (–0.0008 sec^{-1}) and for the background reaction (1.66×10^{-5} sec^{-1}). The correlation coefficient for both straight line fits is > 0.99. ATP, adenosine triphosphate; ADP, adenosine diphosphate.

Z plate analyses,[12] a statistical construct designed to evaluate assay robustness, were employed to assess the suitability of the PS enzyme reaction for a high-throughput format. This experimental design allows for the analysis of both intraplate and interplate variability. Z values obtained for PS in the 96-well assay averaged approximately 0.9, indicating an extremely robust assay. Reagents were stable in solution at room temperature for at least 4 h (data not shown). A similar analysis was done for the "coupling enzyme" assay with similarly robust Z values.

A small proprietary library of 1200 was screened in triplicate at compound concentrations of 10 μg/mL to validate the assay and set the hit threshold. Internal controls consisting of reagents for a full reaction and a background reaction were included in each assay plate; because there are not any known inhibitors of PS, a positive control was not available (**Fig. 2A**). Using these data and past experience with enzyme assays, the hit threshold was set at ≥ 20% inhibition. There were 18 compounds whose average inhibition reached the hit threshold. Of these 18 compounds, 12 (67%) had at least 2 replicates with percent inhibitions ≥ 20%. This correlation was considered sufficient to proceed with the high-throughput campaign.

In total, 2880 compounds from the Prestwick and Microsource collections were screened against PS. These libraries contain known drugs covering several therapeutic areas, experimental bioactives, and natural products. Most of these compounds are bioavailable and safe in humans. Results of screening these libraries at 10 μg/mL for Prestwick and 10 μM for Microsource are presented in **Figure 2B**,**C**. Twenty-nine compounds demonstrated at least some activity against PS, with IC_{50} values in the range of 12 to 67 μM. The dose response of all 29 initial hits was performed in duplicate at compound concentrations ranging from 0.2 to 100 μM (2-fold dilutions) with excellent reproducible results. At the same time, these 29 compounds were evaluated in the coupling enzyme assay to determine whether their inhibitory activity was specific for PS. Only 1 compound was identified as a specific inhibitor of PS: nafronyl oxalate, which is a vasodilator that has been used in the treatment of vascular and cerebral disorders (**Fig. 3**). This compound was evaluated further as a potential lead compound for antimycobacterial activity.

The first step for determining the K_i for nafronyl oxalate was to determine the K_m for the substrate, pantoate. The K_m for pantoate was determined by doing a standard substrate curve, varying the concentration of pantoate from 5 to 0.0625 mM, with 5 mM β-alanine and 10 mM ATP held constant. The K_m experiment was done in duplicate; K_m was determined to be 410 ± 50 mM using the simple ligand-binding analysis in SigmaPlot.

For the K_i experiments, the pantoate concentrations used were 4000, 2000, 1000, 500, and 250 μM, and the nafronyl oxalate concentrations used were 0, 40, 80, 120, and 160 μM. Substrate curves were run for each inhibitor concentration in duplicate. The Enzyme Kinetics Module in SigmaPlot can be used to rank various modes of enzyme inhibition. Under these conditions, we were unable to definitively establish the mode of inhibition because all of the models were equivalent with R^2 for the fits > 0.99. A K_i value of 75 ± 13 μM was obtained for the competitive model. Ambiguity in the model is not unexpected because the models and experimental design are based on a single substrate site on the enzyme, whereas this enzyme is actually a 3-substrate enzyme. Because all of the models other than competitive one produced a substantial (i.e., > 60%) increase in the K_m for pantoate, the competitive model was chosen as the appropriate fit.

The soaked structure of PS with nafronyl oxalate shows an initial $F_o - F_c$ difference electron density in both of the active

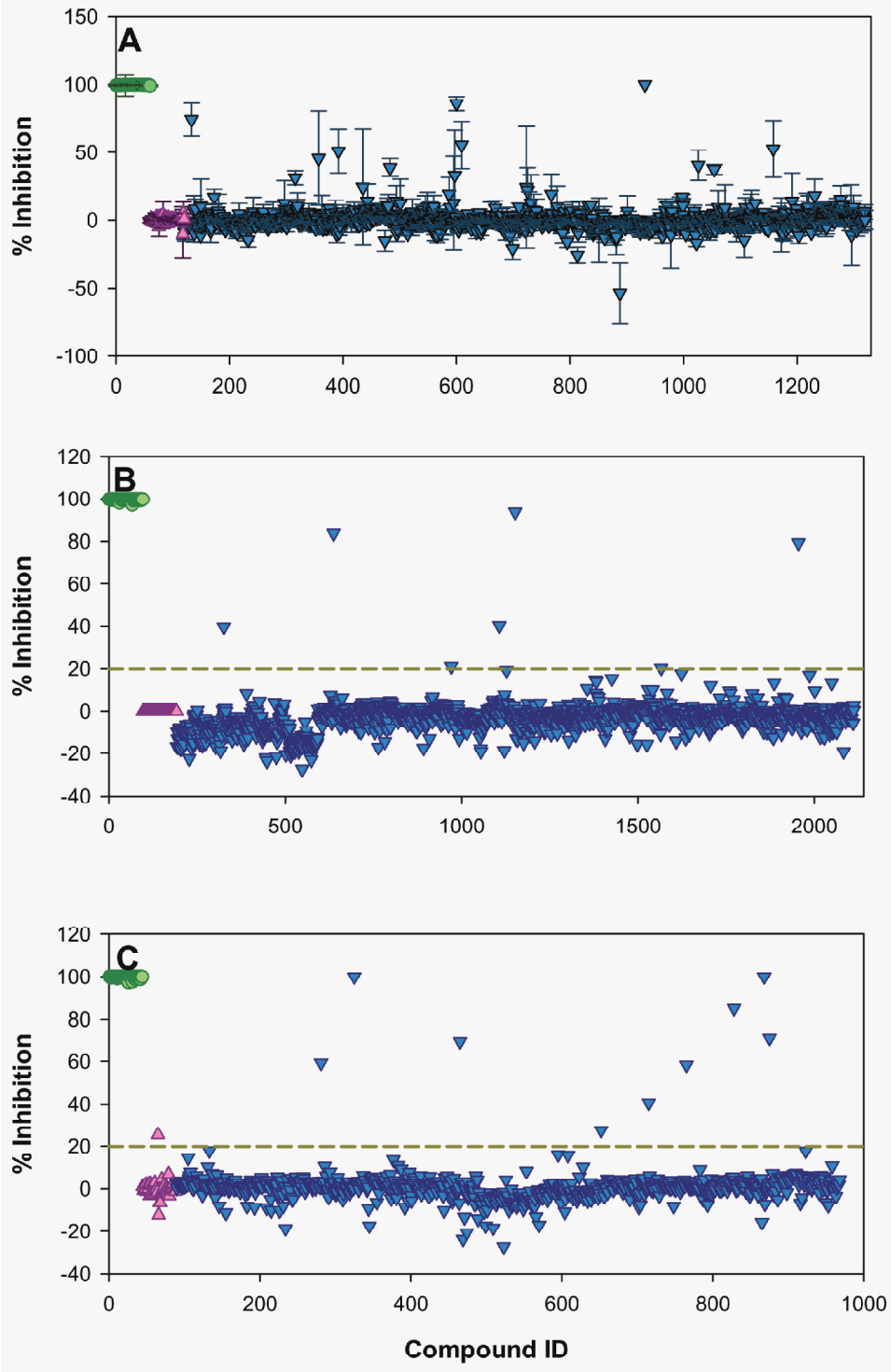

FIG. 2. High-throughput screening (HTS) results. (**A**) A proprietary library of 1200 compounds was run in triplicate in the HTS assay for a total of 45 plates. The screening data are presented as the average of triplicates ± standard deviation. Two commercially available libraries, Microsource Spectrum (**B**) and Prestwick (**C**), were screened in the pantothenate synthetase (PS) assay. Percent inhibition versus compound ID is plotted. Control wells from each plate are grouped at the beginning of each graph for easy visualization. Each plate contained control wells with PS (full reaction, triangles) and without PS (background, circles) and 80 wells containing compound (inverted triangles) from the library at a final concentration of 10 µg/mL for the proprietary library and Prestwick and 10 µM for Microsource.

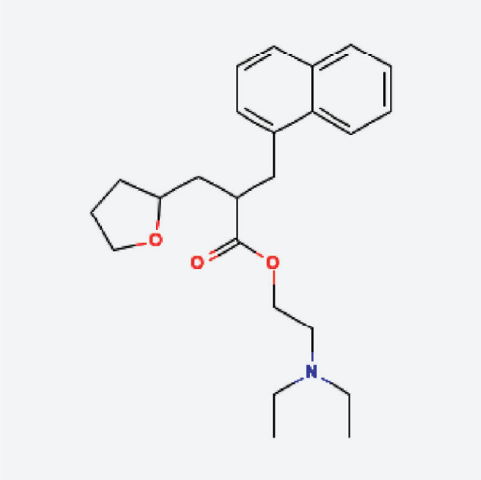

FIG. 3. Structure of nafronyl oxalate.

sites in the PS dimer at a resolution of 2.9 Å. Although the electron density for nafronyl oxalate was weak, we modeled nafronyl oxalate into each active site (**Fig. 4A**). The cocrystal structure of PS bound with the reaction intermediate, pantoyl adenylate, bound tightly in the active site was previously solved[6] and is shown as a comparison to PS bound with nafronyl oxalate (**Fig. 4B**). The comparison of nafronyl oxalate and pantoyl adenylate bound in the active site shows that although the 2 molecules are in a similar position, pantoyl adenylate fits snugly into the active site with a good hydrogen-bonding network in comparison to nafronyl oxalate. The residues that form hydrogen bonds to pantoyl adenylate include His44, His47, Glu72, Asp161, and Gln164.[6,8] The hydrogen-bonding network for nafronyl oxalate in the active site is two ~3.5 Å hydrogen bonds between the oxygen in the furan ring of nafronyl oxalate and the nitrogen atoms on both His44 and His47 (**Fig. 4A**).

AUTODOCK was used in an attempt to position nafronyl oxalate more accurately within the active site of PS. Results showed that the lowest minimal docking energy placed nafronyl oxalate at the exact position in the active site as found in the cocrystal structure of the PS–nafronyl oxalate complex. Thus, the precise orientation of the molecule is unresolved due to the weak electron density for nafronyl oxalate in the cocrystal structure, as well as the resulting 5 different orientations obtained from AUTODOCK, all with the same lowest minimal docking energy. One could postulate that to increase binding infinity of the inhibitor and thus inhibition of PS, the diethylamine and naphthalene groups of nafronyl oxalate could be substituted with charged groups.

When the compound was submitted to the National Institute of Allergy and Infectious Diseases (NIAID) Tuberculosis Antimicrobial Acquisition and Coordinating Facility for antituberculosis evaluation[13] in a supplemented 7H9 broth, it was inactive at the standard test concentration of 6.25 μg/mL (13 μM). The apparent lack of antimicrobial activity might be due to the relatively low concentration, 1/6th K_i concentration, used in the assay, and the compound has been rescheduled for testing at a higher concentration (100 μg/mL). Nafronyl oxalate is apparently well tolerated by humans with a Food and Drug Administration (FDA) maximum recommended therapeutic dose of 10 μg/kg-body weight/day, so the apparent need for higher test concentrations is therapeutically reasonable. Because the standard media used in the antimicrobial testing contain pantothenate, arrangements have been made to determine the minimum inhibitory concentration with and without pantothenate. In addition, 2 mouse animal models, both with aerosolized exposure to MTB, are available through the TAACF.

SUMMARY

A high-throughput method for screening compounds against MTB PS was developed and optimized. From a screen of 2880 compounds, 1 lead compound, nafronyl oxalate, was identified. This compound is a competitive inhibitor of the enzyme with a K_i value one-fifth of the substrate K_m. Early crystallographic studies show that the compound binds at the active site of the enzyme. This is the first published finding of an inhibitor of PS from any organism. Preliminary studies indicate that this compound does not inhibit MTB growth in vitro, but it is still a viable lead in the search for compounds with a novel mechanism of action. Such leads are important in the war against antibiotic-resistant organisms, specifically multidrug-resistant MTB.

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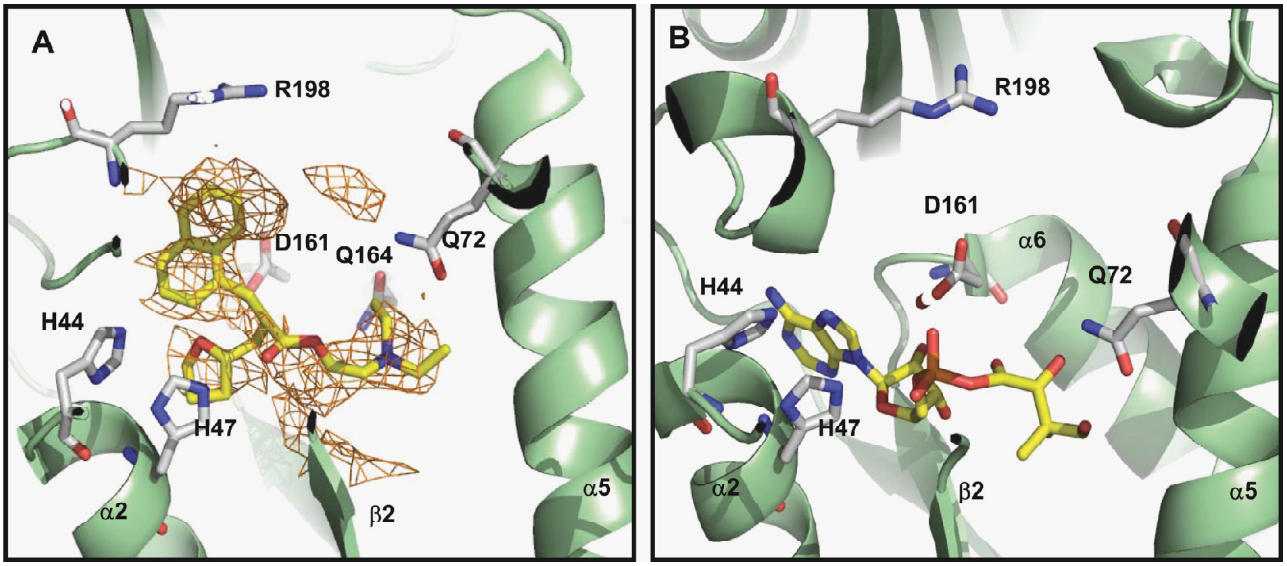

FIG. 4. Cocrystallization of nafronyl with pantothenate synthetase (PS). (**A**) Section of the initial difference electron density map ($F_o - F_c$) in the active site of subunit A of the $P2_1$ crystal showing partial electron density for nafronyl oxalate. The difference density was calculated at 2.9 Å after rigid body refinement with the apoenzyme (PDB code 1MOP) as a model and contoured at the 1.8 Å level. Superimposed on the electron density is the final refined model. The side chains shown have little movement relative to those in the apoenzyme and thus have little initial difference electron density. PS is shown as a green ribbon diagram with residues His44, His47, Gln72, Asp161, Gln164, and Arg198 shown in stick model, with carbon, nitrogen, and oxygen atoms colored in grey, blue, and red, respectively. The inhibitor nafronyl oxalate is shown as a stick model with carbon, oxygen, and nitrogen atoms colored in yellow, red, and blue, respectively. The difference electron density is shown in an orange mesh. (**B**) Shows the same view of PS as (**A**) with PS's reaction intermediate, pantoyl adenylate (shown with carbon atoms in yellow), tightly bound in the active site. These figures were prepared with PYMOL.

REFERENCES

1. Office of Communications and Public Liaison, National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health: Tuberculosis. http://www.niaid.nih.gov/factsheets/tb.htm. Last accessed June 9, 2006.
2. World Health Organization (WHO): links for an overview of tuberculosis worldwide. http://www.who.int/inf-fs/en/fact104.htm. Last accessed June 9, 2006.
3. Abiki Y: Metabolism of coenzyme A. In Greenburg DM (ed): *Metabolic Pathways*. New York: NY Academic Press, 1975:1-25.
4. Sambandamurthy VK, Derrick SC, Jalapathy KV, Chen B, Russell RG, Morris SL, et al: Long-term protection against tuberculosis following vaccination with a severely attenuated double lysine and pantothenate auxotroph of *Mycobacterium tuberculosis*. *Infect Immun* 2005;73:1196-1203.
5. Zheng R, Blanchard JS: Steady-state and pre–steady-state kinetic analysis of *Mycobacterium tuberculosis* pantothenate synthetase. *Biochemistry* 2001;40:12904-12912.
6. Wang S, Eisenberg D: Crystal structures of a pantothenate synthetase from *M. tuberculosis* and its complexes with substrates and a reaction intermediate. *Protein Sci* 2003;12:1097-1108.
7. King HL Jr, Dyar RE, Wilken DR: Ketopantoyl lactone and ketopantoic acid reductases: characterization of the reactions and purification of two forms of ketopantoyl lactone reductase. *J Biol Chem* 1974;249:4689-4695.
8. Wang S, Eisenberg D: Crystal structure of the pantothenate synthetase from *Mycobacterium tuberculosis*, snapshots of the enzyme in action. *Biochemistry* 2006;45:1554-1561.
9. Otwinowski Z, Minor W: Processing of X-ray diffraction data collected in oscillation mode. *Methods Enzymol* 1997;276:307-326.
10. Brunger AT, Adams PD, Clore GM, DeLano WL, Gros P, Grosse-Kunstleve RW, et al: Crystallography & NMR system: a new software suite for macromolecular structure determination. *Acta Crystallogr D Biol Crystallogr* 1998;54:905-921.
11. Morris GM, Goodsell DS, Halliday RS, Huey R, Hart WE, Belew RK, et al: Automated docking using a lamarckian genetic algorithm and empirical binding free energy function. *J Comput Chem* 1998;19:1639-1662.
12. Zhang JH, Chung TD, Oldenburg KR: A simple statistical parameter for use in evaluation and validation of high throughput screening assays. *J Biomol Screen* 1999;4:67-73.
13. Collins L, Franzblau GA: Microplate Alamar Blue assay versus BACTEC 460 system, for high-throughput screening against *Mycobacterium tuberculosis* and *Mycobacterium avium*. *Antimicrob Agents Chemother* 1997;41:1004-1009.

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