The Antituberculosis Drug Ethionamide Is Activated by a Flavoprotein Monoxygenase*

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Ethionamide (ETA), a prodrug that must undergo metabolic activation to exert its cytotoxic effects, is a second line drug against tuberculosis, a disease that infects more than a third of the world’s population. It has been proposed, on the basis of genetic experiments, that ETA is activated in Mycobacterium tuberculosis by the protein encoded by the gene Rv3854c (DeBarber, A. E., Mdluli, K., Bosman, M., Bekker, L.-G., and Barry, C. E., III (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 9677-9682; Baulard, A. R., Betts, J. C., Engohang-Ndong, J., Quan, S., McAdam, R. A., Brennan, P. J., Locht, C., and Besra, G. S. (2000) J. Biol. Chem. 275, 28326-28331). We report here the expression, purification, and characterization of the protein encoded by this gene. Our results establish that the enzyme (EtaA) is an FAD-containing enzyme that oxidizes ETA to the corresponding S-oxide. The S-oxide, which has a similar biological activity as ETA, is further oxidized by EtaA to 2-ethyl-4-amidopyridine, presumably via the unstable doubly oxidized sulfenic acid intermediate. This flavoenzyme also oxidizes thiacetazone, thiobenzamide, and isothionicotinamide and thus is probably responsible, as suggested by the observation of crossover resistance, for the oxidative activation of other thioamide antitubercular drugs.

Tuberculosis continues to be a major worldwide epidemic with approximately one-third of the world population infected with Mycobacterium tuberculosis, 7 million people each year developing the active disease, and 2 million deaths per annum (1, 2). Drugs such as isoniazid (INH)1 and rifampicin have historically been successful in the treatment of tuberculosis infections. In recent history, however, poor compliance with the prolonged and complicated chemotherapeutic regimens currently used to treat the disease (3), in conjunction with the advent of the AIDS epidemic and the increased mobility of human populations, has led to the emergence of numerous multidrug-resistant M. tuberculosis strains (4). Resistance to frontline therapeutics, most notably INH and rifampicin, results in treatment of patients with “second-line” agents that are less effective and/or more toxic. Among these second tier drugs for the treatment of multidrug-resistant tuberculosis, one of the most effective is ethionamide (ETA) (5).

ETA (Fig. 1), like INH, is thought to be a prodrug that must be converted to its active form by the bacterial cell. Both ETA and INH, when activated, appear to disrupt cell wall biosynthesis and have at least one common cellular target, the enoyl-acyl carrier protein reductase InhA (6, 7). Support for a common site of action can be deduced from gene array studies demonstrating that both ETA and INH induce similar patterns of gene expression in M. tuberculosis (8). Despite this evidence for a common site of action, INH and ETA are activated by different mechanisms as resistance to INH does not confer resistance to ETA (9). INH is now known to be oxidized by the bacterial catalase-peroxidase KatG to a reactive species, probably an acyl free radical, that is eventually responsible for its bacterial toxicity (10–13). Mutations in KatG that diminish or annihilate its activity toward INH are responsible for a large proportion of the INH-resistant M. tuberculosis strains. Until recently, however, the enzyme that activates ETA has remained obscure (14, 15). Sequence homology studies using the Rv3854c gene as a template indicate that the protein responsible for ETA activation may be a flavin monooxygenase (14, 15). In vivo mammalian and bacterial studies suggest that the first metabolite of ETA is the thioamide S-oxide (ETA-SO, 2, Fig. 1), which retains the biological activity of the parent drug (16–19). Other metabolites identified in whole cell bacterial systems include the nitrile (3), amidine (4), and alcohol (6) derivatives of ETA (Fig. 1) (14).

Recently two laboratories independently reported identification of a gene, Rv3854c, in the M. tuberculosis genome that codes for a protein that activates ETA (14, 15). Genetic and transfection experiments provided strong evidence for the biological role of the Rv3854c encoded protein (EtaA) in the activation of ETA. However, the actual enzyme was not isolated, and its nature remains speculative. Herein we report cloning, heterologous expression, purification, and characterization of the Rv3854c gene product. Our results establish that the enzyme responsible for ETA activation is an FAD-containing enzyme, provide information on the catalytic and physical properties of this enzyme, and demonstrate that it catalyzes two steps rather than one step in the activation of ETA.

EXPERIMENTAL PROCEDURES

Materials

All chemicals, including NADPH, were purchased from Aldrich or Sigma, were of ACS grade or better, and were used without further purification. All the enzymes used in cloning procedures were from New England Biolabs (Beverly, MA) and were used with buffers from the same company. Protein purification was done at 4 °C. Once purified, the protein was stored at −70 °C and was found to be stable for at least 4 months at that temperature. HPLC columns were purchased from Alltech Associates, Inc.

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1 The abbreviations used are: INH, isoniazid; ETA, ethionamide or 2-ethyl-4-thiocarbamoylpyridine; ETA-SO, ethionamide sulfoxide; EtaA, ethionamide-activating enzyme; HPLC, high-pressure liquid chromatography; ESI, electrospray ionization; MS, mass spectroscopy; FPLC, fast protein liquid chromatography; NTA, nitrilotriacetic acid.
UV-visible spectra were obtained on either a Hewlett Packard model 8254A diode array instrument or a Varian Cary 1E spectrophotometer. Mass spectra were obtained using a Thermoquest Finnigan LCQDECA electrospray ionization (ESI) ion trap spectrometer. HPLC was performed on a Hewlett Packard Series 1090 liquid chromatograph equipped with either a Rheodyne model 7125 manual sample injector or an autosampler.

Kinetic Assays

All activity and steady state kinetic assays were conducted by monitoring the formation of the product ETA-SO (2). Product formation was measured at 350 nm over a time of 5–10 min using the Cary 1E split beam spectrophotometer. The molar extinction coefficient of ETA-SO in the reaction system was experimentally determined to be 6,000 (±5%) M⁻¹ cm⁻¹. The reaction medium contained 50 mM Tris buffer (pH 7.5), 10% methanol, and an NADPH-regenerating system consisting of glucose-6-phosphate dehydrogenase (2 units/ml), glucose 6-phosphate (250 mM), and either NADP⁺ or NADPH. The final concentration of enzyme was typically 500 nM in FAD. ETA stock solutions (25 mM) were freshly prepared in acetonitrile, and the final added volume never exceeded 1% of the total reaction volume. All stock solutions were kept on ice prior to mixing within the cuvette.

The enzymatic reaction was typically initiated by adding substrate (ETA) to the sample cuvette containing the reaction medium plus the solutions (25 mM) were freshly prepared in acetonitrile, and the final added volume never exceeded 1% of the total reaction volume. All stock solutions were kept on ice prior to mixing within the cuvette.

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were: 5’-end, 5’-GGCTGAGCATATGACCGAACCTGCG; 3’-end, 5’-CTAAAGCTGCCTAAGCGCTAAACCCC (the restriction sites are underlined). The insert was ligated into the pCWori expression vector containing a poly-His insert at the 5’-end of the coding sequence just prior to the start codon. Sequencing was used to confirm that the full-length gene without alterations in the sequence was inserted into the expression vector. The expression vector was transformed into commercial (Invitrogen) competent DH5α *Escherichia coli* cells, and protein expression was induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside once the cells grew to an A₆₀₀ of 0.9. Cells were grown for 24 h postinduction at 22 °C and were harvested by centrifugation at 4,000 × g.

The *E. coli* cells were resuspended in 50 mM Tris (300 mM NaCl, 1% (v/v) Triton X-100, 6 mM imidazole, pH = 7.4), lysed, and sonicated. The cytosolic fraction was removed after centrifugation at 25,000 × g. This supernatant was loaded directly onto a Ni-NTA affinity column pre-equilibrated with 50 mM Tris, 500 mM NaCl, 10% glycerol, pH = 7.4. After loading, the column was washed with 10 column volumes of the same buffer, and the protein was eluted with a gradient over 10 column volumes of 0–200 mM imidazole in 50 mM Tris (pH = 7.4) containing 500 mM NaCl and 10% glycerol. The fractions containing the pure Rv3854c gene product (EtaA), as determined by sodium dodecylsulfate polyacrylamide gel electrophoresis, were pooled and concentrated. Imidazole was removed from the protein solution by passing it through a Sephadex G-25 GF size exclusion column (elution buffer: 50 mM Tris, 100 mM KCl, 10% glycerol, pH 7.4) with a final yield of ~15 mg of pure protein/liter of culture.

Fast protein liquid chromatographic analysis of the protein was done on a Superdex 75 FPLC column eluted at a rate of 0.5 ml/min with 50 mM Tris buffer, pH = 7.4, containing 100 mM KCl and 10% glycerol. Thin layer chromatographic comparison of the prosthetic group extracted from the recombinant flavoprotein with authentic FMN and FAD was done on silica gel using water as the elution solvent. The extracted prosthetic group has the retention time of FAD and not FMN. Rv3854c binds one flavin group. Thin layer chromatographic comparison of the extracted prosthetic group (RF_p = 0.9) with authentic FAD (RF_p = 0.9) and FMN (RF_p = 0.1) showed that the prosthetic group has the retention time of FAD and not FMN. Rv3854c clearly codes for a flavoprotein with a single FAD prosthetic group.

**HPLC Identification of Metabolites**

The enzymatic reactions with ETA, thionamides, isothiocyanates, and thiacetazone were analyzed by HPLC, and the metabolites were identified, where possible, by comparison with authentic standards. The enzymatic reaction was run for 3 h at 37 °C using conditions identical to those described for the kinetic assays. The crude reaction mixture was filtered through a 0.22-μm syringe filter (Millex®-GV low protein binding Durapore membrane) and analyzed, without further modification, by HPLC on a Whatman Partisil® C8 reverse-phase column (particle size, 5 μm; 250 × 4.6-mm inner diameter) equipped with a C8 guard column (particle size, 5 μm; 5 × 4.6-mm inner diameter). The solvent system consisted of 0.01% formic acid in water (A) and 350 mM NaH₂PO₄ (B) with a linear gradient of 1% B over 30 min. The eluent was monitored at 284 and 350 nm, and complete UV-visible spectra of the metabolite peaks were collected for comparison with authentic standards.

**RESULTS**

**Cloning and Expression of the Protein**—Genetic experiments, including expression in intact cells, have implicated the protein encoded by Rv3854c in the bioactivation of ETA to a form of the drug with direct antimycobacterial activity (14, 15). The metabolic products of ETA suggest that the protein in question is likely to be a monoxygenase (15). The transformations are consistent with the involvement of either a cytochrome P450 or a flavin monoxygenase, but the protein sequence encoded by Rv3854c is more consistent with a flavoprotein (14, 15). To elucidate the precise nature of the enzyme involved in the activation of ETA and the nature of the transformation that it catalyzes, we have cloned and expressed Rv3854c in *E. coli*. The protein was expressed with a poly-His tag to facilitate purification of the protein. Expression of two constructs, one with the poly-His tag at the amino terminus and the other with the tag at the carboxyl terminus, gives protein with similar catalytic properties, indicating that the poly-His tag does not interfere with catalytic function (all subsequent experiments were conducted with the amino-terminally His-tagged protein). An exploration of expression conditions led to production of the enzyme in yields of 15 mg of purified protein/liter of medium. The protein was highly purified by Ni-NTA affinity chromatography as shown by the fact that even at high concentrations the protein gives rise to a single band on SDS-PAGE (Fig. 2). The specific activity of the protein product at different stages of the purification procedure, measured as indicated under “Experimental Procedures” and as discussed below, is shown in Table 1. Herein the recombinant Rv3854c gene product is denoted as EtaA.

**Physical Characterization of the Protein**—EtaA, as expected from the predicted length of 488 amino acids, migrates with a molecular mass of ~55 kDa (Fig. 2). FPLC analysis of EtaA on a Superdex column shows that the protein elutes as a single peak but at a molecular weight that corresponds to that of an oligomeric (3–4-mer) species, suggesting that EtaA aggregates in solution. UV-visible spectra of solutions of EtaA exhibit maxima at 365 and 440 nm in agreement with its identification as a flavoprotein (Fig. 3). Furthermore, extraction of the prosthetic group by boiling EtaA for 5 min and sedimenting the denatured protein by centrifugation yields a supernatant with a spectrum identical to that of authentic FAD (Fig. 3). Quantitation of the flavin released from denatured EtaA yields a ratio of flavin to protein of 1:1.3, indicating that the protein binds one flavin group. Thin layer chromatographic comparison of the extracted prosthetic group (RF_p = 0.9) with authentic FAD (RF_p = 0.9) and FMN (RF_p = 0.1) showed that the prosthetic group has the retention time of FAD and not FMN. Rv3854c clearly codes for a flavoprotein with a single FAD prosthetic group.

**Catalytic Oxidation of ETA by EtaA**—The identity of the metabolite(s) produced in the reaction of purified EtaA with ETA and NADPH was determined by letting the reaction proceed for 3 h, separating the products by reverse-phase HPLC, and comparing the peaks with authentic (synthetic) standards. The standards that were available were the 3-oxide (2), 2-ethyl-4-cyanopyridine (3), 2-ethyl-4-aminopyridine (4), and 2-ethyl-4-carboxypyridine (5), all of which have been reported as metabolites from the in vivo mycobacterial oxidation of ETA (14, 16). The two major metabolic products observed with this method had retention times of 9.1 and 14.9 min. The two peaks were identified as the amide (4) and ETA-SO (2), respectively, by comparison with the elution time and spectrum of the synthetic standards (RF(4) = 9.1 and RF(2) = 14.9 min) (Fig. 4).

Since ETA-SO exhibits similar antimycobacterial activity as ETA (14, 16), the two major metabolic products observed with this method had retention times of 9.1 and 14.9 min. The two peaks were identified as the amide (4) and ETA-SO (2), respectively, by comparison with the elution time and spectrum of the synthetic standards (RF(4) = 9.1 and RF(2) = 14.9 min) (Fig. 4).

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Flavoprotein Activation of Ethionamide

The flavin appears to partially dissociate from the protein during the last purification step. Preliminary results suggest that the protein can be reconstituted with exogenous flavin.

| Purification step | NADPH consumption | Ethionamide S-oxide formation |
|-------------------|------------------|------------------------------|
| After cell lysis  | $6.21 \times 10^{-3}$ | 0                            |
| Supernatant after lysis | $3.79 \times 10^{-3}$ | $4.84 \times 10^{-4}$ |
| Pellet + Emulgen | $4.87 \times 10^{-3}$ | 0                            |
| Before Ni-NTA    | $1.19 \times 10^{-3}$ | $2.33 \times 10^{-2}$         |
| Pure flavoprotein* | $1.13 \times 10^{-2}$ | $2.59 \times 10^{-3}$         |

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The catalytic activity of purified EtaA with respect to ETA and ETA-SO was confirmed by the corresponding S-oxide and benzamide by comparison with commercially available material. The second metabolite was tentatively identified as the amide by the presence of a consensus N binding domain in the predicted protein sequence (23), is clearly established by the demonstration that the protein possesses a single FAD prosthetic group.

Cloning experiments in M. tuberculosis, Mycobacterium smegmatis, and Mycobacterium bovis BCG have shown that Rv3854c codes for a protein that is critical for the antitubercular activity of ETA (14, 15). These studies also demonstrated that the expression level of Rv3854c is regulated by a suppressor gene, Rv3855, and that resistance to ETA increases with increased expression levels of Rv3855. Conversely increases in the expression levels of Rv3854c confer heightened sensitivity of the bacilli to ETA (14, 15). The work presented here demonstrates that the protein encoded by Rv3854c, EtaA, is a flavoprotein monooxygenase with a single FAD prosthetic group.

**DISCUSSION**

Cloning experiments in M. tuberculosis, Mycobacterium smegmatis, and Mycobacterium bovis BCG have shown that Rv3854c codes for a protein that is critical for the antitubercular activity of ETA (14, 15). These studies also demonstrated that the expression level of Rv3854c is regulated by a suppressor gene, Rv3855, and that resistance to ETA increases with increased expression levels of Rv3855. Conversely increases in the expression levels of Rv3854c confer heightened sensitivity of the bacilli to ETA (14, 15). The work presented here demonstrates that the protein encoded by Rv3854c, EtaA, is a flavoprotein monooxygenase with a single FAD prosthetic group.

The identity of EtaA as a flavoprotein, a classification suggested by the presence of a consensus N-X$_5$D$_X$,G,XGXXG flavin binding domain in the predicted protein sequence (23), is clearly established by the demonstration that the protein possesses a single FAD prosthetic group. The UV-visible spectrum of the pure protein obtained after a final size-exclusion chromatographic step (Fig. 3) is typical of a flavoprotein. The Rv3854c protein is most likely membrane-associated when expressed in the E. coli system as evidenced by its presence predominantly in the pellet fraction of the first centrifugation step following...
FIG. 5. UV-visible spectra of the formation of ETA-SO from ETA during a 5-min incubation with EtaA. Inset, double-reciprocal plot of initial rate measurements performed at [ETA] = 50, 100, 200, and 400 μM. The initial reaction system consisted of EtaA (500 nM), ETA (200 μM), and an NADPH-regenerating system comprised of NADPH (200 μM), glucose-6-phosphate dehydrogenase (2 units/ml), and glucose 6-phosphate (2.5 mM). The reaction was carried out in 50 mM Tris (pH 7.5) in the presence of superoxide dismutase (100 units/ml), catalase (100 units/ml), bovine serum albumin (0.1 mg/ml), and 100 mM KCl at 37°C.

FIG. 6. The structures of analogues of ethionamide that are oxidized by the purified flavoprotein: thiobenzamide (7) with its observed metabolite thiobenzamide S-oxide (8), isothiocyanatamide (9), and thiacetazone (10).

Thiobenzamide, a substrate for the enzyme to another metabolite. Chromatographic comparison of this second metabolite with synthesized standards identify it as 2-ethyl-4-amidopyridine (Fig. 4). This second metabolite is thus a product of the enzymatic action of EtaA on ETA-SO and not a branching product stemming from an alternative reaction with ETA. However, 4 is likely not the actual enzymatic product of EtaA and ETA-SO but rather is a more stable molecule resulting from decomposition of this putative cytotoxic metabolite, possibly a sulfenic acid species (Fig. 1, 2a). Since the final metabolite, 4, has no antitubercular activity, the key species is the reactive intermediate formed by EtaA that is the precursor of the amide.

Whole cell incubations of M. tuberculosis with radiolabeled ETA have indicated that ETA-SO and the nitrile (3) are initially formed and accumulate to a maximum concentration over a couple of hours (14). Following this early increase of ETA-SO and 3, their cellular concentrations begin to decrease, and increases in the amide (4) and alcohol (6) are observed (14). However, the final metabolites produced in the intact bacillus may arise both by alternative metabolic pathways acting on ETA and secondary metabolism of the products generated by EtaA. The link between the ETA-resistant tuberculosis and mutations in the Rv3855 and Rv3854c genes (14, 15) clearly indicates that the activation step is catalyzed by EtaA and therefore that the oxidation of ETA-SO catalyzed by this enzyme is critical for activation of the drug to its cytotoxic metabolite.

The natural substrate for the M. tuberculosis flavoprotein monoxygenase, ETA, is not known. As bacteria containing mutated, presumably inactive, forms of ETA are viable (14), the enzyme cannot be involved in the processing of an essential endogenous substrate. In agreement with the fact that patients infected with ETA-resistant M. tuberculosis strains present cross-resistance to thiacetazone (14), we have shown here that thiobenzamide (7) and isothiocyanatamide (9) are also substrates for EtaA. Thiobenzamide is oxidized by EtaA to thiobenzamide S-oxide (8) and benzamide. The oxidation of isothiocyanatamide (9) by EtaA generates two products, a minor product unambiguously identified as isonicotinamide by comparison with an authentic sample and a major product tentatively identified as the sulfoxide from its HPLC properties. Thus, EtaA, like the mammalian flavoprotein monooxygenases, exhibits a relatively broad substrate specificity with the primary constraint being that an appropriate oxidizable functionality be present.

Thiobenzamide, a substrate for the M. tuberculosis flavopro-
tein monoxygenase (see above), is one of a variety of related compounds that are known to be substrates for the mammalian flavoprotein monoxygenases (24–27). As shown here for the reaction with the mycobacterial enzyme, thiobenzamide is also oxidized to the corresponding S-oxide by the mammalian flavoprotein monoxygenases (17, 18). The thiobenzamide S-oxide is hepatotoxic in rats (24, 28), but the available evidence suggests that this hepatotoxicity requires further transformation of the S-oxide to a secondary reactive metabolite. Thus, rat liver microsomes oxidize thiobenzamide to the Soxide plus a small amount of benzamide (29). Under the same conditions, liver microsomes convert the thiobenzamide S-oxide exclusively to benzamide. This second reaction, which was proposed to involve conversion of the thiobenzamide S-oxide to the unstable S,S-dioxide, appears to also be mediated by the hepatic flavoprotein monoxygenase because it was inhibited by inhibitors of that enzyme (29). Model studies show that thiobenzamide is oxidized to the S-oxide and subsequently to the S,S-dioxide by H2O2 (30) or, much more rapidly, by a hydroperoxyflavin model of flavin monoxygenases (31). The second oxidation, which was much slower with H2O2 rapidly, by a hydroperoxyflavin model of flavin monooxygenases (17, 18), is catalyzed by a flavin monooxygenase (see above), which was much slower with H2O2 rapidly, by a hydroperoxyflavin model of flavin monooxygenases (17, 18). The thiobenzamide S-oxide is hepatotoxic in rats (24, 28), but the available evidence suggests that this hepatotoxicity requires further transformation of the S-oxide to a secondary reactive metabolite. Thus, rat liver microsomes oxidize thiobenzamide to the S-oxide plus a small amount of benzamide (29). Under the same conditions, liver microsomes convert the thiobenzamide S-oxide exclusively to benzamide. This second reaction, which was proposed to involve conversion of the thiobenzamide S-oxide to the unstable S,S-dioxide, appears to also be mediated by the hepatic flavoprotein monoxygenase because it was inhibited by inhibitors of that enzyme (29). Model studies show that thiobenzamide is oxidized to the S-oxide and subsequently to the S,S-dioxide by H2O2 (30) or, much more rapidly, by a hydroperoxyflavin model of flavin monoxygenases (31). The second oxidation, which was much slower with H2O2 than the first S-oxidation, produced the nitrite at pH >8, the benzamide at pH 4.0, and mixtures of the two products at intermediate pH values (30). Thus, EtaA, like the mammalian flavin monoxygenase, is capable of converting thiobenzamide to the corresponding S-oxide and of further oxidizing this intermediate to the amide.

In summary, Rv3854c codes for a flavoprotein containing a single FAD group that catalyzes the NADPH- and O2-dependent monooxygenation of ETA to the corresponding S-oxide. This sulfoxidation is a required step in the activation of ETA to the species directly responsible for the antimycobacterial activity of the drug. EtaA is also capable of further oxidizing ETA-SO to what is believed to be the final cytotoxic species. EtaA is also capable of further oxidizing this intermediate (25). We now plan to explore the further activation of ETA-SO to elucidate the nature of the putative reactive metabolite of ETA and to characterize the Rv3854c mutants known to convey resistance to ETA to evaluate their interaction with the drug. Elucidation of the role of the mutations will make possible a better understanding of the mechanism of action of flavoprotein encoded by Rv3854c.

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