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Bioactivation of Anti-Tuberculosis Thioamide and Thiourea Prodrugs by Bacterial and Mammalian Flavin Monooxygenases

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Abstract

The thioamide and thiourea class of antituberculosis agents encompasses prodrugs that are oxidatively converted to their active forms by the flavin monooxygenase EtaA of *Mycobacterium tuberculosis*. Reactive intermediates produced in the EtaA-catalyzed transformations of ethionamide and prothionamide result in NAD⁺/NADH adducts that inhibit the enoyl CoA reductase InhA, the ultimate target of these drugs. In the case of thiacetazone and isoxyl, EtaA produces electrophilic metabolites that mediate the antibacterial activity of these agents. The oxidation of the thioamide/thiourea drugs by the human flavin monooxygenases yields similar reactive metabolites that contribute to the toxicities associated with these second line antituberculosis drugs.

Keywords

antituberculosis drugs; drug metabolism; flavin monooxygenases; thiacetazone; ethionamide; isoxyl

1.1 Introduction

Tuberculosis infections are treated initially with a cocktail of drugs to prevent the development of drug resistance. The first line drugs normally employed in this cocktail for drug-sensitive tuberculosis are isoniazid (1952), ethambutol (1961), pyrazinamide (1952) and rifampin (1966) [1]. The dates in which these drugs were introduced into the clinic are shown in the parentheses. Second line drugs come into play when the infection is unresponsive to treatment with first line drugs, usually as a result of the development of resistance to one or more of the agents. Resistance is widespread to all the first line drugs and, indeed, to many of the second line drugs as well [2].

An important class of clinically employed second line drugs is composed of ethionamide (**1**, ETA), prothionamide (**2**, PTA), thiacetazone (**3**, TAZ) and isoxyl (**4**, ISO) (Fig. 1). The feature that distinguishes and classifies these drugs is the presence in each of a thioamide or thiourea function. All four of these drugs are pro-drugs in that they must be metabolically converted by mycobacterial enzymes to the active drugs. In this regard they are like isoniazid (INH), a drug of an entirely different class, which is also converted to its active

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form by an enzyme of *Mycobacterium tuberculosis*. However, the transformations that activate INH and the thioamide drugs differ, as do the enzymes that catalyze them.

1.2 Isoniazid (INH) Activation Paradigm

KatG, a catalase peroxidase of *Mycobacterium tuberculosis*, is the enzyme that converts INH into its active form. Close correlations exist between mutations in the gene coding for KatG that decrease the ability of the enzyme to activate INH and mycobacterial resistance to this drug [3]. Oxidative activation of INH produces the NADH adduct shown in Fig. 2A [4]. This adduct reversibly inhibits the enoyl CoA-reductase InhA and thereby interferes with biogenesis of the mycolic acids required for cell wall synthesis in *Mycobacterium tuberculosis*. The crystal structure of the NADH-INH adduct bound in the InhA active site shows that the adduct binds partially in the NADH binding site of the enzyme, blocking binding of this cofactor [4]. The activation of INH by KatG is thought to involve initial oxidation to the diazenyl radical that, upon loss of a molecule of nitrogen gas, produces a carbonyl free radical that adds to the NAD⁺ pyridinium ring (Fig. 3). It is unclear whether this occurs while NADH or NAD⁺ is bound to InhA or in solution. It is also possible that sites other than InhA are subject to attack by the INH radical or to inhibition by the INH-NADH adducts produced by KatG [5,6].

1.3 Ethionamide Activation

The critical clue to identification of the enzyme involved in activation of ETA and other thioamide/thiourea drugs was provided by the finding that EtaA-resistant *M. tuberculosis* strains had mutations in a regulatory factor EtaR, a repressor of the expression of a gene (Rv3854c) coding for a putative flavoprotein EtaA (7,8). These results led to the demonstration that EtaA is the enzyme responsible for activating ETA. Heterologous expression of EtaA yielded a protein that was confirmed to be a flavoprotein that readily oxidized ETA (9).

The oxidation of ETA by EtaA first yields the S-oxide (**5**) (8,9), a metabolite that is known to retain the full anti-tuberculosis activity of ETA (Fig. 4) (8). Further oxidation of the S-oxide then produces the unstable sulfinic acid **6** and, eventually, the 2-ethyl-4-carboxamidopyridine metabolite **7** that has no antimycobacterial activity [9,10]. Thus, the active metabolite of ETA lies between the S-oxide and 2-ethyl-4-carboxamidopyridine and is probably the sulfinic acid or a product of its decomposition to something other than 2-ethyl-4-carboxamidopyridine. The EtaA-dependent oxidation of ETA has been followed by magic angle spinning NMR in living mycobacterial cells [11,12]. These studies have provided evidence for *in situ* formation of the S-oxide and 4-pyridylmethanol metabolites, both of which were found primarily outside the cells, and the accumulation of an unidentified activated intermediate of ETA within the cells that did not appear to leak out of the cells very efficiently. The ionic properties of a sulfinic acid product would be consistent with these observations.

1.4 Site of Action of Ethionamide

The evidence suggests that the ultimate site of action of ethionamide is the same as that of INH. Gene array studies have shown that both INH and ETA induce similar patterns of protein expression [13], and mutations in the *InhA* gene give rise to resistance to both INH and ETA [14,15]. It has also been shown that activated ETH and PTA powerfully inhibit EtaA [16]. The basis for the inhibition of InhA by ETA and PTA was recently provided by the elegant demonstration that the activation of ETA and PTA, like that of INH, gives rise to ETA-NADH (Fig. 2B) and PTA-NADH adducts, respectively, that are very similar to the adduct obtained with INH [16]. The structures of the ETA-NADH and PTA-NADH adducts

bound in the InhA active site are similar to that of the INH-NADH adduct bound in the same site.

The mechanism that results in formation of the ETA and PTA adducts is less clear than that for formation of the INH adduct. As noted, the activated ETA molecule follows formation of the S-oxide and precedes hydrolysis of the putative sulfinic acid to give the amide. The sulfinic acid is a good leaving group and can be displaced by nucleophiles, as evidenced by hydrolysis to the amide, but NAD^+ is an electron deficient ring and is highly unlikely to act as a nucleophile. One possible solution to this problem is to propose that the sulfinic acid undergoes homolytic cleavage to give the carbon radical, as addition of this to the NAD^+ ring followed by hydrolysis of the imine function provides a ready route to the observed adduct. However, homolytic scission of the C-S bond in a sulfinic acid does not have great precedent.

1.5 Activation of TAZ

TAZ is also oxidized by EtaA [17], as might be expected from the clinical history of cross-resistance between ETA and TAZ [18,19] and the observation that TAZ resistance involves mutations in EtaA [7,8]. The oxidation of TAZ by recombinant, purified EtaA produces both the sulfenic (8) and sulfinic (9) acids, as well as carbodiimide 10, as the principal metabolites (Fig. 5) [10,17]. The sulfenic acid is the precursor of the latter two metabolites. At pH 7.4, the sulfinic acid was formed with a $K_m = 131 \pm 29 \mu\text{M}$ and $V_{\max} = 5.1 \pm 0.4 \text{ min}^{-1}$, and the carbodiimide slightly more slowly, with $K_m = 147 \pm 25 \mu\text{M}$ and $V_{\max} = 2.9 \pm 0.2 \text{ min}^{-1}$. Analysis of the pH dependence of the reaction showed that formation of the carbodiimide was favored at basic pH and formation of the sulfinic acid at neutral or acidic pH [17]. In the presence of glutathione, the carbodiimide, but not the sulfinic acid, reacted to give glutathione conjugate 11 (Fig. 5). However, in the presence of a large excess of glutathione from the beginning of the incubation, no detectable metabolites were formed at all, a finding consistent with competitive reduction by glutathione of the initial sulfenic acid intermediate back to the thioamide. The oxidation of TAZ thus produces at least two electrophilic metabolites that are chemically reactive and could account for both the antimycobacterial activity of the drug and its toxicity, depending on the site of formation of the metabolites and the nucleophiles with which they react. In this context it is worth noting that TAZ-NADH adducts analogous to those obtained with ETA and PTA are not formed [16], and that the action of TAZ, although still involving inhibition of mycolic acid biosynthesis, is not exerted at the level of the enoyl CoA reductase InhA [16,20].

1.6 Activation of Other Thioamide Drugs

EtaA appears to be involved in the activation not only of ETA, PTA, and TAZ, but more generally in the activation of the entire class of thioamide prodrugs. EtaA readily oxidizes thiobenzamide to the S-oxide and benzamide, and isothionicotinamide to a metabolite tentatively identified as the S-oxide derivative and isonicotinamide (Fig. 6) [9]. Although these compounds are not antituberculosis drugs, they illustrate the fact that the enzyme has a facility for oxidizing the thioamide group in small organic molecules. The oxidation of the drug isoxyl by EtaA to give an unidentified product has also been reported [20]. Subsequent work established that isoxyl was oxidized by partially purified EtaA to several products [21]. Mass spectrometric analysis suggested the presence of the sulfoxide, the desulfurated urea, and other less well-defined metabolites (Fig. 6C). The activation of isoxyl depended absolutely on activation by EtaA. Activated isoxyl reportedly acts primarily by inhibiting a Δ^9 -desaturase in *M. tuberculosis*, although it also exerts some inhibition of mycolic acid synthesis [22]. In sum, EtaA appears to play a general role in the oxidative metabolism of

thioamide and thiourea antituberculosis drugs, producing reactive intermediates whose final site of action depends on their individual structures.

1.7 Human FMO Enzymes and ETA/TAZ Oxidation

Flavin monooxygenases also oxidize thioamides [23]. For example, 1-phenylthiourea and α -naphthylthiourea are converted to their sulfenic acids that can be reduced back to the starting compounds by glutathione [24]. The consumption of GSH in such a futile cycle produces GSSG and can be the cause of oxidative stress. The reactions catalyzed by the human FMO enzymes are similar to those already discussed for TAZ. Thus, human FMO1, FMO2.1 (an allele of FMO2), and FMO3 have been shown to oxidize both ETA and TAZ to the same sulfenic acid, sulfinic acid, and carbodiimide metabolites produced by *M. tuberculosis* EtaA [10,17]. Determination of the kinetic parameters for the oxidation of TAZ by FMO1, FMO2.1, and FMO3 and EtaA under the same set of conditions reveals that the K_m values for all four enzymes are comparable, but k_{cat} for FMO2.1-catalyzed TAZ oxygenation is much higher than those for FMO1, FMO3, or EtaA (Table 1). FMO2.1, a protein predominantly expressed in the lung, thus catalyzes the oxidation of ETA and TAZ more effectively and will contribute significantly to metabolism of the drug in the lungs [10]. As Europeans and Asians lack FMO2.1, but a substantial fraction of the population in sub-Saharan Africa expresses FMO2.1, interindividual differences in response to TAZ and ETA may arise due to more rapid clearance of the drug in the population expressing FMO2.1.

1.8 Conclusions

The oxidation of thioamide and thiourea groups in both model compounds and antituberculosis drugs produces sulfoxides that, as the result of a second oxidative cycle, are converted to sulfinic acids. The electrophilic sulfinic acids can decompose, possibly to carbon radicals by C-S bond scission, or in thioureas can undergo elimination to produce a carbodiimide. These reactive species in some instances add NAD^+ to generate adducts that reversibly inhibit cell wall synthesis in *Mycobacterium tuberculosis*. Alternatively, they may serve as electrophilic agents that modify proteins in either the mycobacterium or, if produced by host FMO enzymes, in the host tissue. A further complication is that the initial thioamide sulfoxide can be recycled back to the thioamide by glutathione, but at the expense of the consumption of glutathione in a futile cycle that may lead to oxidative stress.

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Abbreviations

ETA	ethionamide
FMO	flavin-containing monooxygenase
GSH	glutathione
INH	isoniazid
ISO	isoxyl
PTA	prothionamide
TAZ	thiacetazone

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Fig. 1.
Structures of the thioamide/thiourea class of antituberculosis drugs.

Fig. 2. Structures of the INH-NADH and ETA-NADH adducts formed in the activation of (A) INH by KatG and (B) ETA by EtaA. Stereoisomers are possible due to the chiral center in the dihydropyridine ring.

Fig. 3.
Schematic mechanism postulated for the activation of INH to an acyl free radical that adds to the pyridinium ring of NAD to give the INH-NADH adduct. A part of the NADH structure is represented by R.

Fig. 4.

Schematic mechanism postulated for the activation of ETA to a reactive species, possibly the acyl or imminium radical, that adds to the pyridinium ring of NAD⁺ to give the INH-NADH adduct. Hydrolysis of the imine would yield the carbonyl group in the final adduct. A part of the NADH structure is represented by R.

Fig. 5. Scheme showing the oxidative metabolism of TAZ by EtaA. Glutathione (GSH) can trap the carbodiimide metabolite and, at higher concentrations, can reduce the S-oxide.

Fig. 6.
Oxidation of (A) thiobenzamide, (B) isothionicotinamide, and (C) isoxyl by EtaA.

Table 1

Kinetic parameters for the oxidation of TAZ by human FMO1, FMO2.1, FMO3, and EtaA [10]

Enzyme	K_m μM	k_{cat} min^{-1}	k_{cat}/K_m $\text{min}^{-1}\text{M}^{-1} (\times 10^3)$
FMO1	6.30 ± 0.80	5.08 ± 0.46	7.94 ± 1.99
FMO2.1	5.80 ± 0.55	80.10 ± 4.42	142.56 ± 18.30
FMO3	7.01 ± 0.53	1.37 ± 0.20	1.96 ± 0.39
EtaA	9.05 ± 0.57	3.02 ± 0.30	3.29 ± 0.81