Redox Regulation of the Human Xenobiotic Metabolizing Enzyme Arylamine N-Acetyltransferase 1 (NAT1)

REVERSIBLE INACTIVATION BY HYDROGEN PEROXIDE*

Received for publication, April 11, 2003, and in revised form, June 26, 2003
Published, JBC Papers in Press, June 27, 2003, DOI 10.1074/jbc.M303813200

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Oxidative stress is increasingly recognized as a key mechanism in the biotransformation and/or toxicity of many xenobiotics. Human arylamine N-acetyltransferase 1 (NAT1) is a polymorphic ubiquitous phase II xenobiotic metabolizing enzyme that catalyzes the biotransformation of primary aromatic amine or hydrazine drugs and carcinogens. Functional and structural studies have shown that NAT1 catalytic activity is based on a cysteine protease-like catalytic triad, containing a reactive cysteine residue. Reactive protein cysteine residues are highly susceptible to oxidation by hydrogen peroxide (H₂O₂) generated within the cell. We, therefore, investigated whether human NAT1 activity was regulated by this cellular oxidant. Using purified recombinant NAT1, we show here that NAT1 is rapidly (k_{inact} = 420 M^{-1} min^{-1}) inactivated by physiological concentrations of H₂O₂. Reducing agents, such as reduced glutathione (GSH), reverse the H₂O₂-dependent inactivation of NAT1. Kinetic analysis and protection experiments with acetyl-CoA, the physiological acetyl-donor substrate of the enzyme, suggested that the H₂O₂-dependent inactivation reaction targets the active-site cysteine residue. Finally, we show that the reversible inactivation of NAT1 by H₂O₂ is due to the formation of a stable sulfenic acid group at the active-site cysteine. Our results suggest that, in addition to known genetically controlled interindividual variations in NAT1 activity, oxidative stress and cellular redox status may also regulate NAT1 activity. This may have important consequences with regard to drug biotransformation and cancer risk.

All organisms respond to harmful stressors, whether of endogenous or environmental origin (e.g. cellular by-products or chemical xenobiotics), by producing stress-related proteins including heat-shock proteins, antioxidant proteins and xenobiotic metabolizing enzymes (XME)1 (1). The acetyl-CoA:arylamino-N-acetyltransferases (NATs; EC 2.3.1.5) are phase II XME that catalyze the transfer of an acetyl moiety from acetyl-CoA to the nitrogen or oxygen atom of primary amines, hydrazines, and their N-hydroxylated metabolites (2). NATs, therefore, play an important role in the detoxification and/or activation of substrates, including arylamine drugs and carcinogens (3,4). NAT enzymes have been identified in several species (5–8). In humans, two functional isoforms of NATs (NAT1 and NAT2) have been described (9). Interindividual genetic variations in their genes have been shown to cause differences in NAT1 and NAT2 protein levels and activity. These variations are a potential source of pharmacological and/or pathological susceptibility (4,10,11). Although the human NAT1 and NAT2 protein sequences are 81% identical (12), their kinetic selectivity for amine-containing acceptor substrates differs markedly (13). The tissue distributions of these two enzymes also differ, with NAT2 present principally in the liver and intestinal epithelium and NAT1 being ubiquitous (2,11). Elucidation of the crystal structures of NATs from Salmonella typhimurium and Mycobacterium smegmatis and homology models of the two human NATs have revealed structural similarity to cysteine proteases and the existence of a conserved cysteine protease-like catalytic triad (Cys-His-Asp) in the catalytic core of NATs (14–17). These structural data show that vertebrate and eubacterial NATs have adapted a catalytic mechanism commonly found in cysteine proteases for use in acetyl-transfer reactions (2,14,15).

Redox-dependent regulation of catalytic activities by reversible oxidation of an active-site cysteine residue has been reported for several enzymes, including protein phosphatases (18–21) and cysteine proteases (22–24). H₂O₂ is one of the oxidants that has been shown to regulate cell function, by oxidizing active cysteine residues in proteins to cysteine sulfinic acid or to disulfide (20,25–27). In vivo, H₂O₂ formation is mainly due to enzymatic reactions such as the superoxide dismutase-dependent dismutation of superoxide (26,28,29). Although superoxide can oxidize proteins (30), H₂O₂, its main product, has appeared as a critical element involved in many cellular functions, including cell signaling (20,27,31). In addition, substantial increases in the intracellular concentration of H₂O₂ are generally associated with deleterious conditions such as apoptosis, necrosis, inflammation, and cancer (20,26,27,31). The induction of H₂O₂ production by xenobiotics has also been described, with potential effects on cysteine proteases [e.g. calpain (22)].

NAT1, human arylamine N-acetyltransferase 1; PAS, p-aminosalicylic acid; PNPA, p-nitrophenylacetate; AcCoA, acetyl-coenzyme A; DTT, dithiothreitol; GST, glutathione S-transferase; PTP, protein-tyrosine phosphatase.

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Redox Regulation of Human NAT1

Materials—p-Aminosalicylic acid (PAS, a NAT1-selective arylamine acceptor substrate), n-propenylacetamide (PAA, a NAT1 acceptor substrate), acetyl-CoA, CoA, hydrogen peroxide (H₂O₂), 5,5-dimethyl-1,3-cyclohexanedione (dimedone), 1,4-dithiothreitol (DTT), reduced glutathione (GSH), imidazole, lysozyme and glutathione-agarose, bovine catalase (5200 units/mL), glucose oxidase (type II from Aspergillus niger, 15,000 units/g), and β-glucose were purchased from Sigma. pEKE-2T vector and Escherichia coli BL21(DE3) cells were supplied by Amaresh Biosciences. The pET28 vector was obtained from Novagen. Nickel-nitrilotriacetic acid superflow resin was purchased from Qiagen. Anti-fluorescein Fab’ fragments conjugated to peroxidase, fluorescein-conjugated iodocetamide, and complete protease inhibitor tablets were obtained from Roche Applied Science. All other reagents were obtained from Sigma or Eurobio (Les Ulis, France). The Bradford protein assay kit was purchased from Bio-Rad.

Expression and Purification of Recombinant Human NAT1—The human NAT1 cDNA, kindly provided by Dr. D. Grant (Toronto, Canada), was subcloned into pEKE-2T and pET28 vectors. The resulting constructs encoded the enzyme as a glutathione S-transferase fusion protein (GST-NAT1) and as a polyhistidine-tagged fusion protein (His-NAT1), respectively. We transformed BL21(DE3) bacteria with these constructs, expressing the human NAT1 cDNA, kindly provided by Dr. D. Grant (Toronto, Canada). We transformed BL21(DE3) bacteria with these constructs, expressing the human NAT1 cDNA, kindly provided by Dr. D. Grant (Toronto, Canada). The recombinant-tagged NAT1 enzyme was expressed in Escherichia coli and purified by a combination of ammonium sulfate precipitation, ion exchange chromatography, and affinity chromatography. The purified enzyme was then dialyzed against 25 mM Tris-HCl, pH 7.5, 1 mM EDTA.

Enzyme Assays—NAT1 enzyme activity was determined spectrophotometrically (410 nm), using PNPA as the acetyl donor and PAS as the NAT1-specific arylamine substrate, as described by Mushtaq et al. (43). Briefly, treated or non-treated samples (10–20 µl) containing NAT1 were assayed in a reaction mixture containing 500 mM PNPA (final concentration) in 25 mM Tris-HCl, pH 7.5, 1 mM EDTA. Reactions were started by adding 125 µM PNPA (final concentration). In all reaction mixtures (total volume of 1 ml), the final concentration of NAT1 was 15 nM. The reaction mixture was incubated for 10 min incubation at 37 °C, and the reaction was then quenched by adding SDS (1% final concentration). β-Nitrophenol, generated by the NAT1-mediated hydrolysis of PNPA in the presence of PAS, was quantified by measuring absorbance at 410 nm with an enzyme-linked immunosorbent assay plate reader (Metertech). One unit of enzyme was defined as the amount of enzyme giving an A410 of 0.5 per 10 min per ml. For the controls, we omitted the enzyme, PNPA, or PAS. All enzyme assays were performed in quadruplicate, in conditions in which the initial rates were linear. Enzyme activities are shown as percentages of control NAT1 activity.

Redox Regulation of Human NAT1 with H₂O₂ in the Presence or Absence of Other Compounds—Concentrations of stock H₂O₂ solutions were determined by measuring absorbance at 240 nm (εH₂O₂ = 44 m M⁻¹cm⁻¹). In all subsequent experiments, the final concentration of enzyme during the oxidation step with H₂O₂ was 1.5 µM, giving a final concentration in the enzyme assay of 15 nM. The total volume of the enzyme assay (1 ml) provided a great enough dilution (1:50 or 1:100) of the various compounds used to prevent these compounds from interfering with NAT1 enzyme activity measurements. NAT1 activity in the absence of H₂O₂ was used as 100% activity.

We assessed the effect of bolus addition of H₂O₂ on NAT1 enzyme activity by incubating purified NAT1 samples (1.5 µM final concentration) with various concentrations of H₂O₂ in 25 mM Tris-HCl, pH 7.5, 1 mM EDTA (total volume of 10 µl) for 10 min at 37 °C. Mixtures were then assayed for NAT1 activity, as described above.

In reactivation experiments, NAT1 (1.5 µM final concentration) was first oxidized by H₂O₂ (200 µM final concentration), as described above, and incubated with catalase (300 unit/ml) for 1 min at 37 °C. The mixture was then incubated for 10 min at 37 °C with various concentrations of DTT or GSH in a total volume of 20 µl. A NAT1 enzyme assay was then carried out. Assays performed in this condition (with catalase) but without H₂O₂ gave 100% NAT1 activity.

In substrate protection experiments, NAT1 (1.5 µM final concentration) was first incubated with various concentrations of AcCoA or CoA (final concentrations of 0.4–4 mM) in 25 mM Tris-HCl, pH 7.5, 1 mM EDTA (total volume of 10 µl) for 5 min at 37 °C. Samples were then incubated with H₂O₂ (200 µM final concentration) in a total volume of 20 µl, as described above, and assayed. Assays performed in these conditions with AcCoA or CoA alone gave 100% NAT1 activity.

In dimeric assays, NAT1 (1.5 µM final concentration) was first incubated with H₂O₂ (200 µM final concentration) in the presence of 10 mM dimedone (a specific reagent of sulfenic acid) in 25 mM Tris-HCl, pH 7.5, 1 mM EDTA (10 µl total volume) for 10 min at 37 °C. It was then incubated with H₂O₂ (200 µM final concentration) in a total volume of 10 µl for 10 min at 37 °C. Residual NAT1 activity was assayed as described above. Assays performed in these conditions, with dimerone alone (10 mM final concentration) gave 100% NAT1 activity.

For the kinetic analysis of H₂O₂-dependent NAT1 inactivation, NAT1 (1.5 µM final concentration) was incubated with H₂O₂ (final concentration of 100–400 µM) in 25 mM Tris-HCl, pH 7.5, 1 mM EDTA at various time intervals, aliquots were removed and assayed for residual activity. The equation for the rate of inactivation of recombinant NAT1 by H₂O₂ can be represented as: d[NAT1]/dt = k inact[NAT1] [H₂O₂], where [NAT1] is the concentration of active enzyme, and k inact is the second-order rate constant. Provided that H₂O₂ is present in substantial excess to NAT1, the apparent first-order inactivation rate constant (k inact = k inact[H₂O₂]) can be calculated for each H₂O₂ concentration from the slope of the natural log (ln) of percent residual activity plotted against time. The second-order rate constant was determined from the slope of k inact plotted against H₂O₂ concentrations.
In Vitro Inactivation of NAT1 by Bolus Addition of H$_2$O$_2$ and by Continuous Generation of H$_2$O$_2$ by the Glucose/Glucose Oxidase System—Several enzymes with catalytic cysteine residues have been shown to be reversibly inactivated by H$_2$O$_2$, a major physiological oxidant (18, 20, 23). Given the reactive nature of the active site cysteine residue of NAT enzymes (47–49), we reasoned that human NAT1 could be a potential target for reversible inactivation by H$_2$O$_2$. We, therefore, produced the human NAT1 enzyme in E. coli and purified it as a fully active GST- or His-tagged protein. Recombinant NAT1 was then used to investigate the effect of physiologically relevant concentrations of H$_2$O$_2$ (26, 45, 50) on NAT1. To this end, we used an approach similar to the one of Lee et al. (20) by assessing the effect on NAT1 of the bolus addition of H$_2$O$_2$ and of the exposure of NAT1 to continuous levels of H$_2$O$_2$ generated by an enzymatic system.

First, reduced NAT1 enzyme (1.5 μM final concentration) was incubated with various concentrations of H$_2$O$_2$ (5–200 μM final concentration) and residual NAT1 activity was measured. NAT1 activity was significantly inhibited by H$_2$O$_2$ in a dose-dependent manner (Fig. 1A). At a concentration of 200 μM, H$_2$O$_2$ inhibited the enzyme by over 90%. An IC$_{50}$ of 45 μM was obtained for an enzyme concentration of 1.5 μM. To make a more realistic and physiologic assessment of the effect of H$_2$O$_2$ on NAT1 activity, we used another source of H$_2$O$_2$, the steady conversion of β-D-glucose to D-gluconolactone and H$_2$O$_2$, which is catalyzed by glucose oxidase (45). As shown in Fig. 1B (filled circles), NAT1 (1.5 μM final) was also inactivated by the constant production of H$_2$O$_2$ through the glucose/glucose oxidase system. In the conditions used, the constant production rate of H$_2$O$_2$ was estimated to be ~6 μM H$_2$O$_2$/min, which is physiologically relevant (45, 51). After a 15-min incubation, residual NAT1 activity was close to 30% of the control. After a 30-min exposure to H$_2$O$_2$, the residual activity was less than 10%. No inactivation of NAT1 by H$_2$O$_2$ generated by the glucose/glucose oxidase system was observed in presence of catalase (300 units/ml) (open circles). Thus, these experiments suggest that NAT1 enzyme is inactivated by bolus addition and constant generation of physiologically relevant levels of H$_2$O$_2$. Detection of H$_2$O$_2$-oxidized Cysteine Residues by Labeling with Fluorescein-conjugated Iodoacetamide—We investigated whether NAT1 contained reactive cysteine residues susceptible to oxidation by physiological concentrations of H$_2$O$_2$, using an approach based on the labeling of cysteine with 5-fluoroacetamidofluorescein (25). Incubation of NAT1 with various concentrations of H$_2$O$_2$ resulted in the dose-dependent modification of cysteine residues, as indicated by the disappearance of...
fluorescein-conjugated iodoacetamide labeling (Fig. 2). Thus, NAT1 cysteine residues are modified by H$_2$O$_2$.

Reactivation of H$_2$O$_2$-inactivated NAT1 by Thiol-reducing Agents—We investigated whether the H$_2$O$_2$-dependent inactivation of NAT1 could be reversed by thiol-reducing agents, as reported for other enzymes (18–20, 23). NAT1 (1.5 μM final concentration) was first inactivated by incubation with H$_2$O$_2$ (200 μM final concentration), and excess H$_2$O$_2$ was removed by catalase (300 units/ml). Inactivated NAT1 was then incubated with DTT or GSH, at various concentrations (1, 5, or 10 mM final concentration), and NAT1 activity was determined (Fig. 3). Both DTT and GSH reactivated H$_2$O$_2$-inactivated NAT1 (Fig. 3). At a concentration of 5 mM DTT, the H$_2$O$_2$-dependent inactivation of NAT1 was completely reversed. In contrast, 5 mM GSH gave ~60% of the NAT1 control activity. A final concentration of 10 mM GSH was able to recover ~100% of the original NAT1 activity. Thus, the H$_2$O$_2$-dependent inactivation of NAT1 is reversible. Our results also suggest that the H$_2$O$_2$-inactivated NAT1 enzyme could be reactivated by GSH.

Inhibition Kinetics and Stoichiometry—We carried out kinetic analysis of the H$_2$O$_2$-dependent inactivation of NAT1 in the presence or absence of various concentrations of H$_2$O$_2$. Semilogarithmic plots of percent residual activity versus time for various concentrations of H$_2$O$_2$ gave straight lines, indicating that inactivation obeyed apparent first-order reaction (Fig. 4A). Replotting the observed pseudo-first-order rate constants (k_{obs}) against H$_2$O$_2$ concentrations gave a straight line that passed very close to the origin (Fig. 4B), consistent with a single-step reaction in which the reverse rate (obtained from B) passed very close to the origin (Fig. 4). The slope was n = 0.87, indicating that the inactivation of NAT1 by H$_2$O$_2$ involved 1:1 stoichiometry. Thus, the oxidative inactivation of NAT1 by H$_2$O$_2$ is a rapid bimolecular process in which one molecule of H$_2$O$_2$ modifies the catalytic cysteine residue, leading to inactivation of the enzyme.

Identification of the Site Modified by H$_2$O$_2$ during NAT1 Inactivation—We then investigated whether the active-site cysteine of NAT1 was the target of H$_2$O$_2$-dependent oxidative inactivation. We included AcCoA (physiological acetyl-donor substrate of NAT1), which forms a covalent acetyl-enzyme intermediate (49), in the reaction to provide protection from inactivation by H$_2$O$_2$. CoA, a product of AcCoA hydrolysis that does not form an acetyl-enzyme intermediate, was used as a control. AcCoA conferred dose-dependent protection (up to 64) of NAT1 from H$_2$O$_2$-induced inactivation, whereas CoA did not (Table I). Of the five cysteine residues present in NAT1, only the active site cysteine has been shown to be conserved in all known NAT sequences and to be critical for enzyme function (5, 7, 48). Although we cannot rule out the possibility that H$_2$O$_2$ modifies other cysteine residues of NAT1, these results clearly demonstrate that the catalytic active site cysteine residue of NAT1 is a target of H$_2$O$_2$-dependent oxidative modification, leading to reversible inactivation of the enzyme.

Determination of the Chemical Nature of the H$_2$O$_2$-oxidized Active-site Cysteine of NAT1—The results reported above led us to investigate the chemical nature of the H$_2$O$_2$-modified catalytic cysteine of the NAT1 enzyme. As H$_2$O$_2$-dependent oxidative inactivation of NAT1 was fully reversible, the oxidized active site cysteine was unlikely to be in the form of a sulfenic (–SOH) or sulfonic acid (–SO$_3$H), neither of which could be reduced by thiol-reducing agents (27, 40). There were two other possibilities. The active-site cysteine residue of the H$_2$O$_2$-inactivated enzyme may be involved in an inter- or intramolecular disulfide bond or form a stable cysteine sulfenic acid (–SOH), any of which could be reduced to give cysteine. Inter- and intramolecular disulfide bonds were ruled out on the basis of electrophoretic mobility shift assays (54). In addition, the cysteine sulfenic acid of human NAT1 has been predicted to be at the base of the active-site pocket and inaccessible to other cysteine residues within the same molecule (15). Dime done has been shown to react specifically with sulfenic acids to form a stable thioether product that cannot be reduced by thiol-reducing agents (22). This compound has been shown to identify cysteine sulfenic acids at the active sites of various enzymes (22, 55, 56). We investigated whether the H$_2$O$_2$-dependent inactivation of NAT1 resulted from the formation of a stable sulfenic acid at the active site cysteine by incubating dimedone (10 mM final concentration) with H$_2$O$_2$-oxidized NAT1 (Fig. 5). The control, in which NAT1 was incubated with 10 mM dimedone alone, showed no inhibition of activity (data not shown). The enzymatic activity of the NAT1 sample treated with H$_2$O$_2$ alone was fully restored by incubation with 5 mM DTT (final concentration). In contrast, NAT1 cotreated with H$_2$O$_2$ and dimedone was only partially reactivated (63% of
We provide here chemical and kinetic evidence to support that human NAT1 activity is regulated by H$_2$O$_2$. Human NAT1 activity was significantly inactivated by both the bolus addition and the constant generation of physiologically relevant levels of H$_2$O$_2$ as reported, in similar conditions, for certain protein-tyrosine phosphatases (PTPs), in particular the isofrom 1B (PTP1B) (18, 20, 57). Indeed, in experimental conditions similar to ours (similar enzyme and H$_2$O$_2$ concentrations, incubation times), IC$_{50}$ values ranging from 60 to 100 µM were reported for inactivation of these PTPs by H$_2$O$_2$ (18, 20, 57). These values are very close to the IC$_{50}$ determined for the inactivation of NAT1 by H$_2$O$_2$ (IC$_{50}$ = 45 µM). Moreover, kinetic analysis showed the rapid oxidation of NAT1 by H$_2$O$_2$ with a second-order rate constant ($k_{\text{inact}}$) for enzyme inactivation of 420 M$^{-1}$min$^{-1}$ (Fig. 5). Interestingly, this value is, again, very close to the $k_{\text{inact}}$ constants ($\approx$600 M$^{-1}$min$^{-1}$) reported for the PTPs mentioned above, the activities of which are regulated in vivo by H$_2$O$_2$ (18, 19, 57). Similar $k_{\text{inact}}$ values were also obtained for caspase 3, a redox-regulated cysteine protease involved in apoptosis (23, 24).

The inactivation of NAT1 by H$_2$O$_2$ was fully reversed by the non physiological thiol reductant DTT and by physiological concentrations of GSH, showing that H$_2$O$_2$-dependent inactivation of NAT1 is reversible. In contrast, physiological concentrations (up to 10 mM) of oxidized glutathione (GSSG) had no effect on NAT1 activity, suggesting that GSSG is unlikely to regulate NAT1 activity in vivo. GSH, a cellular reductant, is

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**DISCUSSION**

H$_2$O$_2$ has been shown to inactivate reversibly reactive cysteine-containing enzymes, such as phosphatases, both in vitro and in vivo (20).

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**FIG. 4. Kinetic analysis of H$_2$O$_2$-induced inactivation of NAT1.** A, NAT1 (1.5 µM) was treated with H$_2$O$_2$ (final concentrations of 100–400 µM) at 37 °C in 25 mM Tris-HCl, pH 7.5, 1 mM EDTA. After various times, aliquots were removed and assayed for residual activity. Plots of the ln of percent residual activity versus time are shown. The apparent first-order inactivation constants ($k_{\text{obs}}$) are calculated from the linear regressions ($r^2 > 0.97$). •, control (no H$_2$O$_2$); ▲, 100 µM; ■, 200 µM; ○, 400 µM. Error bars indicate S.D. values. B, determination of second-order rate constant ($k_{\text{inact}}$) by plotting the apparent first-order inactivation constant ($k_{\text{obs}}$) values versus the H$_2$O$_2$ concentrations (correlation factor $r^2 = 0.99$). Error bars indicate S.D. values. C, determination of reaction order by replotting the ln of $k_{\text{obs}}$ versus the ln of H$_2$O$_2$ concentration (correlation factor $r^2 = 0.99$). Error bars indicate S.D. values.

**TABLE 1**

<table>
<thead>
<tr>
<th>Conditions</th>
<th>% of protection</th>
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<tbody>
<tr>
<td>NAT1 + AcCoA (0.2 mM) + H$_2$O$_2$</td>
<td>17 ± 3$^a$</td>
</tr>
<tr>
<td>NAT1 + AcCoA (0.5 mM) + H$_2$O$_2$</td>
<td>30 ± 5$^a$</td>
</tr>
<tr>
<td>NAT1 + AcCoA (1 mM) + H$_2$O$_2$</td>
<td>46 ± 3$^a$</td>
</tr>
<tr>
<td>NAT1 + AcCoA (2 mM) + H$_2$O$_2$</td>
<td>64 ± 6$^a$</td>
</tr>
<tr>
<td>NAT1 + CoA (0.2 mM) + H$_2$O$_2$</td>
<td>9 ± 4</td>
</tr>
<tr>
<td>NAT1 + CoA (0.5 mM) + H$_2$O$_2$</td>
<td>13 ± 3</td>
</tr>
<tr>
<td>NAT1 + CoA (1 mM) + H$_2$O$_2$</td>
<td>13 ± 6</td>
</tr>
<tr>
<td>NAT1 + CoA (2 mM) + H$_2$O$_2$</td>
<td>13 ± 8</td>
</tr>
</tbody>
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$^a$ p < 0.01 versus H$_2$O$_2$-only treated NAT1.
the major determinant of cellular redox potential, with a concentration of 1–10 mM (52, 53). Thus, our results suggest that GSH could reactivate H2O2-inactivated NAT1 in the cell. GSH levels can be decreased by oxidative stress, in particular by xenobioto-induced oxidative stress, with potential effects on the biotransformation activity of the phase II XME glutathione S-transferase (35).

Our findings are consistent with the specific oxidation of cysteine or methionine residues. Kinetic analysis (Fig. 5C) and protection experiments with AcCoA clearly showed that the essential active-site cysteine of NAT1 was the specific target of H2O2-dependent inactivation and that cysteine thiolate was the reactive species. Thiolates are more susceptible to oxidation by H2O2 in cells than other protein cysteine residues as they are intrinsically stronger nucleophiles (19, 20). Enzymes that contain an essential thiolate in their active site are widely accepted to be potential candidates for reversible oxidation by H2O2 generated within the cell (20, 25, 31). It was then incubated (10 min at 37 °C) in the presence or absence of dithioreitol (10 mM final concentration). Mixtures were then incubated with 5 mM DTT (final concentration) for 10 min at 37 °C, and NAT1 activity was then determined. The values shown are means of three independent experiments in which each treatment was performed in quadruplicate. Error bars indicate S.D. values. Results are presented as percent control NAT1 activity. **, p < 0.01 versus H2O2-inactivated NAT1. ##, p < 0.01 versus H2O2-inactivated NAT1 in the presence of DTT (H2O2/DTT).

The formation of stable sulfenic acid or disulfide, which can be reduced back to cysteine or methionine residues. Kinetic analysis (Fig. 5C) and protection experiments with AcCoA clearly showed that the essential active-site cysteine of NAT1 was the specific target of H2O2-dependent inactivation and that cysteine thiolate was the reactive species. Thiolates are more susceptible to oxidation by H2O2 in cells than other protein cysteine residues as they are intrinsically stronger nucleophiles (19, 20). Enzymes that contain an essential thiolate in their active site are widely accepted to be potential candidates for reversible oxidation by H2O2 generated within the cell (20, 25, 31). It was then incubated (10 min at 37 °C) in the presence or absence of dithioreitol (10 mM final concentration). Mixtures were then incubated with 5 mM DTT (final concentration) for 10 min at 37 °C, and NAT1 activity was then determined. The values shown are means of three independent experiments in which each treatment was performed in quadruplicate. Error bars indicate S.D. values. Results are presented as percent control NAT1 activity. **, p < 0.01 versus H2O2-inactivated NAT1. ##, p < 0.01 versus H2O2-inactivated NAT1 in the presence of DTT (H2O2/DTT).

Fig. 5. Effect of dithiobenzoate on the DTT-dependent reactivation of H2O2-oxidized NAT1. NAT1 (1.5 μM) was incubated with H2O2 (200 μM final concentration) in 25 mM Tris-HCl, pH 7.5, 1 mM EDTA for 10 min at 37 °C. It was then incubated (10 min at 37 °C) in the presence or absence of dithioreitol (10 mM final concentration). Mixtures were then incubated with 5 mM DTT (final concentration) for 10 min at 37 °C, and NAT1 activity was then determined. The values shown are means of three independent experiments in which each treatment was performed in quadruplicate. Error bars indicate S.D. values. Results are presented as percent control NAT1 activity. **, p < 0.01 versus H2O2-inactivated NAT1. ##, p < 0.01 versus H2O2-inactivated NAT1 in the presence of DTT (H2O2/DTT).

Redox Regulation of Human NAT1

The stability of Cys-SOH in proteins depends mainly on the presence of an apolar microenvironment around the Cys-SOH and the absence of proximal cysteine residues (39). Interestingly, the active-site pocket of NAT enzymes, and more specifically human NAT1 and NAT2, has been reported to be apolar with no other cysteine residue being proximal to the catalytic cysteine of these enzymes (2, 8, 15, 16, 59). Thus, the formation of a stable sulfenic acid at the active-site cysteine residue seems to be a plausible mechanism for the reversible inactivation of human NAT1 by H2O2. Conversely, formation of an inter- or intramolecular disulfide formation similar to that observed in some H2O2-regulated proteins (18, 60) is unlikely. First, in NAT family, only the catalytic cysteine is absolutely conserved. Second, the catalytic cysteine residue of human NAT1 is buried at the base of the active-site pocket with no other proximal cysteine residue (15). Third, no electrophototrophic mobility shifts were observed between reduced and H2O2-oxidized NAT1 (data not shown). Finally, dimedone experiments demonstrated the presence of a stable sulfenic acid after H2O2 inactivation.

Our results suggest that human NAT1 could be reversibly inactivated in vitro by H2O2, as shown for other enzymes such as PTPs. Given the importance of oxidative stress in the biotransformation and/or toxicity of many xenobiotics, the inactivation of human NAT1 by H2O2 may be of physiological significance. Our data suggest that, in addition to polymorphic variation of the NAT1 gene (61), redox conditions could regulate NAT1 functional activity. This supports recent reports (62, 63) suggesting that non-genetic factors, such as substrate-dependent inhibition, may also contribute to overall NAT1 activity.

Acknowledgments—We thank Dr. Sebastian Mueller (University of Heidelberg) for helpful discussions in particular concerning the glucose/glucose oxidase assay.

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J. Biol. Chem. 2003, 278:35086-35092. doi: 10.1074/jbc.M303813200 originally published online June 27, 2003

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