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

REVERSIBLE INACTIVATION BY HYDROGEN PEROXIDE*

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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 (\(H_2O_2\)) 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 \((k_{\text{inact}} = 420 \text{ min}^{-1})\) inactivated by physiological concentrations of \(H_2O_2\). Reducing agents, such as reduced glutathione (GSH), reverse the \(H_2O_2\)-dependent inactivation of NAT1. Kinetic analysis and protection experiments with acetyl-CoA, the physiological acetyl-donor substrate of the enzyme, suggested that the \(H_2O_2\)-dependent inactivation reaction targets the active-site cysteine residue. Finally, we show that the reversible inactivation of NAT1 by \(H_2O_2\) 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:arylamine 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). \(H_2O_2\) is one of the oxidants that has been shown to regulate cell function, by oxidizing active cysteine residues in proteins to cysteine sulfenic acid or to disulfide (20, 25–27). In vivo, \(H_2O_2\) 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), \(H_2O_2\), its main product, has appeared as a critical element involved in many cellular functions, including cell signaling (20, 27, 28, 31). In addition, substantial increases in the intracellular concentration of \(H_2O_2\) are generally associated with deleterious conditions such as apoptosis, necrosis, inflammation, and cancer (20, 26, 27, 31). The induction of \(H_2O_2\) production by xenobiotics has also been described, with potential effects on cysteine proteases.

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\†† The abbreviations used are: XME, xenobiotic metabolizing enzyme; PNPA, \(p\)-nitrophenylacetate; AcCoA, acetyl-coenzyme A; DTT, dithiothreitol; GST, glutathione \(S\)-transferase; PTP, protein-tyrosine phosphatase.

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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.
Redox Regulation of Human NAT1

We investigated here whether the catalytic activity of human NAT1 was regulated by H2O2. We found that NAT1 activity was reversibly inhibited by physiologically relevant concentrations of H2O2. We demonstrate that H2O2 oxidized rapidly and irreversibly via the Fe(II)/S→Fe(III)/S pathway, leading to oxidative inactivation of the enzyme. This H2O2-dependent inactivation of NAT1 probably results from oxidative modifications of the essential catalytic cysteine residues of the enzyme, as shown for other enzymes (39–41). The inactivated NAT1 was reactivated by physiological thiols, such as reduced glutathione. Our results suggest that in addition to the polymorphic-dependent variation of NAT1 activity, H2O2 and, more broadly, cellular redox status, could also regulate NAT1 activity, which may have important consequences with regard to drug toxicity and cancer risk.

EXPERIMENTAL PROCEDURES

Materials—p-Aminosalicylic acid (PAS, a NAT1-selective arylamine acceptor substrate), p-nitrophenylacetate (PNPA, an acceptor substrate), acetyl-CoA, acetyl donor), acetyl-enzyme A (AcCoA, acetyl donor), coenzyme A (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/mg), glucose oxidase (type II from Aspergillus niger, 15,500 units/g), and β-glucosides were purchased from Sigma. PGEX-2T vector and Escherichia coli BL21(DE3) cells were supplied by Merck 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 iododecametate, 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 pGEX-2T and pET28 vectors. The resulting constructs encoded the enzyme as a glutathione S-transferase fusion protein (GST-NAT1) and as a polystyrene-tagged fusion protein (His-NAT1), respectively. We transformed BL21(DE3) bacteria with these constructs, induced expression of the transgene with 0.1 mM isopropyl-β-D-galactopyranoside, and incubated the cells for 4 h at 37°C. The bacteria were pelleted by centrifugation (6,000 × g, 30 min), washed with cold phosphate-buffered saline, and harvested by centrifugation (6,000 × g, 30 min). Pellets were stored at −80°C until required.

For the purification of recombinant proteins, pellets (from 1-liter cultures) were resuspended in 40 ml of 50 mM Tris-HCl, pH 8, 150 mM NaCl (lysis buffer) containing lysozyme (1 mg/ml final concentration) and protease inhibitors. Following incubation (1 h at 4°C), protease inhibitors, DNAse I (20 μg/ml final concentration), and 0.2% Triton X-100 (final concentration) were added, and the suspension was incubated for an additional hour at 4°C. Lysates were then subjected to sonication on ice (five pulses of 10 s each), centrifuged (12,000 × g, 30 min), and the supernatant collected.

For the purification of GST-NAT1, this supernatant was added to glutathione-agarose beads (40 mg/liter of original culture) for 2 h at 4°C. Beads were then poured into a column and washed successively with 5 volumes of lysis buffer containing 0.2% Triton X-100 followed by 5 volumes of lysis buffer alone. GST-NAT1 was then eluted with 10 mM reduced glutathione (final) in 50 mM Tris-HCl, pH 8, 1 mM EDTA. The purified enzyme was reduced by incubation with 10 mM DTT (final concentration) for 10 min at 4°C and was then dialyzed against 25 mM Tris-HCl, pH 7.5, 1 mM EDTA.

The His-NAT1 protein was prepared in a similar manner to GST-NAT1. Briefly, supernatants from bacterial lysates were incubated with 1.5 ml of nickel-nitrilotriacetic acid Superflow resin in the presence of 20 μM imidazole (final concentration) for 2 h at 4°C. The resin was then pelleted by centrifugation and washed successively with lysis buffer containing 0.2% Triton X-100 and lysis buffer containing 50 mM imidazole (final concentration). His-NAT1 was eluted with 300 mM imidazole (final concentration) in lysis buffer. Purified His-NAT1 was reduced by incubation with 10 mM DTT (final concentration) for 10 min at 4°C and was then dialyzed against 25 mM Tris-HCl, pH 7.5, 1 mM EDTA. SDS-PAGE analysis was carried out at each stage of purification, and protein concentrations were determined using a standard Bradford assay.

Recombinant-tagged NAT1 enzyme was used in a previous study for functional characterization of the NAT1 enzyme (42). In this study, we used both GST-NAT1 and His-NAT1 as sources of purified recombinant NAT1 enzyme, and similar results were obtained with both proteins in all experiments.

Enzyme Assay—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 Mustag et al. (43). Brieﬂy, treated or non-treated samples (10–20 μl) containing NAT1 protein were assayed in a reaction mixture containing 500 mM PAS (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 μM. 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 analyzer (Metertech). One unit of enzyme was deﬁned as the amount of enzyme giving an A410 value 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.

Reaction of NAT1 with H2O2 in the Presence or Absence of Other Substrates—Concentrations of stock H2O2 solutions were determined by measuring absorbance at 240 nm (ε240 = 44 m M−1 cm−1). In all subsequent experiments, the final concentration of enzyme during the oxidation step with H2O2 was 1.5 μM, giving a final concentration of 15 μM. 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 set as 100% activity (as measured using Sigma’s online calculator).

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 activity assay was then carried out. Assays performed in these conditions (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 dimered assays, NAT1 (1.5 μM ﬁnal concentration) was then assayed 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 assayed with DTT (5 mM ﬁnal concentration). 20 μl total volume) for 10 min at 37°C. Residual NAT1 activity was assayed as described above. Assays performed in these conditions, with dimered 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 37°C. 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, the apparent first-order rate constants (k_app = 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_app plotted against H2O2 concentrations.
Redox Regulation of Human NAT1

**RESULTS**

*In Vitro Inactivation of NAT1 by Bolus Addition of H₂O₂ and by Continuous Generation of H₂O₂ by the Glucose/Glucose Oxidase System*—Several enzymes with catalytic cysteine residues have been shown to be reversibly inactivated by H₂O₂, 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 H₂O₂. 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 H₂O₂ (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 H₂O₂ and of the exposure of NAT1 to continuous levels of H₂O₂ generated by an enzymatic system.

First, reduced NAT1 enzyme (1.5 μM final concentration) was incubated with various concentrations of H₂O₂ (5–200 μM final concentration) and residual NAT1 activity was measured. NAT1 activity was significantly inhibited by H₂O₂ in a dose-dependent manner (Fig. 1A). At a concentration of 200 μM, H₂O₂ inhibited the enzyme by over 90%. An IC₅₀ 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 H₂O₂ on NAT1 activity, we used another source of H₂O₂, the steady conversion of β-n-glucose to β-glucuronolactone and H₂O₂, which is catalyzed by glucose oxidase (45). As shown in Fig. 1B (filled circles), NAT1 (1.5 μM final concentration) was also inactivated by the constant production of H₂O₂ through the glucose/glucose oxidase system. In the conditions used, the constant production rate of H₂O₂ was estimated to be ~6 μM H₂O₂/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 H₂O₂, the residual activity was less than 10%. No inactivation of NAT1 by H₂O₂ 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 H₂O₂.

Detection of H₂O₂-oxidized Cysteine Residues by Labeling with Fluorescein-conjugated Iodoacetamide—We investigated whether NAT1 contained reactive cysteine residues susceptible to oxidation by physiological concentrations of H₂O₂, using an approach based on the labeling of cysteine with 5-iodoacetamido fluorescein (25). Incubation of NAT1 with various concentrations of H₂O₂ resulted in the dose-dependent modification of cysteine residues, as indicated by the disappearance of...
oxidation of NAT1 could be reversed by thiol-reducing agents, as cysteine of NAT1 was the target of H$_2$O$_2$-dependent oxidative modification. Thus, the oxidative inactivation of NAT1 by H$_2$O$_2$ is a rapid bimolecular process in which one molecule of H$_2$O$_2$ (200 μM final concentration) in 25 mM Tris-HCl, pH 7.5, 1 mM EDTA for 10 min at 37 °C. The reaction mixture was incubated with fluorescein-conjugated iodoacetamide (20 μM final concentration) for 10 min at 37 °C. Samples were subjected to SDS-PAGE under reducing conditions, followed by Western blotting using an anti-fluorescein antibody. For the control, NAT1 was not treated with 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 the y intercept) was close to zero. The slope gave the apparent second-order rate constant ($k_{inact}$) of H$_2$O$_2$-dependent NAT1 inactivation as 420 μM$^{-1}$-min$^{-1}$. For each H$_2$O$_2$ concentration, the first-order rate constant can be expressed as $k_{obs} = k_{inact}[H_2O_2]$, where $n$ is the order of H$_2$O$_2$ in its reaction with NAT1. Thus, replotting ln of $k_{obs}$ versus ln of H$_2$O$_2$ concentration gave a straight line (Fig. 4C). 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 (-SO$_2$H) 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 (-SO$_2$H), any of which could be reduced to give cysteine. Intramolecular and intermolecular disulfide bonds were ruled out on the basis of electrophoretic mobility shift assays (54). In addition, the catalytic cysteine residue 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 dimerone (10 mM final concentration) with H$_2$O$_2$-oxidized NAT1 (Fig. 5). The control, in which NAT1 was incubated with 10 mM dimerone 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 dimerone was only partially reactivated (63% of...
control activity (100%).

determined from comparison of percent of residuals activities with 
$r$ (correlation factor $r$inact) by plotting the apparent first-order inactivation constant ($k_{\text{obs}}$) 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 plotting 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.

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 plotting 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.

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 isoform 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 (~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

**TABLE I**

Protection of AcCoA and CoA against H$_2$O$_2$-dependent inhibition of NAT1

Purified NAT1 (1.5 μM final) was reacted with H$_2$O$_2$ (200 μM final) in presence of different concentrations (final) of AcCoA or CoA as described under “Experimental Procedures.” Percent of protection were determined from comparison of percent of residuals activities with NAT1 control activity (100%).

| Conditions | % of protection |
|------------|----------------|
| NAT1 + AcCoA (0.2 mM) + H$_2$O$_2$ | 17 ± 3$^a$ |
| NAT1 + AcCoA (0.5 mM) + H$_2$O$_2$ | 30 ± 5$^a$ |
| NAT1 + AcCoA (1 mM) + H$_2$O$_2$ | 46 ± 3$^a$ |
| NAT1 + AcCoA (2 mM) + H$_2$O$_2$ | 64 ± 6$^a$ |
| NAT1 + CoA (0.2 mM) + H$_2$O$_2$ | 9 ± 4 |
| NAT1 + CoA (0.5 mM) + H$_2$O$_2$ | 13 ± 3 |
| NAT1 + CoA (1 mM) + H$_2$O$_2$ | 13 ± 6 |
| NAT1 + CoA (2 mM) + H$_2$O$_2$ | 13 ± 8 |

$^a$ p < 0.01 versus H$_2$O$_2$-only treated NAT1.

control activity), consistent with the formation of a stable sulfenic acid at the active site cysteine. The partial, but significant, effect of dimedone on H$_2$O$_2$-treated NAT1 was probably due to the low reactivity of dimedone with sulfenic acids. This partial effect of dimedone has been reported elsewhere for inactivation of these PTPs 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 (~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).

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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).
Enzymes that contain an essential thiolate in their active site as they are intrinsically stronger nucleophiles (19, 20, 58). via oxidation of its catalytic cysteine residue (18, 57).

The major determinant of cellular redox potential, with a concentration of 1–10 mM (52, 53). Thus, our results suggest that GSH could reoxidize H$_2$O$_2$-inactivated NAT1 in the cell. GSH levels can be decreased by oxidative stress, in particular by xenobiotic-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 H$_2$O$_2$-dependent inactivation and that cysteine thiolate was the reactive species. Thiolates are much more susceptible to oxidation by H$_2$O$_2$ in cells than other protein cysteine residues as they are intrinsically stronger nucleophiles (19, 20, 58). Enzymes that contain an essential thiolate in their active site are widely accepted to be potential candidates for reversible oxidation by H$_2$O$_2$ generated within the cell (20, 25, 31). For instance, PTP1B, a phosphatase mentioned above, has been shown to be reversibly inactivated in vitro and in vivo by H$_2$O$_2$ via oxidation of its catalytic cysteine residue (18, 57).

H$_2$O$_2$ can oxidize cysteine residues in proteins to give cysteine sulfenic acid or disulfide, which can be reduced back to cysteine by cellular reductants, such as GSH (19, 20). We provide strong evidence that the H$_2$O$_2$-dependent inactivation of NAT1 involved the formation of a stable sulfenic acid at the active-site cysteine (Cys-SOH) of the enzyme. Interest in this oxidative modification of cysteine residues has increased, since it was shown to play an important role in redox-regulated processes (41). More specifically, the formation of stable sulfenic acid at the active-site cysteine of various enzymes, such as tyrosine phosphatase and glutathione reductases, has been suggested to play a key role in the reversible inhibition of these enzymes by oxidative or nitrosative stress (39, 40). 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 H$_2$O$_2$. Conversely, formation of an inter- or intramolecular disulfide formation similar to that observed in some H$_2$O$_2$-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 electrophoretic mobility shifts were observed between reduced and H$_2$O$_2$-oxidized NAT1 (data not shown). Finally, dimedone experiments demonstrated the presence of a stable sulfenic acid after H$_2$O$_2$ inactivation.

Our results suggest that human NAT1 could be reversibly inactivated in vivo by H$_2$O$_2$, 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 H$_2$O$_2$ 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.

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Fig. 5. Effect of dimedone on the DTT-dependent reactivation of H$_2$O$_2$-oxidized NAT1. NAT1 (1.5 μM) was incubated with H$_2$O$_2$ (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 dimedone (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 activity. **, p < 0.01 versus H$_2$O$_2$-inactivated NAT1. #, p < 0.01 versus H$_2$O$_2$-inactivated NAT1 in the presence of DTT (H$_2$O$_2$/DTT).
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