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Characterization of Hamster Recombinant Monomorphic and Polymorphic Arylamine *N*-Acetyltransferases

BIOACTIVATION AND MECHANISM-BASED INACTIVATION STUDIES WITH *N*-HYDROXY-2-ACETYLAMINOFLUORENE

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ABSTRACT. The purified hamster recombinant arylamine *N*-acetyltransferases (NATs), rNAT1-9 and rNAT2-70D, were characterized for their capabilities to bioactivate *N*-hydroxy-2-acetylaminofluorene (*N*-OH-AAF) to DNA binding reactants and for their relative susceptibilities to mechanism-based inactivation by *N*-OH-AAF. The rate of DNA adduct formation resulting from rNAT1-9 bioactivation of [^{14}C]*N*-OH-AAF was more than 30 times greater than that of rNAT2-70D-catalyzed bioactivation of [^{14}C]*N*-OH-AAF. This result is consistent with substrate specificity data indicating that *N*-OH-AAF is a much better acetyl donor for hamster NAT1 than NAT2. Previous studies indicated that *N*-OH-AAF is a mechanism-based inactivator of hamster and rat NAT1. In the presence of *N*-OH-AAF, both rNAT1-9 and rNAT2-70D underwent irreversible, time-dependent inactivation that exhibited pseudo first-order kinetics and was saturable at higher *N*-OH-AAF concentrations. The enzymes were partially protected from inactivation by the presence of cofactor and substrates. The limiting rate constants (k_i) and dissociation constants (K_I) for inactivation by *N*-OH-AAF were determined. The second-order rate constants (k_i/K_I) were 22.1 min^{-1} mM^{-1} for rNAT1-9 and 1.0 min^{-1} mM^{-1} for rNAT2-70D, indicating that rNAT1-9 is approximately 20 times more susceptible than rNAT2-70D to inactivation by *N*-OH-AAF. The kinetic parameters for rNAT1-9 were nearly identical to values previously reported for partially purified hamster NAT1. Partition ratios were 504 for inactivation of rNAT1-9 by *N*-OH-AAF and 137 for inactivation of rNAT2-70D. Thus, a turnover of almost 4 times as many *N*-OH-AAF molecules is required to inactivate each molecule of rNAT1-9 than is needed to inactivate rNAT2-70D. The partition ratio data are consistent with the finding that rNAT1-9 catalyzes a higher rate of DNA adduct formation by *N*-OH-AAF than rNAT2-70D. The combined results indicate that the recombinant enzymes are catalytically and functionally identical to hamster NATs and, therefore, will be a useful resource for studies requiring purified NATs. BIOCHEM PHARMACOL **56**;1:47–59, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. arylamine *N*-acetyltransferase; recombinant NATs; *N*-hydroxy-2-acetylaminofluorene; mechanism-based inactivation; bioactivation

NATs§ (EC 2.3.1.5; acetyl CoA:arylamine *N*-acetyltransferase) are cytosolic enzymes that are present in a variety of mammalian tissues [1, 2]. NATs catalyze the acetylation of drugs, carcinogens, and other xenobiotics, leading to their detoxification and/or bioactivation. Arylamines, hydrazines, and hydrazides are metabolized by NAT-mediated *N*-acetylation. *N*-Acetylation (NAT; Fig. 1a) is usually considered a detoxification process because it renders the substrate nitrogen atom less susceptible to oxidation. A majority of *N*-acetylation reactions require acetyl CoA as a cofactor. Some NATs, however, can also utilize *N*-arylhydroxamic acids as acetyl donors. *N*-Arylhydroxamic acid-dependent *N*-acetylation of arylamines is designated *N*,*N*-acetylation (*N*,NAT; Fig. 1b). NATs can also catalyze the bioactivation of arylhydroxylamines and arylhydroxamic acids. Acetyl CoA-dependent *O*-acetylation (OAT) of arylhydroxylamines and intramolecular *N*,*O*-acetylation (*N*,OAT) of *N*-arylhydroxamic acids produce reactive *N*-acetoxyarylamine intermediates (Fig. 1, c and d). *N*-Acetoxy esters are considered ultimate carcinogens because they are capable of forming covalent adducts with nucleic acids and proteins [3, 4].

The two human NAT genes, *NAT1* and *NAT2*, are

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§ *Abbreviations:* AAB, 4-aminoazobenzene; DEAE, diethylaminoethyl; DHFR, dihydrofolate reductase; DTT, dithiothreitol; LB, Luria-Bertani medium; NAT, *N*-acetyltransferase; NAT1, monomorphic *N*-acetyltransferase; NAT2, polymorphic *N*-acetyltransferase; *N*-OH-AAF, *N*-hydroxy-2-acetylaminofluorene; PABA, *p*-aminobenzoic acid; and PE, potassium phosphate buffer.

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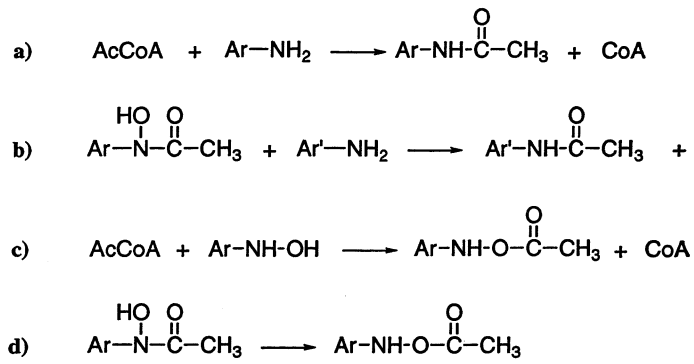

FIG. 1. Reactions catalyzed by mammalian arylamine NATs. (a) Acetyl CoA-dependent *N*-acetylation (NAT). (b) Arylhydroxamic acid-dependent *N*-acetylation (N,NAT). (c) Acetyl CoA-dependent *O*-acetylation (OAT). (d) Arylhydroxamic acid N,*O*-transacetylation (N,OAT).

polymorphically expressed. Variations at the *NAT2* locus are responsible for the classic acetylation polymorphism that categorizes individuals as rapid, intermediate, or slow acetylators [5]. Certain variations at the *NAT1* locus (*NAT1*10* and *NAT1*17*) result in elevated *N*-, *O*- and/or *N*,OAT acetylation activities compared with wild-type NAT1 (*NAT1*4*) [6, 7]. Differences in the abilities of NAT1 and NAT2 allozymes to bioactivate or detoxify arylamines and their derivatives have been associated with various susceptibilities to arylamine-induced toxicities [3, 8]. Epidemiological studies have suggested that slow NAT2 acetylators are more susceptible to bladder cancer than rapid acetylators [9, 10]. Also, preliminary reports have suggested that individuals inheriting the *NAT1*10* genotype are at an increased risk for bladder cancer [11]. Postmenopausal women who are slow NAT2 acetylators and who smoke tobacco may have an increased risk for breast cancer [12]. Some investigators have suggested that NAT2 acetylation capacity in combination with other factors, such as the rapid CYP1A2 phenotype, smoking, and a high consumption of meat, provides an increased risk for colorectal cancer [13–15]. Preliminary data also have suggested an increased risk for colorectal cancer among individuals possessing the *NAT1*10* genotype, and the risk was most apparent among NAT2 rapid acetylators [16]. However, the lack of an association between the presence of the *NAT1*10* allele and colorectal adenomas has been observed [17].

Acetylation polymorphisms have been described in the rabbit, mouse, hamster, and rat [5]. Two *NAT* genes have been cloned from rabbits, rats, and hamsters, while mice possess three *NAT* genes [2, 18]. The genetic basis for the observed polymorphisms differs both within and between species [2, 19]. In humans, variant *NAT2* alleles contain at least one of several point mutations, which result in NAT2 allozymes with decreased acetylation capability. Mutations in the hamster *NAT2* locus result in truncated allozymes with decreased acetylation ability, and in the slow acetylator rabbit the polymorphic *NAT* is deleted [2]. Most NAT1 and NAT2 allozymes from humans and animals catalyze *N*-, *O*-, and *N*,*O*-acetylation reactions to varying degrees. However, there are significant differences in isozyme substrate selectivity among species. For example, PABA is classified as an NAT1 substrate in humans [20] and rabbits, but is an NAT2 substrate for mouse, hamster, and rat liver NATs [5, 21].

As illustrated in Fig. 1d, *N*-arylhydroxamic acids can be activated by NATs. *N*-OH-AAF is a proximate carcinogenic hydroxamic acid that is biotransformed to an *N*-acetoxy ester by NAT-mediated *N*,*O*-transacetylation (*N*,OAT). *N*-Acetoxyarylamines bind covalently to DNA, primarily forming C-8 deoxyguanosine adducts [4]. Studies on the *in vitro* bioactivation of arylhydroxamic acids by hamster and rat hepatic NATs revealed that *N*-OH-AAF and certain other hydroxamic acids are mechanism-based inactivators of NAT activity, and that the bioactivation is catalyzed mainly by the NAT1 isozymes [1, 22, 23].

Detailed biophysical studies and kinetic characterization of NATs will provide insight into the molecular basis of their catalytic mechanism and substrate specificities. Such studies require large quantities of purified enzymes. A system for the large-scale expression of hamster recombinant NAT2 as a fusion protein to a mutant DHFR is available [24]. Described herein is a modification of the previously reported expression system that allows the production and purification of large quantities of hamster recombinant NAT1 (rNAT1-9). Hamster rNAT1-9 and rNAT2-70D are analogous to NAT1 8 and NAT2 15, respectively [2]. This report also describes the characterization of rNAT1-9 and rNAT2-70D with regard to their capacities for bioactivation of *N*-OH-AAF to DNA binding reactants and with regard to their susceptibilities to mechanism-based inactivation by *N*-OH-AAF.

MATERIALS AND METHODS

Chemicals

Expression constructs pPH8 [25] and pPH70D [24] have been described previously. *Escherichia coli* Ultracompetent XL2-Blue cells were purchased from Strategene. Spherilose A-500 weak anion exchanger is manufactured by Isco, Inc. All other materials used for the construction of plasmid pPH9D, the expression of the fusion protein, and the purification of rNAT1-9 were obtained as described previously [24]. Sephadex G-25 M PD-10 columns were pur-

chased from Pharmacia Biotech. 2-Nitro-[9-^{14}C]fluorene was purchased from Chemsyn Science Laboratories.

Synthetic Procedures

N-OH-AAF was synthesized as previously described [26], and [^{14}C]*N*-OH-AAF was synthesized from 2-nitro-[9-^{14}C]fluorene by a similar method [27]. 1,5-Dimethyl-4-imidazole disulfide was synthesized by a modification of the method of Holler [28].

Construction of Plasmid pPH9D

Plasmids pPH8 [25] and pPH70D [24] were each subjected to digestion with *Kpn*I and *Xba*I to obtain *rNAT1* insert DNA, and the modified *pFLAG-L54F DHFR* vector DNA, respectively. DNA was visualized by staining with ethidium bromide after separation on 1% agarose gels, and subsequently purified from the gels with the use of the Wizard PCR Prep DNA purification system (Promega). Vector DNA was treated with shrimp alkaline phosphatase and ligated to *rNAT1* insert DNA. Ultracompetent XL2-Blue cells were transformed with the ligation mix, and plasmid-containing clones were selected on LB agar plates by their ampicillin resistance. The presence and proper orientation of the *rNAT1* insert in the resulting plasmid, pPH9D, were confirmed by restriction mapping with *Kpn*I and *Bgl*II.

Expression of rNAT1-F-D-T-9

Non-K-12-derived *E. coli* strain TOPP3 cells were made competent by the method of Hanahan [29] before being transformed with pPH9D. Plasmid-containing clones were selected on LB/ampicillin/tetracycline plates. Overnight cultures (10 mL) were grown from single colonies, and a 1% inoculum was added to 1 L of LB containing 0.4% glucose and 100 μg of ampicillin per mL in a 2-L flask. Cultures were grown aerobically at 37° to an A_{600} of 0.5, at which time IPTG (isopropyl-β-D-thiogalactopyranoside) was added to a final concentration of 100 μM. After additional incubation for 3 hr, the cells were harvested by centrifugation at 5000 *g* for 15 min at 4°. The cell pellets were frozen and stored at −80°.

Purification of rNAT1-9

The purification of rNAT1-9 is similar to that previously described for rNAT2-70D [24]. All purification steps were performed at 4° in degassed buffers and under an argon atmosphere unless otherwise stated. The cell pellets from 10 L of TOPP3/pPH9D culture were suspended in 5 mL of lysis buffer (50 mM of Tris, pH 8.0; 5 mM of EDTA; 2 mM of DTT) per g of cells. Lysozyme (1 mg/mL) was added, and the suspension was incubated for 15 min at room temperature. The viscous lysate was sonicated with a VirTis Virsonic 300 Cell Disruptor fitted with a 0.5-in. disruptor horn. After tuning, the cell disruptor was set at 8 (50% power), and the bacterial cells were lysed in two 5-sec bursts with a 1-min cooling period on ice between bursts. The lysate was centrifuged at 25,000 *g* for 30 min to pellet the cell debris.

The soluble fraction was then dialyzed against 6 L (three 2-L portions) of PE (20 mM, pH 7.4; 1 mM of EDTA; 1 mM of DTT) purged with argon. The dialyzed soluble fraction was applied to a 2.5 × 50 cm DEAE anion exchange column that had been equilibrated with PE. The column was eluted with 50 mL of PE followed by a 1-L 0 to 0.5 M KCl gradient in PE. Fractions (10 mL) were collected at a flow rate of 0.5 mL/min. The absorbance at 280 nm was determined for each fraction, and an aliquot of selected fractions was assayed for *N*-OH-AAF/AAB transacetylation activity. The fractions containing the highest levels of acetylation activity were combined and concentrated in a Centriplus 30 (Amicon) unit to at least 1 mg of protein/mL for optimum thrombin cleavage. This DEAE chromatography step resulted in the partial purification of the fusion protein rNAT1-F-D-T-9. Glycerol was added to a final concentration of 10% to stabilize the enzyme activity, and the partially purified fusion protein was stored at −80°.

The partially purified fusion protein was then dialyzed against 6 L (three 2-L portions) of thrombin cleavage buffer (50 mM of Tris, pH 8.0; 0.1 M of NaCl; 2.5 mM of $CaCl_2$; 1 mM of DTT) purged with argon. Cleavage of the partially purified fusion protein was accomplished by overnight incubation at 4° with 5 U of human thrombin/mg of protein. Thrombin cleavage was terminated by dialysis, as described above, into PE purged with argon.

To separate rNAT1-9 from FLAG-L54F DHFR and other contaminating proteins, the dialyzed, thrombin-cleaved sample was applied to a 1.5 × 63 cm DEAE Spherilose anion exchange column that had been equilibrated with PE. The column was eluted with 50 mL of PE followed by an 800-mL 0 to 0.25 M KCl gradient in PE. Fractions (10 mL) were collected at a flow rate of 0.5 mL/min. Total protein was determined for each fraction by the Bradford assay [30], and an aliquot of selected fractions was assayed for *N*-OH-AAF/AAB transacetylation activity. The fractions containing the highest levels of activity were combined and concentrated in a Centriplus 10 unit to at least 3.5 mL. This second DEAE chromatography step resulted in the purification of rNAT1-9 to near homogeneity. The nearly homogeneous recombinant protein can be stored at −80° as a 10% glycerol-PE solution.

To remove a few high molecular weight contaminants, gel filtration chromatography was performed by applying the concentrated DEAE eluate to a 1.5 × 62 cm column of Sephadex G75 that had been equilibrated with PE. The column was eluted with PE at a flow rate of 0.5 mL/min while collecting 3-mL fractions. Total protein was determined for each fraction by the Bradford assay, and an aliquot of selected fractions was assayed for *N*-OH-AAF/AAB transacetylation activity. The fractions containing the highest levels of activity were combined and concentrated in a Centriplus 10 unit to at least 0.5 mg of

protein/mL. The eluate from the gel filtration column contained homogeneous rNAT1-9. Enzyme activity was stabilized by addition of a three-fold excess of BSA and glycerol to a concentration of 10%. The protein solution was divided into portions for storage at −80°.

Electrophoresis

SDS-PAGE and immunoblot analysis were performed as previously described [24].

Protein Assay

Protein concentrations were determined by the method of Bradford [30], with BSA as the standard. The Bradford assay was performed with the Coomassie Protein Assay Reagent according to the manufacturer's instructions.

Spectrophotometric Assays for NAT Activity

N-OH-AAF/AAB ASSAY. The assay was conducted as described previously except that the protein concentrations were either 0.2 mg of protein extract/mL or 2 μg of pure rNAT1/mL [31, 32].

ACETYL COA/PABA ASSAY. The assay was performed as described previously [31, 33]. Reaction mixtures contained 0.1 mL of the enzyme preparation (final concentrations were 3 μg of protein extract/mL or 0.5 μg of pure rNAT2/mL).

Assay for Covalent Binding to DNA

The procedure for the DNA binding assay is based on previously reported methods [27, 34]. Incubation tubes (15 mL) contained calf thymus DNA (2 mg), 1 mM of DTT, purified rNAT1-9 (~0.003 mg) or purified rNAT2-70D (0.008 mg), [^{14}C]*N*-OH-AAF (25 μM; 1.88 mCi/mmol) and sufficient tetrasodium pyrophosphate buffer (50 mM, pH 7.0) to make a total volume of 1.0 mL. Calf thymus DNA was dissolved in tetrasodium pyrophosphate buffer (50 mM, pH 7.0), DTT was dissolved in deionized water, and [^{14}C]*N*-OH-AAF was dissolved in 95% ethanol. The final concentration of ethanol in the reaction mixture was less than 1%. Purified rNAT1-9 in PE was stabilized with 1 mM of DTT, 10% glycerol, and three-fold BSA. The rate of adduct formation in the presence and absence of BSA did not differ, and, thus, the presence of the BSA was determined to have no effect on adduct formation. Purified rNAT2-70D in PE was stabilized with 1 mM of DTT and 10% glycerol.

After an incubation period of 2 min at 37°, the substrate, [^{14}C]*N*-OH-AAF, was added to initiate the reaction. The mixtures were vortexed briefly and incubated in air in a Dubnoff metabolic shaker at 37° for either 1.0 min (rNAT1-9) or 5.0 min (rNAT2-70D). The reactions were terminated by precipitating the modified DNA with 6 vol. of cold ethanol. The tubes were centrifuged at 3000 *g*. The precipitate was dissolved in 2 mL of 0.5 M NaCl and extracted four times with 1 mL of butanol saturated with deionized water. The aqueous layer was extracted twice with 1 mL of 0.1% (w/v) 8-hydroxyquinoline in phenol saturated with 10 mM of Tris buffer (pH 8.0) containing 1 mM of EDTA and twice with 1 mL of anhydrous ethyl ether. The DNA was precipitated as described above with cold ethanol, the mixture was centrifuged, and the precipitate was redissolved in 1 mL of tetrasodium pyrophosphate buffer (50 mM, pH 7.0). The radioactivity was determined by liquid scintillation. The DNA concentration was measured by the absorbance at 260 nm, and the purity was estimated by the absorbance ratio of 260 nm and 280 nm. Control incubations performed in the absence of enzyme resulted in less than 0.5 pmol of radiolabel bound/mg DNA/min.

When potential trapping agents were included in the incubation mixtures, they were added as solutions (5–40 μL) in tetrasodium pyrophosphate buffer (50 mM, pH 7.0; 1 mM of DTT) with the following exception. 1,5-Dimethyl-4-imidazole disulfide was dissolved in tetrasodium pyrophosphate buffer (50 mM, pH 7.0) containing 50 mM of DTT. According to Holler [28], 1,5-dimethyl-4-imidazole disulfide is converted to 1,5-dimethyl-4-imidazolethiol in the presence of excess DTT. The final concentrations of DTT in the incubation mixtures containing 1,5-dimethyl-4-imidazolethiol ranged from 1.2 to 3.0 mM. In all cases, simultaneous control experiments were conducted in which potential trapping agents were omitted from the incubation mixtures. Control experiments with 3.0 mM of DTT were also performed. An *N*-OH-AAF/AAB transacetylation activity assay performed in the presence of each trapping agent confirmed that the trapping agents did not inhibit enzyme activity.

Mechanism-based Inactivation

Concentration-dependent inactivation of rNAT1-9 and rNAT2-70D was determined by incubation of the enzyme with *N*-OH-AAF, followed by filtration through a PD-10 column. PD-10 eluates were assayed for protein content and residual enzyme activity. Incubation tubes (1.5 mL) contained either purified rNAT1-9 or rNAT2-70D (8 μg; 0.24 nmol), *N*-OH-AAF (0 to 0.75 mM), and sufficient tetrasodium pyrophosphate buffer (50 mM, pH 7.0; 0.1 mM DTT) to make a total volume of 0.1 mL. Purified rNAT1-9 was stabilized with 1 mM of DTT, three-fold BSA, and 10% glycerol in PE. Inactivation reactions were performed in the presence and absence of BSA. The presence of BSA was determined to have no effect on the rate of mechanism-based inactivation. Purified rNAT2-70D was stabilized with 1 mM of DTT and 10% glycerol in PE. *N*-OH-AAF was dissolved in 95% ethanol. The final concentration of ethanol in the reaction mixture was 5%, and it did not affect enzyme activity.

rNAT1-9 or rNAT2-70D was incubated for 5 min at 37°

with 5 μL of an *N*-OH-AAF solution. The incubations were conducted in air in a heat block. The reactions were terminated by application to a PD-10 gel filtration column. PD-10 columns were eluted with 2.6 mL of tetrasodium pyrophosphate buffer (50 mM, pH 7.0; 1.0 mM of DTT) before an additional 1.0 mL of buffer was applied to the column, and the eluate containing the rNAT was collected. Typically, 65–80% of the protein was recovered by this method. The concentration of protein in the PD-10 eluate was determined prior to assaying for residual *N*-OH-AAF/AAB (rNAT1-9) or acetyl CoA/PABA (rNAT2-70D) acetylation activity. The percentage of activity remaining was calculated with respect to controls containing 95% ethanol in place of *N*-OH-AAF. The concentration of rNAT1-9 used in the *N*-OH-AAF/AAB activity assay following elution from the PD-10 column was based on the assumption that the BSA co-eluted with the rNAT1-9.

When potential protecting agents were included in the incubation mixtures, they were added as solutions (5 μL) in tetrasodium pyrophosphate buffer (50 mM, pH 7.0; 0.1 mM of DTT). The enzyme and potential protecting agent were incubated for 2 min at 37° before the addition of *N*-OH-AAF in 50% ethanol and a further 4-min incubation at 37°. The incubation mixtures were filtered through a PD-10 column before residual activity was determined. In all cases, simultaneous control experiments were conducted in which the potential protecting agents and/or *N*-OH-AAF were omitted from the incubation mixture. Control experiments contained buffer in place of the protecting agent and 50% ethanol in place of *N*-OH-AAF.

Kinetics of rNAT1-9 and rNAT2-70D Inactivation by N*-OH-AAF*

Concentration- and time-dependent inactivation were determined by incubation of the enzymes with *N*-OH-AAF, followed by an assay to determine residual enzyme activity. For inactivation of rNAT1-9 by *N*-OH-AAF, incubation tubes (1.5 mL) contained purified rNAT1-9 (1 μg), *N*-OH-AAF (0–35 μM), and sufficient tetrasodium pyrophosphate buffer (50 mM, pH 7.0; 1 mM of DTT) to make a total volume of 0.49 mL. Purified rNAT1-9 was stabilized with 1 mM of DTT, three-fold BSA, and 10% glycerol in PE buffer. *N*-OH-AAF was dissolved in 95% ethanol. The final concentration of ethanol was less than 2%, and it did not affect enzyme activity. Incubation at 37° in a heat block was initiated by the addition of a 10-μL solution of *N*-OH-AAF. After incubation (0.5 to 2.0 min), 10 μL of an *N*-OH-AAF/AAB stock solution (final concentrations: 25 mM of *N*-OH-AAF/7.5 mM of AAB; 0.5 mM of *N*-OH-AAF/0.15 mM of AAB) in 95% ethanol was added. The *N*-OH-AAF/AAB assay was conducted as described above under "spectrophotometric assays for NAT activity." The percent activity remaining after each incubation was calculated with respect to controls containing 95% ethanol in place of *N*-OH-AAF. Kinetic constants (K_I and k_i) were determined as described previously [22, 35]. The correlation coefficient for the fit of the points to the line generated in the double-reciprocal plot was 1.0.

For inactivation of rNAT2-70D by *N*-OH-AAF, incubation tubes (1.5 mL) contained purified rNAT2-70D (5 μg), *N*-OH-AAF (0 to 0.5 mM), and sufficient tetrasodium pyrophosphate buffer (50 mM, pH 7.0; 1 mM of DTT) to make a total volume of 0.1 mL. Purified rNAT2-70D was stabilized with 1 mM of DTT and 10% glycerol in PE. *N*-OH-AAF was dissolved in 50% ethanol. The final concentration of ethanol was 2.5%, and it did not affect enzyme activity. Incubation at 37° in a heat block was initiated by the addition of 5 μL of *N*-OH-AAF. After incubation (2–7 min), a 10-μL aliquot of the incubation mixture was diluted into 90 μL of buffer (10× dilution). Immediately after dilution, a 16.7-μL aliquot of the 10× dilution was further diluted into 83.3 μL of buffer and was assayed for residual acetyl CoA/PABA activity by the addition of 66.7 μL of an acetyl CoA/PABA substrate solution (final concentrations: 1.5 mM of acetyl CoA/0.25 mM of PABA; 0.6 mM of acetyl CoA/0.1 mM of PABA; 100× total dilution of enzyme). The acetyl CoA/PABA assay was conducted as described above under "spectrophotometric assays for NAT activity." The percent activity remaining after each incubation was calculated with respect to controls containing 50% ethanol in place of *N*-OH-AAF. Kinetic constants (K_I and k_i) were determined as described above. The correlation coefficients for the fit of the points to the lines generated in the double-reciprocal plots was 0.99.

Determination of Partition Ratio

The partition ratios, *r*, for rNAT1-9 and rNAT2-70D inactivation by *N*-OH-AAF were determined by titration of the enzyme with the inactivator. Increasing concentrations of *N*-OH-AAF (0 to 50 μM for rNAT1-9; 0 to 0.5 mM for rNAT2-70D) were incubated with a fixed amount of the enzyme (0.06 μM of rNAT1-9; 1.46 μM of rNAT2-70D). The incubations were as described for the kinetics of inactivation except that the incubation continued until no additional inactivation was observed (2.5 to 15 min). A plot of percent remaining activity versus the ratio of inactivator concentration to enzyme concentration was constructed. The partition ratio was calculated from the x-intercept of the line (x-intercept = 1 + *r*) [36].

RESULTS

Cloning, Expression and Purification of rNAT1-9

Construction of plasmid pPH9D was accomplished by replacement of the hamster *rNAT2* gene in plasmid pPH70D with the hamster *rNAT1* gene, which was excised from plasmid pPH8 (Fig. 2a). Recombinant NAT1 was expressed in *E. coli* as a fusion protein to a mutant DHFR linked to rNAT1 by a thrombin-sensitive linker (Fig. 2b). The fusion protein was partially purified from bacterial lysates by anion exchange chromatography before it was

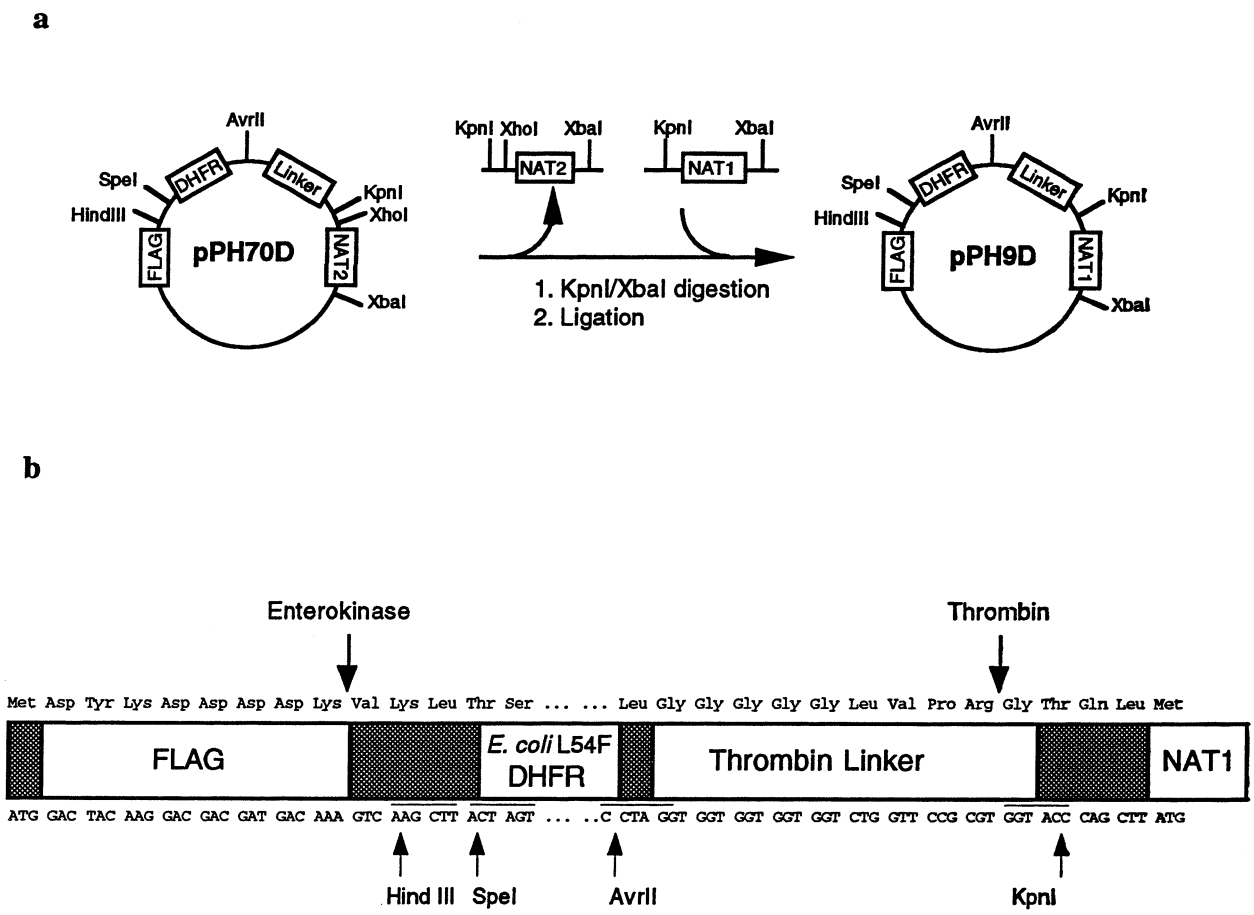

FIG. 2. Schematic of expression construct pPH9D. (a) Construction of bacterial expression plasmid pPH9D. The plasmid pPH70D was digested with *Kpn*I and *Xba*I to obtain the modified *pFLAG-L54F DHFR* vector DNA. *rNAT1* insert DNA was obtained by digestion of plasmid pPH8 (see Materials and Methods). The linearized vector was ligated to the *rNAT1* insert DNA, and the resulting plasmid was designated pPH9D. (b) DNA and protein sequences of the rNAT1-F-D-T-9 fusion protein linker region. The recombinant NAT1-9 produced by thrombin cleavage contains the Gly-Thr-Gln-Leu peptide appended at the amino terminus of the NAT1 sequence.

enzymatically cleaved by incubation with thrombin. The rNAT1 portion of the cleaved fusion protein (rNAT1-9) was purified to homogeneity by anion exchange chromatography followed by gel filtration. Almost 11 mg of homogeneous rNAT1-9 was obtained from 10 L of bacterial culture (Table 1). In addition, more than 50% of the *N*-OH-AAF/AAB activity in the bacterial lysate was recovered as pure rNAT1-9.

As previously observed with other NAT fusion proteins [25], SDS-PAGE analysis of TOPP3/pPH9D lysates revealed that the DHFR-rNAT1 fusion protein was expressed at a lower level than that observed for the DHFR-rNAT2 fusion protein (result not shown). Therefore, 10 L of TOPP3/pPH9D bacterial culture were used to obtain quantities of pure rNAT1-9 comparable to those obtained from 1 L of bacterial culture expressing the rNAT2 fusion protein. Because of the large volume of bacterial cells to be lysed and the large volume of lysate to be applied to the initial anion exchange column, the previously described lysozyme lysis procedure [24] was modified. The use of

TABLE 1. Purification of rNAT1-9*

Purification step	Total protein (mg)	Specific activity† (μmol/mg/min)	Total activity† (μmol/min)	% Recovery	Fold purification
Lysate	2150	0.21	454	100	1.0
DEAE	308	1.56	480	106	7.4
DEAE	23.4	16.8	393	87	80.0
Sephadex G75	10.9	23.1	252	56	110.0

*These results were obtained from 10 L of cell culture. The purification was performed in aliquots.
†Activities are expressed as the rate of *N*-OH-AAF-dependent acetylation of AAB.

TABLE 2. Bioactivation of *N*-OH-AAF by recombinant NATs: DNA adduct formation

Enzyme	Rate of adduct formation (nmol *N*-OH-AAF bound/ mg DNA/min/mg rNAT)
rNAT1-9*	707.5 ± 20.1
rNAT2-70D†	22.3 ± 2.8

*rNAT1-9 was stabilized with 10% glycerol and three-fold BSA. Incubations were performed as described in Materials and Methods for 1 min with 65 nmol/min of *N*-OH-AAF/AAB activity (~0.003 mg rNAT1-9). Values are means ± SD of four separate experiments, each performed in duplicate.

†rNAT2-70D was stabilized with 10% glycerol. Incubations were performed as described for 5 min with 0.008 mg rNAT2-70D. Values are means ± SD of three separate experiments, each performed in duplicate.

sonication to aid cell disruption reduced the volume of lysate by approximately 65%, significantly increasing the efficiency of the purification procedure.

Following purification of rNAT1-9 to homogeneity, it was observed that losses of activity sometimes occurred as a result of freezing and thawing the sample. To prevent loss of activity, the enzyme was stabilized with a 3-fold excess of BSA and 10% glycerol. With this procedure, more than 95% of the *N*-OH-AAF/AAB transacetylation activity of rNAT1-9 was retained during multiple cycles of freezing and thawing.

DNA Adduct Formation

The rate of covalent adduct formation was measured after incubation of radiolabeled *N*-OH-AAF with either purified rNAT1-9 or rNAT2-70D and calf thymus DNA. The dependence of DNA adduct formation upon incubation time and *N*-OH-AAF concentration was determined in the presence of rNAT1-9. The substrate concentration that gave the maximal adduct formation was used in the assays, and the incubation time was chosen from the linear portion of the product (adduct) versus time curve for fixed protein, DNA, and substrate concentrations. Previous studies have indicated that *N*-OH-AAF is a poor substrate for both hamster NAT2 and rNAT2-70D-mediated *N*-OH-AAF/AAB *N*,*N*-acetyltransferase activity [34, 24]. Thus, the concentration of enzyme used in the rNAT2-70D experiments was approximately twice that used in the rNAT1-9 incubations. The rates of adduct formation for rNAT1-9 and rNAT2-70D are shown in Table 2. The rate of DNA adduct formation resulting from rNAT1-9 bioactivation of *N*-OH-AAF was more than 30 times greater than that from rNAT2-70D-catalyzed bioactivation. The rNAT1-9 enzyme preparation used for these studies was stabilized with BSA and glycerol (see Materials and Methods). The rate of adduct formation was similar with rNAT1-9 preparations stabilized only with glycerol, indicating that the presence of BSA had no effect on the bioactivation of *N*-OH-AAF to DNA reactive products (data not shown). The rate of adduct formation in the absence of enzyme was negligible.

TABLE 3. Bioactivation of *N*-OH-AAF by rNAT1-9: Effect of nucleophiles and antioxidants on DNA adduct formation*

Agent [1.0 mM]	Relative DNA adduct formation (%)
None	100†
Glutathione	101.7 ± 4.0
Cysteine	91.0
N-Acetylmethionine	56.2 ± 2.5
Thiourea	36.1 ± 1.7
1,5-Dimethyl-4-imidazolethiol	33.1 ± 0.5
Ascorbic acid	28.8 ± 0.5
Potassium-*O*-ethylxanthate	24.7 ± 1.5

*Incubations were carried out as described in Materials and Methods. Results are expressed as a percentage of the rate of DNA adduct formation in the absence of the trapping agents. Values represent the means ± range of two separate experiments, each performed in duplicate, except for the value for cysteine, which is the result of one experiment performed in duplicate.

†The control value obtained in the absence of nucleophiles was 2.45 ± 0.3 nmol of *N*-OH-AAF bound/mg of DNA/min (mean ± SD; values are the means from three separate experiments, each conducted in duplicate).

Effect of Nucleophiles and Antioxidants on the Rate of DNA Adduct Formation

Various agents were evaluated for their abilities to compete with DNA for the reactive species generated by incubation of rNAT1-9 with *N*-OH-AAF. Similar studies with a partially purified hamster NAT1 have been reported [27]. In experiments with 1-mM concentrations of each agent, glutathione and cysteine had little effect on DNA adduct formation. *N*-Acetylmethionine reduced adduct formation by about 45%, and ascorbic acid, thiourea, and potassium-*O*-ethylxanthate reduced DNA binding by 60–75% (Table 3). These results obtained with purified rNAT1-9 are comparable to those reported by Boteju and Hanna [27]. The only exceptions were that potassium-*O*-ethylxanthate and thiourea each inhibited DNA adduct formation by approximately 20% more than that observed by Boteju and Hanna [27]. The ability of 1,5-dimethyl-4-imidazolethiol to trap reactive species before they react with DNA has not been investigated previously. In this study, the compound was approximately as effective as ascorbic acid, thiourea, and potassium-*O*-ethylxanthate. The concentration of 1,5-dimethyl-4-imidazolethiol was estimated by assuming 100% conversion of 1,5-dimethyl-4-imidazole disulfide to the thiol in the presence of excess DTT (see Materials and Methods). The effect of DTT on the rate of DNA adduct formation also was investigated. The rates of DNA adduct formation in the presence of 0.1 mM and 3.0 mM of DTT were within 10% of the control rate determined in the presence of 1.0 mM of DTT (data not shown).

Mechanism-based Inactivation

Incubation of *N*-OH-AAF with either purified rNAT1-9 or rNAT2-70D resulted in irreversible, time-dependent loss of enzyme activity. Inactivation of both rNAT1-9 (*N*-OH-AAF/AAB activity) and rNAT2-70D (acetyl CoA/PABA activity) was concentration dependent at low *N*-OH-AAF

TABLE 4. Inactivation of recombinant NATs by *N*-OH-AAF: Effect of cofactor, substrates, products, and nucleophiles*

Agent	Concentration (mM)	% Remaining activity†	
		rNAT1-9‡	rNAT2-70D§
None		6.1 ± 5.5	7.8 ± 5.0
Acetyl CoA	0.6	80.8 ± 4.2	99.4 ± 1.6
	1.0	90.6 ± 3.1	ND‖
AAB	0.25	56.9 ± 3.4	ND
N-Acetyl-AAB	0.25	27.1 ± 7.7	ND
PABA	1.0	ND	24.6 ± 0.4
N-Acetyl-PABA	1.0	ND	6.0 ± 0.9
Cysteine	1	47.0 ± 6.5	38.6 ± 1.5
	10	100.8 ± 2.1	93.3 ± 8.5
Glutathione	1	14.8 ± 4.9	29.2 ± 0.1
	10	56.8 ± 5.2	55.3 ± 3.6
N-Acetylcysteine	1	9.8 ± 9.9	14.5 ± 4.1
	10	21.0 ± 9.7	29.6 ± 0.3
N-Acetylmethionine	1	34.3 ± 5.1	11.8 ± 6.4
	10	74.7 ± 4.7	41.8 ± 5.5

*Incubations were performed as described under Materials and Methods. Enzyme was incubated with agent for 2 min prior to the addition of *N*-OH-AAF, followed by a 4-min incubation period. Reaction mixtures were filtered through a PD-10 column prior to determination of residual enzyme activity.

†Results are expressed as the percentage of enzyme activity in the absence of the agent and *N*-OH-AAF. All agents were determined to have no effect on enzyme activity in the concentrations used. Values represent the means ± range of two separate experiments, with each assay performed in triplicate, except for the effect of 0.6 mM of acetyl CoA and the effect of *N*-acetylcysteine on rNAT1-9 inactivation, which are the means ± SD for three experiments, performed in triplicate.

‡A concentration of 0.05 mM of *N*-OH-AAF was used to inactivate rNAT1-9. The control *N*-OH-AAF/AAB activity was 19.3 ± 0.8 μmol/mg/min (mean ± SD, N = 23 experiments, with each activity assay performed in triplicate). The result for inactivation of rNAT1-9 in the absence of agent represents the mean of 21 experiments, with each activity assay performed in triplicate.

§A concentration of 0.5 mM of *N*-OH-AAF was used to inactivate rNAT2-70D. The control acetyl CoA/PABA activity was 68.8 ± 5.8 μmol/mg/min (mean ± SD, N = 18 experiments, with each activity assay performed in triplicate). The result for inactivation of rNAT2-70D in the absence of agent represents the mean of 18 experiments, with each activity assay performed in triplicate.

‖ND, not determined.

concentrations and saturable at higher concentrations, indicating an enzyme-mediated process. The saturation concentration for inactivation of rNAT1-9 was 0.05 mM of *N*-OH-AAF, while a concentration of 0.5 mM of *N*-OH-AAF was required to achieve the maximal rate of inactivation of rNAT2-70D (results not shown). The rNAT1-9 enzyme preparation used for these studies was stabilized with BSA and glycerol (see Materials and Methods). The rate and extent of enzyme inactivation in the presence of BSA were similar to those of rNAT1-9 preparations stabilized only with glycerol, indicating that the presence of BSA had no effect on *N*-OH-AAF-mediated inactivation.

The effect of cofactor, substrate, and product on inactivation of rNAT1-9 and rNAT2-70D by *N*-OH-AAF was investigated. Inclusion of acetyl CoA in the incubation mixture resulted in protection of both rNAT1-9 and rNAT2-70D from inactivation by *N*-OH-AAF (Table 4). When AAB (0.25 mM), the acetyl acceptor in the *N*-OH-AAF/AAB transacetylation assay, was incubated with rNAT1-9 and *N*-OH-AAF, about 50% of the *N*-OH-AAF/AAB transacetylation activity was protected from inactivation (Table 4). In contrast, *N*-acetyl-AAB provided only about 21% protection. Substrate and product of rNAT2-70D were less effective as protecting agents. The substrate, PABA, provided less than 20% protection of rNAT2-70D from inactivation by *N*-OH-AAF, and no protection was observed when *N*-acetyl PABA was included in the incubation mixture (Table 4).

Previously it was demonstrated that nucleophiles provide partial protection from *N*-OH-AAF-mediated inactivation of partially purified hamster *N*-OH-AAF/AAB activity [22, 26]. Therefore, *N*-OH-AAF was incubated with the purified recombinant NATs in the presence and absence of nucleophiles prior to determination of residual enzyme activity. The results are presented in Table 4. The nucleophiles examined in this study protected both rNAT1-9 (*N*-OH-AAF/AAB activity) and rNAT2-70D (acetyl CoA/PABA activity) to similar extents, with the exception that *N*-acetylmethionine provided substantially more protection of rNAT1-9 than of rNAT2-70D. For both rNAT1-9 and rNAT2-70D, cysteine provided the greatest protection, whereas *N*-acetylcysteine provided the least protection. Each nucleophile was examined at 1- and 10-mM concentrations. At the higher concentration of nucleophile, a 2- to 5-fold increase in protection was observed.

The effect of nucleophiles on mechanism-based inactivation (Table 4) was determined in buffer containing 0.1 mM of DTT. The effect of DTT on mechanism-based

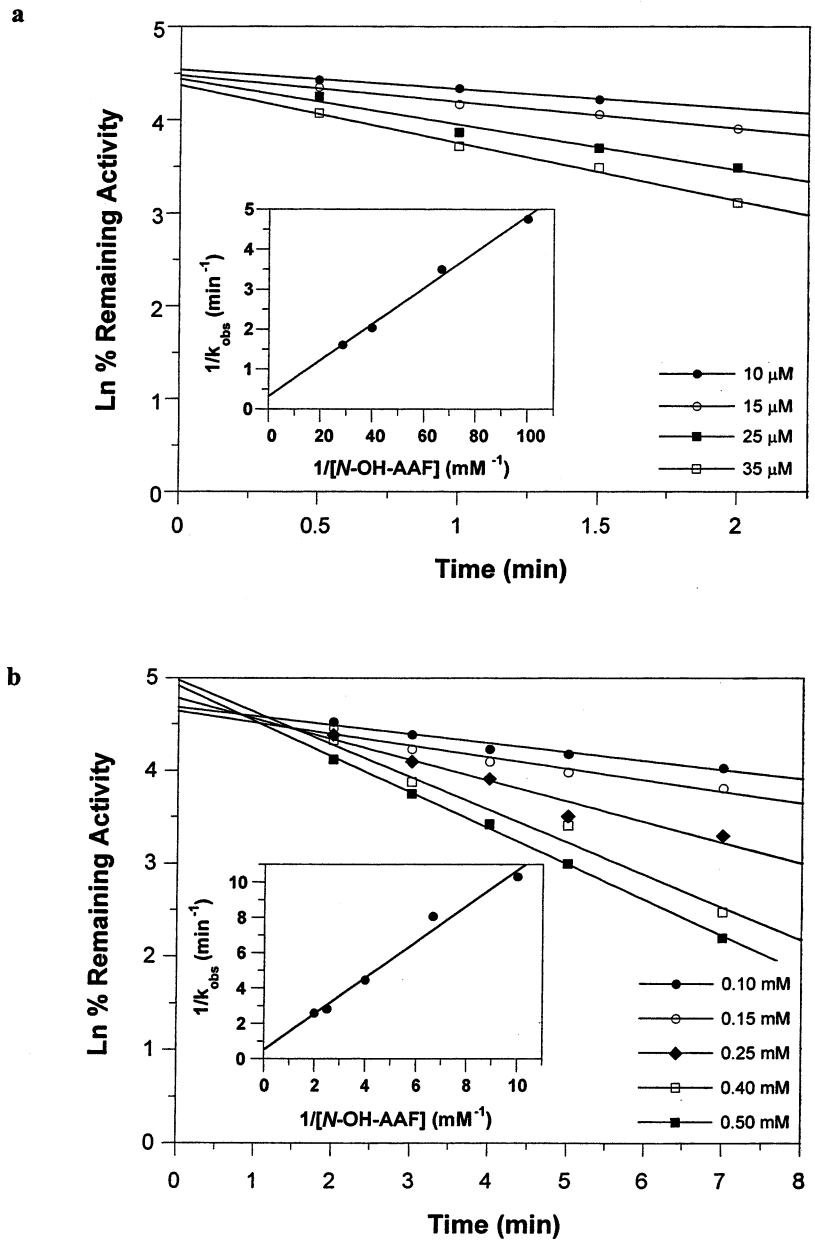

FIG. 3. Concentration- and time-dependent inactivation of rNAT1-9 and rNAT2-70D by N-OH-AAF. rNAT1-9 or rNAT2-70D was incubated for various time periods with the indicated concentrations of *N*-OH-AAF. At the indicated times, the remaining acetylation activity was determined as described in Materials and Methods. Data are the averages of two experiments, each performed in triplicate. *Inset*: Double-reciprocal plot of the apparent rate of inactivation (k_{obs}) as a function of N-OH-AAF concentration. (a) Inactivation of rNAT1-9 by N-OH-AAF. Control N-OH-AAF/AAB transacetylation activities of rNAT1-9 were 19.74 ± 0.5, 16.96 ± 0.9, 18.58 ± 1.9, and 20.46 ± 0.9 μmol/mg/min (mean ± range; N = 2, with each experiment performed in triplicate) for 0.5, 1.0, 1.5, and 2.0 min, respectively. The correlation coefficients for the fit of the points to the lines were 0.98 to 1.0. The correlation coefficient for the fit of the points to the line shown in the *inset* plot was 1.0. (b) Inactivation of rNAT2-70D by N-OH-AAF. Control acetyl CoA/PABA acetylation activities of rNAT2-70D were 68.46 ± 6.5, 65.84 ± 5.2, 68.15 ± 6.0, 70.50 ± 6.4, and 67.89 ± 5.0 μmol/mg/min (mean ± SD; N = 10, with each experiment performed in triplicate) for 2, 3, 4, 5, and 7 min, respectively. The correlation coefficients for the fit of the points to the lines were 0.98 to 1.0. The correlation coefficient for the fit of the points to the line shown in the *inset* plot was 0.99.

inactivation of rNAT1-9 by *N*-OH-AAF was investigated. Preliminary experiments indicated that increasing concentrations of DTT (0.1, 1.0, and 2.5 mM) resulted in lower percentages of rNAT1-9 activity being lost (95, 79, and 28%, respectively) (data not shown). Thus, concentrations of DTT greater than 0.1 mM appear to provide a significant protective effect independent of that afforded by the presence of other nucleophiles.

Determination of Kinetic Parameters for Inactivation of rNAT1-9 and rNAT2-70D by N-OH-AAF

Several concentrations of *N*-OH-AAF were incubated with either rNAT1-9 or rNAT2-70D for various time periods, and linear semilog plots of percent remaining activity versus time were obtained (Fig. 3). First-order inactivation rate constants (k_{obs}) were calculated from the slopes of the lines, and limiting rates of inactivation (k_i) and dissociation constants (K_I) were obtained from the double-reciprocal plots of k_{obs} versus inhibitor concentration according to the method of Kitz and Wilson [35] (Fig. 3). The values of the kinetic constants for inactivation by *N*-OH-AAF are listed in Table 5. There was no substantial difference between the kinetic parameters previously reported for partially purified NAT1 from hamster tissue and the homogeneous rNAT1-9. This further confirms the catalytic and functional relationship of the recombinant enzyme with NAT1. The limiting rate constants (k_i) varied less than two-fold between rNAT1-9 and rNAT2-70D; however, the dissociation constant (K_I) varied by more than 10-fold between the two recombinant enzymes. The k_i/K_I ratio is a second-order rate constant and represents

TABLE 5. Inactivation of NATs by *N*-OH-AAF: Kinetic parameters and partition ratios*

Enzyme	k_i (min^{-1})	K_I (mM)	k_i/K_I (min^{-1} mM^{-1})	r
NAT1†	1.95	0.089	21.9	
rNAT1-9	3.10	0.140	22.1	504
rNAT2-70D	1.86	1.88	1.0	137

*The kinetic constants, k_i (limiting rate constant) and K_I (inactivation affinity constant), were determined from the double-reciprocal plots of k_{obs} versus [*N*-OH-AAF] for several concentrations of *N*-OH-AAF (Fig. 3). The partition ratio, *r*, was determined from the plot of percent remaining activity versus the ratio of [*N*-OH-AAF] to [enzyme] (Fig. 4).

†Ref. 22. Hamster hepatic NAT1 was purified 2- to 3-fold from cytosol by fractionation with ammonium sulfate.

the selectivity of the inactivation process. Thus, rNAT1-9 was over 20 times more susceptible than rNAT2-70D to inactivation by *N*-OH-AAF.

Determination of Partition Ratios

The partition ratio, *r*, is the ratio of product release to inactivation events, and represents the efficiency of the mechanism-based enzyme inactivator [37]. Both purified rNAT1-9 and rNAT2-70D were incubated with increasing concentrations of *N*-OH-AAF until no additional inactivation occurred. A plot of the percentage of activity remaining versus the ratio of *N*-OH-AAF concentration to enzyme concentration was constructed (Fig. 4). The lower ratio data points were used to extrapolate a straight line through the x-axis. Assuming there is a 1:1 stoichiometry of inactivator and enzyme, the x-intercept gives the turnover number, i.e. the number of inactivator molecules required to inactivate each enzyme molecule. Since the turnover number includes the one molecule of inactivator needed to inactivate the enzyme, the partition ratio is the turnover number minus one [37]. The *r* value for rNAT2-70D inactivation by *N*-OH-AAF was about 4 times lower than that for rNAT1-9, indicating that fewer molecules of *N*-OH-AAF are required to inactivate one molecule of rNAT2-70D than of rNAT1-9 (Table 5).

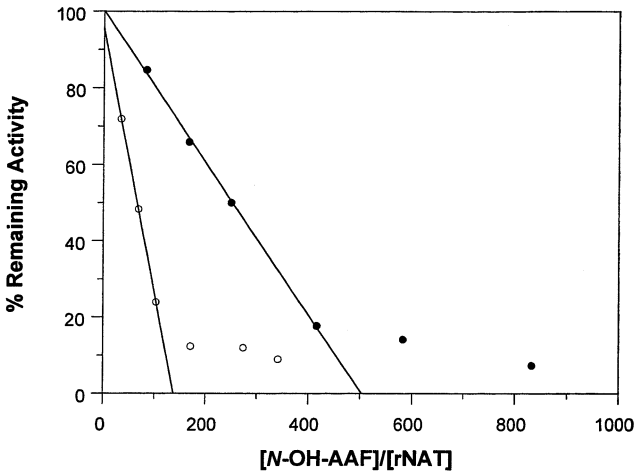

FIG. 4. Loss of *N*-OH-AAF activity as a function of the ratio of *N*-OH-AAF to rNAT1-9 (●) and rNAT2-70D (○). rNAT1-9 (1 μg, 0.06 μM) or rNAT2-70D (5 μg, 1.46 μM) was incubated with increasing concentrations of *N*-OH-AAF (5–50 μM, rNAT1-9; 0.05–0.5 mM, rNAT2-70D) until no additional inactivation occurred (2.5–10 min, rNAT1-9; 7–15 min, rNAT2-70D). When inactivation was complete, the remaining acetylation activity was determined as described in Materials and Methods. Each point represents the mean of two experiments, each performed in triplicate. The correlation coefficient for the fit of the points to the line was 1.0 for both rNAT1-9 and rNAT2-70D. The partition ratio was determined from the x-intercept as described in Materials and Methods.

DISCUSSION

Detailed biophysical studies, kinetic characterization, and x-ray crystallography of enzymes require a reliable source of large quantities of homogeneous protein. It has been a goal of this laboratory to clone, express, and purify large quantities of hamster recombinant NAT1 and NAT2. Our laboratory recently reported the large-scale expression of hamster rNAT2 as a fusion protein to a mutant DHFR. After the DHFR-rNAT2 fusion protein was cleaved, the rNAT2 portion was purified to homogeneity and designated rNAT2-70D [24]. The protocol allows the routine purification of more than 8 mg of homogeneous rNAT2-70D from 1 L of bacterial culture. To obtain comparable amounts of hamster recombinant NAT1, the expression system was modified by exchanging the *rNAT2* gene sequence for the hamster *rNAT1* gene sequence (Fig. 2a). Expression of the fusion protein and purification of the cleaved rNAT1 were carried out under conditions nearly identical to those described for rNAT2-70D [24].

The recombinant NAT1 (rNAT1-9) produced by thrombin cleavage of the fusion protein contains the amino acids Gly-Thr-Gln-Leu appended at the N-terminus of the native *NAT1* gene sequence (Fig. 2b). The rNAT1-9 amino acid sequence is identical to that of the previously reported rNAT1-8 derived from plasmid pPH8. The addition of the tetrapeptide appeared to have no appreciable effect on the activity or substrate specificity of rNAT1-8 [25]. As expected, the relative substrate specificity of rNAT1-9 was similar to that of NAT1 from hamster tissue and rNAT1-8 (data not shown). The expression and purification methods described herein for hamster rNAT1-9 afford a better yield and recovery than either the immunoaffinity based protocol for purification of hamster rNAT1-8 [25] or the protocol for purification of human rNAT1 [38]. It is also less labor intensive, inexpensive, and is able to accommodate large-scale preparations.

In the course of characterizing the enzymes, the relative abilities of rNAT1-9 and rNAT2-70D to catalyze the formation of DNA binding reactants from *N*-OH-AAF were determined (Table 2). Covalent adducts were produced at a much greater rate in the presence of rNAT1-9 than rNAT2-70D, a result consistent with substrate specificity data, indicating that hamster NAT2 does not readily catalyze reactions with *N*-OH-AAF as the acetyl donor [24,

34, 39]. Although rNAT2-70D did bioactivate *N*-OH-AAF to DNA reactive intermediates, the data suggest that NAT2 may not play a substantial role in the production of arylamine-DNA adducts by arylhydroxamic acids, such as *N*-OH-AAF, in hamster tissues.

The ability of various nucleophiles to compete with DNA for electrophilic species formed by rNAT1-9-mediated bioactivation of [^{14}C]*N*-OH-AAF was examined and compared with previous results with partially purified, unresolved hamster NATs [27]. The results were similar, indicating that the recombinant enzyme bioactivates *N*-OH-AAF to the same types of reactive species as the native enzyme. Two thiols, glutathione and cysteine, had little effect on DNA adduct formation, while adduct formation in the presence of ascorbic acid, thiourea, or potassium-*O*-ethylxanthate was only 25–36% of that formed when no agent was present in the incubation (Table 3). Ethylxanthate is particularly useful for *in vitro* electrophile trapping experiments because it is highly nucleophilic and, unlike glutathione and cysteine, it exists only in the negatively charged state at pH 7.0 [40]. 1,5-Dimethyl-4-imidazolethiol, a compound whose structure is based on antioxidant chemicals (ovothiols) isolated from sea urchin eggs, was the only thiol that inhibited DNA adduct formation. Ovothiols are both more nucleophilic and more reactive as one-electron donors than glutathione at physiological pH [41]. The preliminary data (Table 3) indicate that ascorbic acid, thiourea, potassium-*O*-ethylxanthate, and 1,5-dimethyl-4-imidazolethiol have similar effectiveness in protecting DNA against adduct formation. Due to their toxicities, thiourea and potassium-*O*-ethylxanthate are unlikely to be useful for preventing DNA adduct formation *in vivo*, but further studies with 1,5-dimethyl-4-imidazolethiol and structurally related agents may be warranted to evaluate their potential for *in vivo* use.

N-OH-AAF is a mechanism-based inactivator of hamster [22, 39] and rat [23, 42] NAT1. Previous studies with partially purified enzyme have not detected inactivation of NAT2 by *N*-OH-AAF and other arylhydroxamic acids [39]. In this study, the susceptibility of purified rNAT1-9 and rNAT2-70D to inactivation by *N*-OH-AAF was examined. Both rNAT1-9 and rNAT2-70D underwent irreversible, time-dependent inactivation that exhibited pseudo first-order kinetics and was saturable at higher *N*-OH-AAF concentrations. The maximal rate of inactivation of rNAT1-9 was obtained with 0.05 mM of *N*-OH-AAF, whereas 0.5 mM of *N*-OH-AAF was required to achieve saturation with rNAT2-70D. Preincubation with acetyl CoA resulted in nearly complete protection of both rNAT1-9 and rNAT2-70D from inactivation, and a decrease in the extent of inactivation also was observed when NAT substrates were included in the incubation mixtures (Table 4). Protection by cofactor and substrate is consistent with the criteria for a mechanism-based inactivation process [37]. It was demonstrated previously that mechanism-based inactivation of partially purified rat NAT1 with [^{14}C]*N*-OH-AAF resulted in covalent binding of radiolabel to protein, which correlated with loss of enzyme activity [42]. In preliminary experiments with rNAT1-9 that had been 95% inactivated by treatment with [^{14}C]*N*-OH-AAF, it was found that approximately 0.7 mol of ^{14}C remained associated to the enzyme after gel filtration and resolution of BSA and rNAT1-9 by HPLC (data not shown). This provides further evidence that formation of one or more covalent protein adducts is responsible for inactivation.

Previous studies provided evidence that mechanism-based inactivation of NAT1 by *N*-OH-AAF involves molecular species that react while complexed within the active site as well as electrophiles that diffuse away from the active site prior to reaction with NAT [22, 26]. Thus, experiments were conducted to determine the effect of nucleophiles on the extent of inactivation of the recombinant NATs by *N*-OH-AAF. Presumably, nucleophilic agents that protect the enzyme from inactivation do so by trapping electrophilic reactants that diffuse away from the active site, thereby preventing them from contributing to inactivation of the enzyme. In previous studies with hamster hepatic NAT1 that had been purified 2- to 3-fold, the presence of cysteine afforded 30–40% protection from inactivation by *N*-OH-AAF [22, 26]. However, 10 mM cysteine prevented all but 10–15% of loss of activity by a more highly purified rat NAT1 that was treated with *N*-OH-AAF [42] and, in the present study with homogenous recombinant NATs, cysteine almost completely prevented enzyme inactivation by *N*-OH-AAF (Table 4). The latter results may indicate that almost all loss of NAT activity is due to electrophiles that react with the protein after diffusing away from the active site. Alternatively, cysteine may be capable of trapping electrophiles while they are complexed with the active site, but before they can react with nucleophilic residues on the protein.

The rank order of effectiveness of the three thiol nucleophiles, cysteine, glutathione, and *N*-acetylcysteine (Table 4), was the inverse of the order of the pK_a values of their respective thiol groups [43]. This is consistent with their reaction primarily as thiolates with the electrophile(s) produced from *N*-OH-AAF. Further, the ineffectiveness of cysteine and glutathione in preventing DNA adduct formation (Table 3), in contrast to their protective effects against NAT inactivation, is consistent with the conclusion that different chemical intermediates are responsible for DNA adduct formation and NAT inactivation by bioactivated *N*-OH-AAF.

The kinetic parameters k_i and K_I were determined previously for inactivation of partially purified hamster NAT1 by *N*-OH-AAF [22]. The values obtained with partially purified NAT1 and the data generated in the present study with purified rNAT1-9 are strikingly similar, further confirming the identity and functionality of the recombinant enzyme and the utility of *N*-OH-AAF-mediated inactivation as a probe for comparison of NATs. The relative magnitudes of the second-order rate constants (k_i/K_I) indicate that rNAT1-9 is approximately 20 times more susceptible than rNAT2-70D to inactivation by

N-OH-AAF, and the 13-fold difference in K_I values establishes that the differential susceptibility results from the lower affinity of *N*-OH-AAF for rNAT2-70D. Determination of the partition ratios revealed that, although rNAT1-9 is more susceptible to inactivation than rNAT2-70D, inactivation of each molecule of rNAT1-9 requires approximately 4 times as many molecules of inactivator than is required for inactivation of rNAT2-70D. The higher turnover number determined for rNAT1-9 is consistent with the greater amount of DNA adduct formed from activation of *N*-OH-AAF by this enzyme (Table 2).

In summary, an efficient method for expression and purification of large quantities of hamster rNAT1 (rNAT1-9) has been developed. The purified recombinant enzymes rNAT1-9 and rNAT2-70D were characterized for their relative abilities to bioactivate *N*-OH-AAF to DNA binding reactants and were examined for their relative susceptibility to inactivation by *N*-OH-AAF. Consistent with substrate specificity data indicating that *N*-OH-AAF is preferentially a hamster NAT1 substrate, rNAT1-9 afforded a higher rate of DNA adduct formation by bioactivation of *N*-OH-AAF and was more susceptible than rNAT2-70D to mechanism-based inactivation by *N*-OH-AAF. The effect of nucleophiles on DNA binding and mechanism-based inactivation by *N*-OH-AAF indicates that the reactive species responsible for DNA adduct formation are different from those responsible for mechanism-based inactivation.

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