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Affinity Alkylation of Hamster Hepatic Arylamine *N*-Acetyltransferases: Isolation of a Modified Cysteine Residue

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SUMMARY

N-Acetyltransferases (NATs) play key roles in the detoxification and/or bioactivation of arylamines, arylhydroxylamines, arylhydroxamic acids, and hydrazines in mammalian tissues. In the present study, two hamster hepatic NATs (NAT I and NAT II) were separated, and each was purified >2000-fold by sequential ammonium sulfate fractionation, DEAE anion exchange chromatography, Sephadex G-75 gel filtration chromatography, aminoazobenzene-coupled affinity chromatography, and DEAE anion exchange high performance liquid chromatography. Both NAT I and NAT II were purified to near-homogeneity. The molecular masses of NAT I and NAT II were estimated to be 30.5 kDa and 32.6 kDa, respectively. 2-(Bromoacetylamino)fluorene (Br-AAF) and bromoacetanilide were synthesized and evaluated as affinity labels for NAT I and NAT II. Whereas Br-AAF was a highly selective inactivator of NAT II, bromoacetanilide inactivated both NAT I and NAT II in a similar fashion. Inactivation of NAT II by both Br-AAF and bromoacetanilide, and inactivation of NAT I by bromoacetanilide, followed pseudo-first-order kinetics. Relative rate constants (k_{obs}/[I]) for the two compounds indicate that Br-AAF is approximately 25 times more potent than bromoacetanilide as an inactivator of NAT II. Both acetylcoenzyme A (CoASAc) and 2-acetylaminofluorene protected NAT II from inactivation by Br-AAF, and CoASAc provided protection of both NAT I and NAT II activities from inactivation by bromoacetanilide, indicating that the inactivation by both bromoacetanilide and Br-AAF is active site directed. The irreversibility of the inactivation of NATs by Br-AAF and bromoacetanilide was demonstrated by the failure to recover transacetylase activities after gel filtration of enzyme preparations that had been preincubated with Br-AAF or bromoacetanilide. Preincubation of NAT II with CoASAc significantly reduced the incorporation of [^{14}C]Br-AAF into the enzyme, providing further evidence that the labeling is active site directed. In addition, pretreatment of NAT II with *N*-ethylmaleimide completely prevented the labeling of NAT II with [^{14}C]Br-AAF, which suggests that a cysteine thiol is the target nucleophile of Br-AAF. High performance liquid chromatography analysis of the hydrochloric acid hydrolysate of [^{14}C]Br-AAF-labeled NAT II revealed that 70% of total radioactivity is associated with *S*-carboxymethyl-L-cysteine, indicating that Br-AAF reacts primarily with a cysteine residue at the active site. These studies provide direct evidence that hamster hepatic NAT II contains an essential cysteine residue at the active site, and they establish the potential utility of Br-AAF for determining amino acid sequences in the active site of hamster hepatic NAT II.

NATs (EC 2.3.1.5) constitute a class of conjugation enzymes that are important because of their involvement in the metabolic activation/detoxification of arylamine and hydrazine drugs, as well as arylamine carcinogens. In addition, NATs exhibit a genetic polymorphism, which may have profound effects on drug therapy and on susceptibility to arylamine-induced carcinogenesis (1, 2).

NATs, which catalyze the transfer of the acetyl group from endogeneous CoASAc to the amino group of arylamines, are widely distributed in tissues of many mammalian species, including humans (3, 4). The NATs exist in multiple forms, which differ in their physical and biochemical properties and in the genetic regulation of their expression (2). Two NAT isozymes have been identified in hamster (5–7), rat (8), mouse (9–11), and human liver (12–15). Two NATs have been partially purified from hamster intestinal cytosol (6), and evidence supporting the existence of two NATs in rapid acetylator rabbit liver and intestine has been presented (16, 17).

In the hamster, one isozyme (NAT I) is polymorphically expressed and catalyzes CoASAc/PABA and CoASAc/AF transacetylation activities.[1] The other isozyme (NAT II) is monomorphically expressed and catalyzes virtually all of the arylhy-

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[1] The enzyme referred to as NAT I in the present paper corresponds to the polymorphic enzyme designated as AT-II in Ref. 7. NAT II corresponds to the monomorphic enzyme designated as AT-I in Ref. 7. Similarly, NAT I corresponds to the enzyme designated as polymorphic NAT in Refs. 5, 18, and 19 and as PABA NAT in Ref. 6. NAT II corresponds to the enzyme designated as monomorphic in Refs. 5, 18, and 19 and as AHAT in Ref. 6.

ABBREVIATIONS: NAT, *N*-acetyltransferase; CoASAc, acetylcoenzyme A; AAB, 4-aminoazobenzene; HPLC, high performance liquid chromatography; Br-AAF, 2-(bromoacetylamino)fluorene; 2-AAF, 2-acetylaminofluorene; AF, 2-aminofluorene; NEM, *N*-ethylmaleimide; CMC, *S*-carboxymethyl-L-cysteine; N,NAT, *N,N*-acetyltransferase; PABA, *p*-aminobenzoic acid; N-OH-AAF, *N*-hydroxy-2-acetylaminofluorene; VFK, 1-(fluoren-2-yl)-2-propen-1-one; DTT, DL-dithiothreitol; DMSO, dimethylsulfoxide; TCA, trichloroacetic acid; PITC, phenylisothiocyanate; PTC, phenylthiocarbamoyl; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

droxamic acid *N,O*-acyltransferase activity, N-OH-AAF/AAB N,NAT activity, and CoASAc/procainamide and CoASAc/sulfamethazine NAT activities, as well as some of the CoASAc/AF NAT activity (6, 7, 18, 19). Fig. 1 illustrates the NAT, *N,O*-acyltransferase, and N,NAT reactions that are catalyzed by hamster transacetylases. In addition, NATs can catalyze the *O*-acetylation of *N*-hydroxyarylamines (20–23). The *N,O*-acyltransferase and *O*-acetyltransferase reactions are bioactivation processes that result in the production of reactive electrophiles that bind covalently to cellular constituents.

The molecular basis of the hereditary acetylation polymorphism has been the subject of a number of investigations. It has been established that the gene for polymorphic NAT is absent from the slow acetylator rabbit phenotype (16, 24) but that mutations of the polymorphic gene are responsible for acetylation polymorphism in humans (13–15). Western blot analysis with purified hamster hepatic NATs demonstrated that the content of the monomorphic isozyme is relatively constant among individual animals, whereas the content of the polymorphic isozyme correlates with the phenotypic expression of PABA and AF NAT activities (7). Thus, the hamster acetylation polymorphism may result from structural variants of the polymorphic enzyme that are present in decreased levels in slow acetylator animals (7, 25).

Despite the advances in understanding of the acetylation polymorphism, there have been few reported studies on the amino acid residues involved in either the substrate binding or the catalytic mechanism of NATs. Although the precise role of the residues was not characterized, previous studies have suggested the presence of essential cysteine, arginine, and histidine residues in the hamster hepatic NATs (26, 27). The latter studies also provided some insight into possible differences in the active sites of hamster hepatic NAT I and NAT II. The results showed that the two isozymes differ, in that essential arginine residue(s) may be located in the active site of NAT I but not of NAT II (26).

Affinity labeling techniques have been widely utilized to study enzyme active site topology, to elucidate enzyme mechanisms, and to identify essential amino acid residues (28). Previously, it was shown that VFK, synthesized in this laboratory, is a selective affinity label for rat hepatic NAT II (29). Reports by Andres *et al.* (30) indicated that bromoacetanilide (Fig. 2) is an active site-directed inhibitor of rabbit hepatic NAT, and the target amino acid was identified as cysteine. For the present study, Br-AAF, which is structurally similar to VFK but differs in the type of reactive group (Fig. 2), has been synthesized as a potential affinity labeling agent for NATs. Affinity labeling studies with both bromoacetanilide and the novel compound Br-AAF were carried out with NATs isolated from outbred hamster liver. The results demonstrate that Br-AAF is a selective affinity labeling agent for hamster hepatic NAT II, whereas

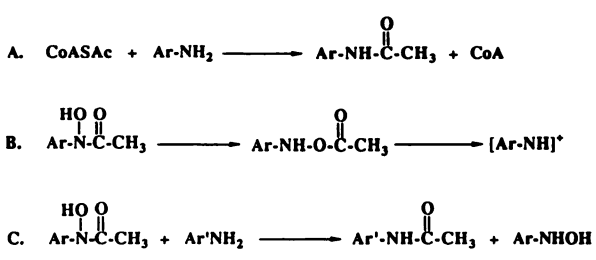

Fig. 1. Acetyltransferase-catalyzed reactions. A, CoASAc-dependent arylamine NAT. B, *N*-Arylhydroxamic acid *N,O*-acyltransferase. C, *N*-arylhydroxamic acid-dependent N,NAT.

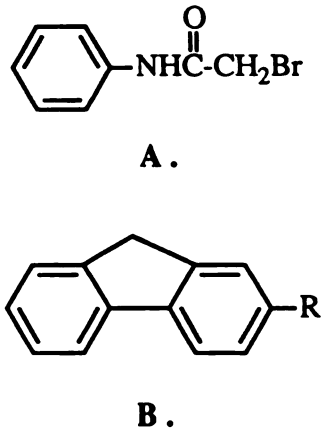

Fig. 2. A, Structure of bromoacetanilide. B, Structures of N-OH-AAF (R = $NOHCOCH_3$), VFK (R = $COCH = CH_2$), and Br-AAF (R = $NHCOCH_2Br$).

bromoacetanilide acts as an affinity labeling agent for both NAT I and NAT II. Purification procedures were developed for NAT I and NAT II, and the NAT II-selective affinity label [^{14}C]Br-AAF was used to identify an active site cysteine residue of NAT II. Some of these results have been reported in abstract form (31).

Experimental Procedures

Materials. PABA (sodium salt), DTT, CoASAc (trilithium salt), bovine serum albumin, tetrasodium pyrophosphate, ammonium sulfamate, NEM, Sephadex G-75–120, and hydrochloric acid solution (constant boiling) were purchased from Sigma Chemical Co. (St. Louis, MO). AAB and *N*-1-(naphthyl)ethylenediamine dihydrochloride were purchased from Eastman Kodak Co. (Rochester, NY). Epoxy-activated Sepharose 6B and PD-10 columns (1.5 × 5 cm) prepacked with Sephadex G-25M were purchased from Pharmacia Chemical Co. (Piscataway, NJ). Sodium nitrite, TCA, EDTA, bromophenol blue (free acid), ammonium persulfate, Brilliant Blue R-250, *N,N,N′,N′*-tetramethylethylenediamine, 2-mercaptoethanol, bisacrylamide, acrylamide, formaldehyde solution, DMSO, acetonitrile, SDS, Tris (free base), and glycine were purchased from Fisher Scientific Co. (Fairlawn, NY). 2-AAF, CMC, and triethylamine were obtained from Aldrich Chemical Co. Inc. (Milwaukee, WI). DEAE-Biogel A, Bio-Rad dye reagent concentrate, and SDS-PAGE molecular weight standards were obtained from Bio-Rad Laboratories (Richmond, CA). Ultrafiltration membranes (YM-10) were obtained from Amicon Corp. (Danvers, MA). Fluoro-Hance (autoradiography enhancer) was obtained from Research Products International Corp. (Mount Prospect, IL). PITC was obtained from Pierce (Rockford, IL). Immobilon P transfer membranes were obtained from Millipore (Bedford, MA). Ecoscint A was obtained from National Diagnostics (Manville, NJ). N-OH-AAF was synthesized as previously described (32). Coupling of AAB to epoxy-activated Sepharose 6B was performed as previously described (6, 33); dimethylformamide (15 ml) was used as a co-solvent for AAB. The efficiency of AAB coupling was 42–62% (34).

Enzyme preparation. Male Golden Syrian hamsters (50–70 g), obtained from Harlan Sprague Dawley (Indianapolis, IN), were lightly anesthetized with diethyl ether before decapitation. Livers were removed immediately, and gall bladders were dissected away from the livers. Livers were rinsed with cold 0.05 M sodium pyrophosphate buffer (pH 7.0, containing 1 mM DTT) and homogenized in 1 ml of buffer per g of liver, with a motor-driven Teflon/glass homogenizer. The homogenate was centrifuged at 105,000 × *g* for 60 min at 4°, and the resulting supernatant fraction was subjected to ammonium sulfate fractionation in an ice bath, as previously described (35). The remaining steps were carried out at 4°.

DEAE ion exchange chromatography. The ammonium sulfate pellets from the 35–50% fraction were dissolved in 0.02 M potassium phosphate buffer (pH 7.4, containing 1 mM DTT and 1 mM EDTA), to bring the protein concentration to approximately 50 mg/ml, and the solution (6 ml) was applied to a PD-10 column of Sephadex G-25M, to remove ammonium sulfate; a volume of 2.5 ml was applied to each PD-10 column, and the eluent was discarded. Then, the sample was eluted with 3.5 ml of 0.02 M potassium phosphate buffer (pH 7.4, containing 1 mM DTT and 1 mM EDTA), and the eluent was collected. Approximately 90% of the protein and NAT activity was recovered in the 3.5 ml of eluent. The combined eluent (10 ml) was applied to a DEAE ion exchange column (2.5 × 45 cm) that had been equilibrated with degassed 0.02 M potassium phosphate buffer (pH 7.4, containing 1 mM DTT and 1 mM EDTA). The column was then eluted with 30 ml of 0.02 M potassium phosphate buffer (pH 7.4, containing 1 mM DTT and 1 mM EDTA). Fractions (6 ml) were collected at a flow rate of 30 ml/hr. At fraction 6, application of a potassium chloride gradient (0–0.35 M) in degassed 0.02 M potassium phosphate buffer (total volume, 700 ml) was begun. This purification step resulted in the separation of NAT I and NAT II. The CoASAc/PABA NAT and N-OH-AAF/AAB N,NAT activities of each fraction were determined as described in Enzyme assays. The fractions that contained the peak of NAT I activity were pooled and concentrated under nitrogen to approximately 20% of the original volume, with an Amicon ultrafiltration cell fitted with a YM-10 membrane. Glycerol (10% final concentration) was added to stabilize enzymatic activity, and the NAT I solutions were stored at −70°. The fractions that contained the peak of NAT II activity were pooled, concentrated, and stored at −70° in 20% glycerol.

Sephadex G-75 gel filtration chromatography. The NAT I preparations purified by DEAE ion exchange chromatography (5.2 mg/ml; 1.3 ml) were applied to a column (1.5 × 30 cm) of Sephadex G-75, which had been equilibrated with degassed 0.02 M potassium phosphate buffer (pH 7.4, containing 1 mM DTT and 1 mM EDTA). The column was eluted with 150 ml of degassed buffer, and 3-ml fractions were collected at a flow rate of 30 ml/hr. The CoASAc/PABA NAT activity of each fraction was determined as described in Enzyme assays. Fractions containing the majority of the CoASAc/PABA transacetylase activity were combined.

AAB-coupled affinity chromatography. The pooled fractions of NAT I activity from Sephadex G-75 gel filtration chromatography (0.08 mg/ml; 10 ml) were applied to an AAB-coupled epoxy-activated Sepharose affinity column (1.5 × 15 cm) equilibrated with degassed 0.02 M potassium phosphate buffer (pH 7.4, containing 1 mM DTT and 1 mM EDTA). The column was eluted with 100 ml of degassed potassium phosphate buffer. Five-milliliter fractions were collected at a flow rate of 20 ml/hr. Fractions containing the peaks of the CoASAc/PABA transacetylation activity were combined, concentrated under nitrogen with an Amicon ultrafiltration cell with a YM-10 membrane, and stored at −70° in 10% glycerol. The NAT II preparation (1.31 mg/ml; 12 ml), separated and purified by DEAE ion exchange chromatography, was filtered through a PD-10 column of Sephadex G-25M. The column was eluted with 0.02 M potassium phosphate buffer (pH 7.4, containing 1 mM DTT and 1 mM EDTA), and the eluent, containing the majority of N-OH-AAF/AAB N,NAT activity, was collected. The eluent (0.58 mg/ml; 24 ml) was loaded onto an AAB affinity column (1.5 × 15 cm) equilibrated with degassed 0.02 M potassium phosphate buffer (pH 7.4, containing 1 mM DTT and 1 mM EDTA). The column was eluted with 150 ml of degassed buffer, and fractions (4 ml) were collected at a flow rate of 30 ml/hr. At fraction 31, application of a potassium chloride gradient (0–0.4 M) in degassed 0.02 M potassium phosphate buffer (total volume, 200 ml) was begun. The fractions that contained the majority of N-OH-AAF/AAB N,NAT activity were pooled, concentrated, and stored at −70° in 30% glycerol.

DEAE ion exchange HPLC. The NAT I preparation purified by AAB affinity chromatography was subjected to DEAE ion exchange HPLC with a Beckman HPLC system. The preparation (0.03 mg/ml; 2 ml) was applied to a HPLC DEAE Spherogel TSK 3SW column (7.5 mm × 7.5 cm). At 2 min, a potassium chloride gradient (0–0.8 M) in 0.02 M potassium phosphate buffer (pH 7.4, containing 1 mM DTT and 1 mM EDTA) was started, and it was continued over a 50-min period, at a flow rate of 0.3 ml/min. UV absorbance was monitored at 280 nm, and 1-min fractions were collected with a fraction collector. An aliquot of each fraction was assayed for CoASAc/PABA NAT activity, as described in Enzyme assays, and the fraction containing the peak of the activity was stored overnight in the elution buffer at −70°. The NAT II preparation purified by AAB affinity chromatography was passed through a PD-10 column of Sephadex G-25M, and the eluent, which contained the majority of the N-OH-AAF/AAB N,NAT activity, was collected. The eluent (2 ml; 0.08 mg/ml) was injected onto the DEAE HPLC column, and a potassium chloride gradient (0–0.8 M) in 0.02 M potassium phosphate buffer (pH 7.4, containing 1 mM DTT and 1 mM EDTA) was applied over a 50-min period. Flow rate was maintained at 0.3 ml/min, and 1-min fractions were collected. The fraction that contained the peak of N-OH-AAF/AAB N,NAT activity was stored overnight in the elution buffer, at −70°.

SDS-PAGE. SDS-PAGE of the NAT I and NAT II isozymes (5–10 μg) purified through DEAE ion exchange HPLC was carried out by the method of Laemmli (36), with a Tris-glycine buffer system, pH 8.3, containing 0.1% SDS. The acrylamide concentration was 12.5% in the resolving gel and 5% in the stacking gel. Because potassium ions cause the precipitation of SDS (37), protein samples were passed through a PD-10 column of Sephadex G-25M, eluted with 0.05 M sodium pyrophosphate buffer (pH 7.0, containing 1 mM DTT), and then concentrated with an Amicon Centricon 10 microconcentrator. Samples were resuspended in buffer containing 0.125 M Tris, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.002 M EDTA, 1 mM DTT, and 0.01% bromophenol blue. After denaturation of samples by heating at 95° for 3 min, samples were applied to the stacking gel. Electrophoresis was performed with a constant voltage of 150 V/gel for 4 hr, at which time the bromophenol blue dye front reached the bottom of the gel. Protein bands were detected by Coomassie blue staining.

Molecular weight determination. The molecular weights of the NAT I and NAT II isozymes were estimated from the calibration curves constructed for relative mobility versus logarithm of the molecular weight of the standard proteins. The calibration standards and their molecular weights were as follows: phosphorylase *b*, M_r 97,400; bovine serum albumin, M_r 66,200; ovalbumin, M_r 42,699; carbonic anhydrase, M_r 31,000; soybean trypsin inhibitor, M_r 21,500; and lysozyme, M_r 14,400. The molecular weights of the purified NAT I and NAT II were calculated by linear regression of the calibration curves, based on their mobility under the same electrophoretic conditions.

Inactivation of NAT I by bromoacetanilide and Br-AAF. To determine the concentration dependence of the inactivation of NAT I by either bromoacetanilide or Br-AAF, partially purified NAT I (final protein concentration, 3.13 mg/ml) in 0.05 M sodium pyrophosphate buffer (pH 7.0, containing 1 mM DTT) was incubated with 0.01 ml of either an ethanolic solution of bromoacetanilide (0.25–1.6 mM final concentration) or a DMSO solution of Br-AAF (0.5–1.0 mM final concentration) at 37°, in a final volume of 0.12 ml. The final concentrations of ethanol (3%) and DMSO (4%) had no effect on the control NAT activities. At the end of the incubation period (1 min with Br-AAF and 2 min with bromoacetanilide), 8-μl aliquots were withdrawn from the incubation mixtures and placed into assay tubes, which contained 0.6 mM CoASAc, 0.1 mM PABA, and 0.05 M sodium pyrophosphate buffer (pH 7.0, containing 1 mM DTT), in a volume of 0.492 ml (62.5-fold dilution). A 62.5-fold dilution of the incubation mixtures was sufficient to terminate the inactivation of NAT I by bromoacetanilide. The remaining CoASAc/PABA NAT activity was determined as described in Enzyme assays. To examine the time dependence of the inactivation of NAT I by bromoacetanilide, incubation mixtures containing 0.11 ml of partially purified NAT I (final protein concentration, 3.13 mg/ml) in 0.05 M sodium pyrophosphate buffer (pH 7.0, containing 1 mM DTT) and 0.01 ml of 14.4 mM bromoacetanilide were incubated at 37°. At the indicated times, 8-μl aliquots were withdrawn and assayed

for the remaining CoASAc/PABA NAT activity, as described above. A semilogarithmic plot of percentage of remaining activity versus incubation time was constructed for 1.2 mM bromoacetanilide, and the k_{obs} value was obtained from the slope of the plot. The effect of CoASAc on the inactivation of NAT I by bromoacetanilide was examined to determine whether the inactivation is active site directed. Incubation mixtures contained 0.1 ml of partially purified NAT I (final protein concentration, 3.13 mg/ml) and 0.01 ml of CoASAc (0.01–0.5 mM final concentration). After incubation for 1 min at 37°, 0.01 ml of 14.4 mM bromoacetanilide was added and incubated for an additional 2 min. Then, 8-μl aliquots were removed from the incubation mixtures, and the remaining CoASAc/PABA NAT activity was measured as described in Enzyme assays.

Inactivation of NAT II by bromoacetanilide and Br-AAF. Incubation mixtures contained NAT II that had been partially purified by DEAE ion exchange chromatography, with a sufficient amount of 0.05 M sodium pyrophosphate buffer (pH 7.0, containing 1 mM DTT) to bring the protein concentration to 2.50 mg/ml, in a final volume of 0.12 ml. Incubations were started by the addition of 0.01 ml of either bromoacetanilide (0.25–0.9 mM final concentration) or Br-AAF (0.01–0.07 mM final concentration) at 37°. At the end of the incubation period (1 min with Br-AAF and 2 min with bromoacetanilide), 0.02-ml aliquots were withdrawn from the incubation mixtures and placed into assay tubes, which contained 0.46 ml of 0.05 M sodium pyrophosphate buffer (pH 7.0, containing 1 mM DTT) and 0.02 ml of N-OH-AAF/AAB (final concentrations of 0.5 and 0.15 mM, respectively, in 95% ethanol solution) (25-fold dilution). A 25-fold dilution of the incubation mixtures was sufficient to stop the inactivation of NAT II by either bromoacetanilide or Br-AAF. The remaining N-OH-AAF/AAB N,NAT activity was determined as described in Enzyme assays. For the time-dependence experiments, partially purified NAT II (final protein concentration, 2.50 mg/ml) in 0.05 M sodium pyrophosphate buffer (pH 7.0, containing 1 mM DTT) was incubated with either 0.7 mM bromoacetanilide or 0.05 mM Br-AAF at 37°, in a final volume of 0.12 ml. At various time intervals, 0.02-ml aliquots were withdrawn and analyzed for remaining N-OH-AAF/AAB N,NAT activity as described in Enzyme assays. The ability of substrates or products to protect NAT II against inactivation by bromoacetanilide and Br-AAF was studied by preincubating the enzyme preparation in the presence of CoASAc or 2-AAF before the addition of either bromoacetanilide or Br-AAF. Partially purified NAT II (final protein concentration, 2.50 mg/ml) in 0.05 M sodium pyrophosphate buffer (pH 7.0, containing 1 mM DTT) was incubated with 0.01 ml of either CoASAc (0.05–1.0 mM final concentration) or an ethanolic solution of 2-AAF (0.5–1.0 mM final concentration) at 37°, in a final volume of 0.11 ml. At the end of a 1-min incubation, 0.01 ml of either 8.4 mM bromoacetanilide or 0.6 mM Br-AAF was added, and incubations were continued for either 2 min (bromoacetanilide) or 1 min (Br-AAF). Aliquots (0.02 ml) were removed at the end of the incubation periods and placed into assay tubes. The remaining N-OH-AAF/AAB N,NAT activity was determined as described in Enzyme assays.

Inactivation and gel filtration of NATs. Partially purified NAT I (final protein concentration, 2.72 mg/ml) in 0.05 M sodium pyrophosphate buffer (pH 7.0, containing 1 mM DTT) was incubated at 37° with 1.2 mM bromoacetanilide, in a final volume of 0.75 ml. After incubation for 2 min, 0.7-ml aliquots were removed and passed through a PD-10 column of Sephadex G-25M. The column was eluted with two portions (2.3 and 1.3 ml) of sodium pyrophosphate buffer (0.05 M). The fraction that contained the majority of enzyme activity (1.3 ml) was collected and kept on ice. The protein concentration of this fraction was determined and adjusted for analysis of the remaining CoASAc/PABA NAT activity, as described in Enzyme assays. To examine whether the inactivation of NAT I by bromoacetanilide is irreversible over an extended time period, the inactivated enzyme fractions were then incubated at 4°, and the remaining CoASAc/PABA NAT activity was measured at various time intervals. To investigate the irreversibility of the inactivation of NAT II by either bromoacetanilide or Br-AAF, incubation mixtures contained 0.7 ml of partially purified NAT II (final protein concentration, 2.50 mg/ml) in 0.05 M sodium pyrophosphate buffer (pH 7.0, containing 1 mM DTT). Incubations at 37° were started by addition of 0.05 ml of either 8.4 mM bromoacetanilide or 0.6 mM Br-AAF. At the end of the incubation period (2 min with bromoacetanilide and 1 min with Br-AAF), incubation mixtures (0.7 ml) were passed through PD-10 columns of Sephadex G-25M and eluted with two portions (2.3 and 1.3 ml) of 0.05 M sodium pyrophosphate buffer (pH 7.0, containing 1 mM DTT). The second portion of the eluent (1.3 ml), which contained the majority of enzyme activity, was then incubated at 4°, and the remaining N-OH-AAF/AAB N,NAT activity was determined at various time intervals, as described in Enzyme assays.

Affinity labeling of NAT II with [^{14}C]Br-AAF. NAT II (0.17 mg/ml), partially purified by AAB affinity chromatography, was incubated at 37° with a DMSO solution (0.05 ml) of [^{14}C]Br-AAF (0.02 mM final concentration; specific activity, 1 mCi/mmol) in 0.05 M sodium pyrophosphate buffer (pH 7.0, containing 1 mM DTT). The final volume was 0.5 ml. The content of DMSO in the incubation mixture was 3%, which had no effect on the control NAT activity. After incubation for 5 min, proteins were precipitated by the addition of 0.5 ml of 20% TCA. Samples were kept on ice for 15 min and then centrifuged for 15 min. The protein pellets were redissolved in 50 μl of buffer containing 0.125 M Tris, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.002 M EDTA, 1 mM DTT, and 0.01% bromophenol blue and were subjected to SDS-PAGE as described above. To examine the effect of CoASAc and NEM on the labeling of the enzyme with [^{14}C]Br-AAF, partially purified NAT II was preincubated with either 0.5 mM CoASAc or 2 mM NEM for 1 min at 37°. After further incubation for 5 min in the presence of [^{14}C]Br-AAF (0.02 mM), 0.5 ml of 20% TCA was added. After centrifugation of the mixture, the precipitated protein was dissolved in 0.125 M Tris buffer and subjected to SDS-PAGE as described above. Autoradiographs of the SDS gel were obtained with Kodak X-Omat AR X-ray film, in the presence of enhancer (Fluoro-Hance). Exposure was for 2 weeks at −80°. The radiolabeled bands in the autoradiographs were digitized and analyzed by using the Image software program on a MacIntosh II.

Identification of the amino acid(s) radiolabeled by Br-AAF. SDS-PAGE of the NAT II protein labeled with [^{14}C]Br-AAF (18 mCi/mmol) was carried out as described above, except that 2-mercaptoethanol and DTT were omitted from the 0.125 M Tris buffer. Electroblotting of the protein from the SDS gel to the Immobilon P membrane was performed at a constant voltage of 80 V, for 3 hr, with a Tris-glycine buffer system (0.25 mM Tris, 1.92 mM glycine) in 15% methanol. The protein band on the membrane was visualized by staining with 0.5% Coomassie Blue in 40% methanol for 15 min.

The radiolabeled NAT II band was cut from the Immobilon P membrane. Complete acid hydrolysis of NAT II was conducted with constant boiling 6 N HCl at 105° for 24 hr, in an evacuated and sealed hydrolysis tube. After cooling of the hydrolysates, the amino acids were extracted from the membrane with 0.1 N HCl, 30% methanol (3 × 200 μl), as described previously (38). The amino acid hydrolysates were then completely dried with a Speed Vac concentrator, to remove the hydrochloric acid, and were derivatized with 5 μl of PITC, by reaction for 5 min at room temperature (39). After the derivatized solution was dried with a Speed Vac concentrator, the resulting PTC-amino acids were dissolved in 60 μl of 0.14 M sodium acetate (pH 6.35) containing 0.05% triethylamine (solvent A)/60% acetonitrile in water (solvent B) (9:1).

The PTC-amino acids were analyzed by reverse phase HPLC, with a Beckman HPLC system. The PTC-amino acid mixtures (20 μl) were applied to a C18 Ultrasphere reverse phase HPLC column (4.6 mm × 15 cm), and a gradient of acetonitrile was applied at a flow rate of 1 ml/min, at room temperature. The gradient used was a linear increase from 10% solvent B to 53% solvent B over 20 min, followed by 53% solvent B to 100% solvent B over the next 5 min. The eluate was monitored for absorbance at 254 nm, and 0.5-min fractions were collected manually. Aliquots (0.2 ml) of each fraction were dissolved in

10 ml of Ecoscint A, and radioactivity was quantified by liquid scintillation counting by using a Beckman 5800 liquid scintillation counter, which has a machine efficiency of 75%. In a separate experiment, CMC was derivatized with PITC and subjected to HPLC analysis in the same way as described for the hydrolysates of the labeled NAT II.

Enzyme assays. For the CoASAc/PABA NAT assay, reaction mixtures contained either 0.1 ml of NAT I, partially purified by DEAE ion exchange chromatography, or 0.01-ml aliquots of fractions from the other chromatographic columns, 0.0167 ml of 6 mM CoASAc (final concentration, 0.6 mM), 0.05 ml of 0.333 mM PABA (final concentration, 0.1 mM), and a sufficient amount of 0.05 M sodium pyrophosphate buffer (pH 7.0, containing 1 mM DTT) to bring the incubation volume to 0.1667 ml. Incubations at 37° were initiated by addition of PABA and were terminated after 2 min by addition of 0.333 ml of cold 5% TCA. After 10 min of centrifugation, the supernatant was analyzed spectrophotometrically by Weber's modification of the Bratton-Marshall procedure, as previously described (40). CoASAc or enzyme was omitted from control incubations.

For the N-OH-AAF/AAB N,NAT assay, incubation mixtures contained either 0.48 ml of NAT II, partially purified by DEAE ion exchange chromatography, or 0.1-ml aliquots of fractions from the other chromatographic columns, 0.02 ml of N-OH-AAF/AAB solution (final concentrations of 0.5 and 0.15 mM, respectively) in 95% ethanol, and a sufficient amount of 0.05 M sodium pyrophosphate buffer (pH 7.0, containing 1 mM DTT) to bring the incubation volume to 0.5 ml. The reactions were started at 37° by addition of substrates. After incubation for 2 min, the reactions were terminated by addition of 0.5 ml of cold 20% TCA (in ethanol/water, 1:1). After centrifugation of the mixtures, acetylation of AAB was determined spectrophotometrically, according to the method of Booth (41). Enzyme was omitted from control incubations.

Protein assay. Protein concentrations were determined by the dye binding method of Bradford (42), with bovine serum albumin as the standard.

Synthesis of bromoacetanilide and [^{14}C]Br-AAF. Bromoacetanilide was prepared as described by Andres *et al.* (30), except that bromoacetyl bromide was used rather than bromoacetyl chloride. For the synthesis of [^{14}C]Br-AAF, 2-bromo[1-^{14}C]acetic acid (5.64 mg, 0.5 mCi, 12.3 mCi/mmol) was dissolved in anhydrous tetrahydrofuran (3 ml) and was transferred to a three-neck flask containing bromoacetic acid (63.8 mg, 0.46 mmol). The solution was cooled to −5°, and hydroxybenzotriazole (67.5 mg, 0.5 mmol) in tetrahydrofuran (3 ml) was added, followed by slow dropwise addition of 2-aminofluorene (90.62 mg, 0.5 mmol) in tetrahydrofuran (3 ml). Dicyclohexylcarbodiimide (113.3 mg, 0.5 mmol) in tetrahydrofuran (3 ml) was added in a slow dropwise manner. The reaction mixture was stirred at 0° for 1.5 hr and at room temperature for 2 hr. The tetrahydrofuran was evaporated to dryness under reduced pressure at 37°. The residue was stirred with acetone (30 ml). The resulting suspension was filtered, and the residue was washed with acetone (5 ml) and tetrahydrofuran (5 ml). The washings were combined with the filtrate and were evaporated to dryness under reduced pressure. The product was obtained by column chromatography of the residue (silica gel 60, 60–200, 6 g), with chloroform as the eluent. Recrystallization from cold chloroform/petroleum ether (30–60°) gave [^{14}C]Br-AAF as brown-yellow crystals (122.7 mg; 81%; mp, 197°, decomposed); thin layer chromatography (silica gel/chloroform) gave a single spot (R_f, 0.37; radiochemical purity, >99%; specific activity, 1 mCi/mmol). The compound was identical in all respects to an authentic sample of nonradiolabeled Br-AAF.

Results

Purification of NATs. To accomplish the goals of this research, it was necessary to achieve a high degree of purification of NAT I and NAT II, so that the proteins could be readily distinguished by chromatographic means and so that the results of affinity labeling of NAT II with [^{14}C]Br-AAF could be interpreted with certainty. Sequential ammonium sulfate fractionation, DEAE anion exchange chromatography, Sephadex G-75 gel filtration chromatography, AAB affinity chromatography, and DEAE HPLC resulted in purification of the two isozymes to near-homogeneity. Both NAT I and NAT II activities were purified >2000-fold from the 105,000 × *g* supernatant fraction, with overall yields of 4%. Tables 1 and 2 summarize typical purifications.

DEAE ion exchange chromatography resulted in the separation of NAT I and NAT II, as previously reported (5). Also, as previously reported by Hein *et al.* (5), the peak NAT I and NAT II activities were eluted at approximately 0.27 M and 0.33 M KCl, respectively. Although Sepharose 6B-AAB was initially designed as an affinity column for NAT II, to separate the NAT I and NAT II isozymes (6), AAB affinity chromatography also contributed to significant purification of NAT I and was, therefore, included in the purification sequence for NAT I. An improvement in the NAT II purification by affinity chromatography was the application of a potassium chloride gradient (0–0.4 M) instead of the previously used isocratic solution of 0.4 M KCl (6). Sephadex G-75 gel filtration chromatography was used in the purification of NAT I, to remove proteins of high molecular mass, but this step was not required for achievement of the required purification of NAT II. DEAE anion exchange HPLC was successful in removing most of the remaining traces of contaminating proteins from both NAT I and NAT II.

The purity of the NAT I and NAT II isozymes was examined by SDS-PAGE and Coomassie Blue staining. As shown in Fig. 3, both NAT I and NAT II isozymes obtained by HPLC were nearly homogeneous. The molecular masses of NAT I and NAT II were estimated by comparing their electrophoretic mobility with that of standard proteins of known molecular mass and were calculated to be 30.5 kDa and 32.6 kDa, respectively.

The objective of the studies described in the following sections was to obtain data that would support the development of Br-AAF and/or bromoacetanilide as radiolabeled probes for characterization of the active site properties of NAT I and NAT II. Partially purified NAT I and NAT II, obtained by DEAE ion exchange chromatography (Tables 1 and 2), were used for all experiments with nonradiolabeled bromoacetanilide and Br-AAF.

Inactivation of NAT I by bromoacetanilide and Br-AAF. Preliminary experiments indicated that NAT I activity was inhibited by relatively low concentrations of bromoacetanilide. Thus, the concentration and time dependence of the bromoacetanilide-mediated inactivation processes was studied. As shown in Fig. 4A, inactivation of NAT I activity by bromoacetanilide was dependent upon the concentration of bromoacetanilide. Saturation of the inactivation process was observed at 1.2 mM bromoacetanilide, implying that the process involves reversible binding of the reagent at the active site before irreversible inactivation. Under prolonged incubation (10 min), complete inactivation of NAT I activity was obtained with 0.5 mM bromoacetanilide (results not shown).

Incubation of 1.2 mM bromoacetanilide with NAT I resulted in a time-dependent loss of enzymatic activity, which followed pseudo-first-order kinetics. A pseudo-first-order rate constant (k_{obs}) of 0.65 min^{-1} was determined from the slope of the semilogarithmic plot of percentage of control activity versus time (Fig. 4A, *inset*). To demonstrate that bromoacetanilide is an active site-directed inhibitor for NAT I, the ability of

TABLE 1
Purification of hamster hepatic NAT I

Purification step	Total protein	Total activity	Specific activity[a]	Purification	Recovery
	mg	*μmol/min*	*μmol/mg/min*	*fold*	*%*
105,000 × *g* supernatant	8970	160	0.018	1.00	100
35–50% $(NH_2)_2SO_4$	2410	110	0.047	2.63	65.3
DEAE ion exchange	116	94.2	0.820	45.9	55.5
G-75 Sephadex	5.49	16.2	4.76	266	30.0
AAB affinity	0.35	10.8	30.4	1700	19.5
HPLC DEAE	0.04	1.45	36.4	2038	4.09

[a] Activities are expressed as CoASAc/PABA transacetylation rates.

TABLE 2
Purification of hamster hepatic NAT II

Purification step	Total protein	Total activity	Specific activity[a]	Purification	Recovery
	mg	*μmol/min*	*μmol/mg/min*	*fold*	*%*
105,000 × *g* supernatant	8970	147	0.016	1.00	100
35–50% $(NH_4)_2SO_4$	2410	74.5	0.030	1.82	47.2
DEAE ion exchange	55.9	22.2	0.439	26.8	16.5
AAB affinity	0.84	15.3	19.2	1170	8.60
HPLC DEAE	0.05	1.15	42.0	2560	3.83

[a] Activities are expressed as N-OH-AAF/AAB transacetylation rates.

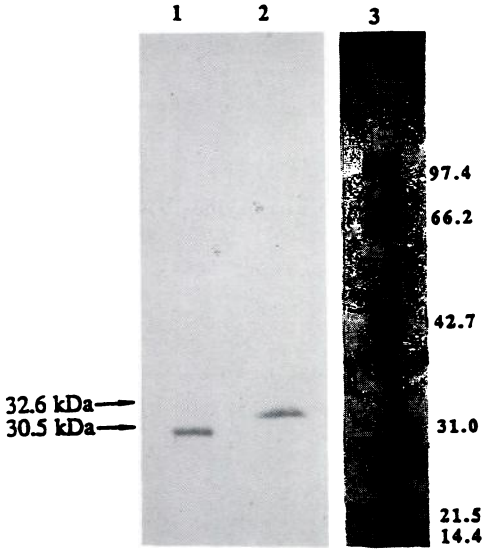

Fig. 3. SDS-PAGE of purified hamster hepatic NAT I and NAT II. SDS-PAGE was performed as described in Experimental Procedures. Protein bands were detected by Coomassie Blue staining. *Lane 1*, 4 μg of NAT I purified by DEAE ion exchange HPLC. *Lane 2*, 4 μg of NAT II purified through DEAE ion exchange HPLC. *Lane 3*, molecular weight markers.

CoASAc to protect NAT I from inactivation by bromoacetanilide was examined. Shown in Fig. 5A are the results of the CoASAc protection experiment. The presence of CoASAc (0.1 mM) provided complete protection of NAT I against inactivation, suggesting that the inactivation of NAT I by bromoacetanilide is active site directed.

Experiments also were conducted to determine the effect of Br-AAF on NAT I activity. A high concentration of Br-AAF (1.0 mM) was required to produce 50% inactivation, indicating that Br-AAF is not a potent inactivator of NAT I (data not presented). Concentrations of >1.0 mM could not be used, due to the insolubility of Br-AAF. Thus, further studies on the inactivation of NAT I by Br-AAF were not conducted.

Inactivation of NAT II by bromoacetanilide and Br-AAF. Partially purified NAT II was inactivated by bromoacetanilide in a concentration-dependent fashion, and the inactivation process was saturated at 0.7 mM bromoacetanilide (Fig. 4A). The inactivation of NAT II with 0.7 mM bromoacetanilide exhibited pseudo-first-order kinetics, as indicated by the linear semilogarithmic plot of percentage of control activity versus time (Fig. 4A, *inset*). The pseudo-first-order rate constant (k_{obs}) for inactivation of NAT II under these conditions was determined, from the slope of the plot shown in Fig. 4A (*inset*), to be 1.15 min^{-1}. To determine whether inactivation of NAT II by bromoacetanilide is due to modification of the active site, the ability of CoASAc to protect the NAT II activity from inactivation was tested. Results presented in Fig. 5A show that CoASAc provided, in a concentration-dependent manner, protection of NAT II from inactivation by bromoacetanilide, with complete protection being achieved at 1.0 mM CoASAc.

In contrast to the effect of Br-AAF on NAT I activity, preliminary studies showed that NAT II was inactivated rapidly by low concentrations of Br-AAF. Therefore, the concentration and time dependence of the inactivation of NAT II by Br-AAF was investigated. Incubation of NAT II with Br-AAF resulted in a concentration-dependent inhibition of the NAT II activity, with a complete loss of activity being produced within 1 min by 0.05 mM Br-AAF (Fig. 4B). As shown in Fig. 4B (*inset*), inactivation of NAT II by 0.05 mM Br-AAF appeared to follow pseudo-first-order kinetics. The pseudo-first-order rate constant (k_{obs}) obtained for Br-AAF from the data shown in Fig. 4B (*inset*) is 2.12 min^{-1}. The effect of CoASAc and the *N*-acetylation product 2-AAF on the inactivation of NAT II by Br-AAF was evaluated, to determine whether the inactivation is an active site-directed reaction. Fig. 5B shows that the presence of either CoASAc or 2-AAF afforded substantial protection against the Br-AAF-mediated inactivation of NAT II. The abilities of CoASAc and 2-AAF to reduce the extent of inactivation of NAT II by Br-AAF strongly suggest that Br-AAF is an active site-directed reagent.

Irreversibility of NAT inactivation. After treatment with either Br-AAF or bromoacetanilide, the NAT preparations were subjected to gel filtration, as described in Experimental Procedures. The failure to reverse the inhibition of either NAT

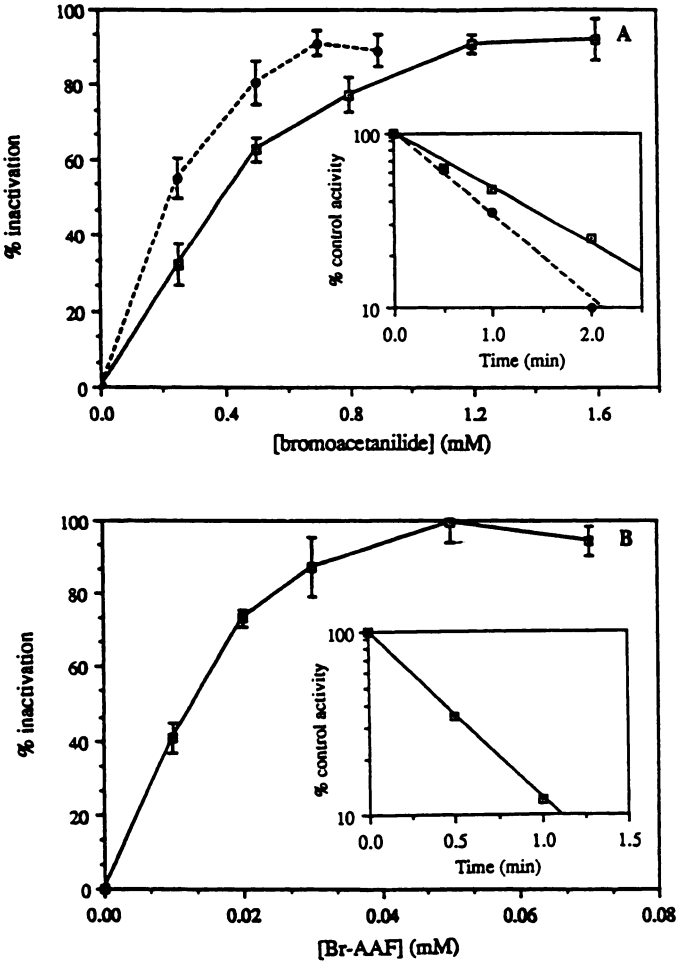

Fig. 4. Concentration-dependent inactivation of hamster hepatic NATs by bromoacetanilide and Br-AAF. A, Incubations of partially purified hamster hepatic NAT I or NAT II were conducted at 37° in the presence of various concentrations of bromoacetanilide (0.25–1.6 mM with NAT I, 0.25–0.9 mM with NAT II). At the end of a 2-min incubation, aliquots of the incubation mixtures were withdrawn and transferred to the assay tubes. The remaining CoASAc/PABA NAT (□) and N-OH-AAF/AAB N,NAT (●) activity was determined as described in Experimental Procedures. Each data point represents the mean ± standard deviation of three determinations. Control activities (determined in the absence of bromoacetanilide and expressed in units of nmol/mg of protein/min) were 671.4 ± 24.6 (mean ± standard deviation, five experiments) for CoASAc/PABA NAT activity and 283.5 ± 16.9 (mean ± standard deviation, three experiments) for N-OH-AAF/AAB N,NAT activity. *Inset*, time-dependent inactivation of NATs by bromoacetanilide. Partially purified hamster hepatic NAT I (□) and NAT II (●) were incubated with bromoacetanilide (1.2 mM and 0.7 mM, respectively). Aliquots were withdrawn at the indicated time points and assayed for remaining enzyme activity. B, Incubations of the partially purified hamster hepatic NAT II were conducted at 37° in the presence of various concentrations of Br-AAF (0.01–0.07 mM). At the end of a 1-min incubation, aliquots were withdrawn and assayed for the remaining N-OH-AAF/AAB N,NAT activity, as described in Experimental Procedures. Each data point represents the mean ± standard deviation of three determinations. Control activities (determined in the absence of Br-AAF) were 284.3 ± 31.7 nmol/mg of protein/min (mean ± standard deviation, three experiments). *Inset*, time-dependent inactivation of NAT II by Br-AAF. Partially purified hamster hepatic NAT II was incubated with Br-AAF (0.05 mM). Aliquots were withdrawn and assayed for the remaining N-OH-AAF/AAB N,NAT activity, as described in Experimental Procedures.

I or NAT II activity in these experiments suggests that the enzymes had been covalently modified. As a test of the resistance to hydrolysis of the covalent modification produced by Br-AAF and bromoacetanilide, the inactivated and gel-filtered enzyme preparations were incubated for an extended period at 4°. The experiments were conducted at 4° because prolonged incubation of untreated control preparations of NAT I and NAT II at higher temperatures results in a substantial loss of activity. Thus, it was necessary to conduct the experiments under conditions that would allow the detection of any activity that resulted from reactivation of the inactivated enzymes. Incubation for 22–27 hr produced no recovery of NAT I activity

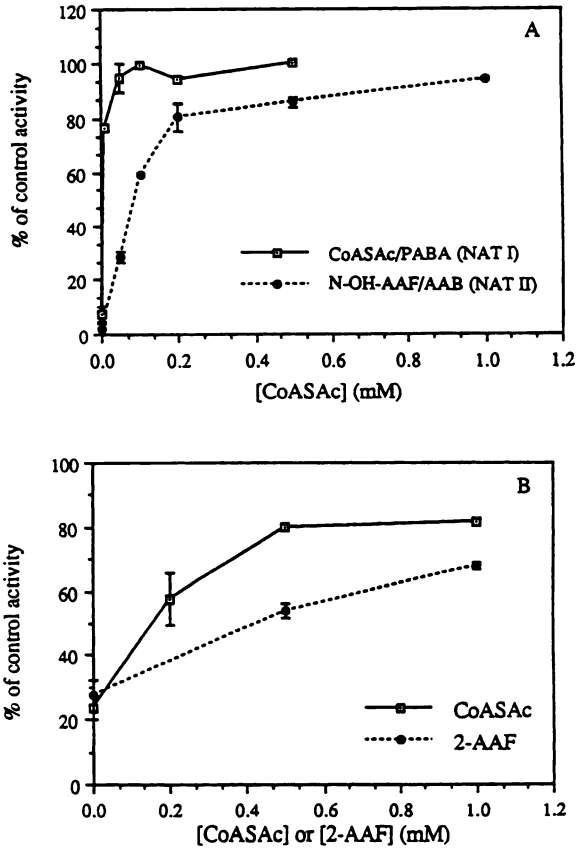

Fig. 5. A, Inactivation of hamster hepatic NAT I and NAT II by bromoacetanilide and effect of CoASAc. Incubations of hamster hepatic NAT I and NAT II, purified by DEAE ion exchange chromatography, were conducted in the presence of either 1.2 mM bromoacetanilide (NAT I) or 0.7 mM bromoacetanilide (NAT II) and the indicated concentrations of CoASAc. Aliquots were removed at the end of a 2-min incubation and were assayed for the remaining CoASAc/PABA NAT or N-OH-AAF/AAB N,NAT activity, as described in Experimental Procedures. Data points represent the mean ± range of two experiments, each conducted in triplicate. Control activities (determined in the absence of bromoacetanilide) were 588.4 ± 61.6 nmol/mg of protein/min (mean ± standard deviation, five experiments) for CoASAc/PABA NAT activity and 242.0 ± 20.2 nmol/mg of protein/min (mean ± standard deviation, six experiments) for N-OH-AAF/AAB N,NAT activity. B, Inactivation of hamster hepatic NAT II by Br-AAF and effect of CoASAc and 2-AAF. Hamster hepatic NAT II, partially purified by DEAE ion exchange chromatography, was incubated at 37° with 0.05 mM Br-AAF in the presence of either CoASAc or 2-AAF. At the end of the incubation period, aliquots were withdrawn and assayed for the remaining N-OH-AAF/AAB N,NAT activity, as described in Experimental Procedures. Data points represent the mean ± range of two experiments, each conducted in triplicate. Control activities (determined in the absence of Br-AAF and expressed in units of nmol/mg of protein/min) were 297.6 ± 4.1 (mean ± standard deviation, three experiments).

after treatment with bromoacetanilide and no recovery after treatment of NAT II with either Br-AAF or bromoacetanilide (data not presented).

Affinity labeling of NAT II with [^{14}C]Br-AAF. The use of HPLC-purified preparations of NAT II for affinity labeling studies with Br-AAF was hampered due to the low recovery of the enzyme. Thus, NAT II that had been carried through the AAB affinity chromatography step was used as an alternative. Preliminary studies indicated that incubation of NAT II, partially purified by AAB affinity chromatography, with 0.02 mM Br-AAF caused complete inactivation of the enzyme (data not presented). The selectivity of Br-AAF for NAT II, compared with other proteins in the preparation, was examined by labeling partially purified NAT II with 0.02 mM [^{14}C]Br-AAF. Shown in Fig. 6 is the result of both the SDS-PAGE and the autoradiogram obtained from the SDS-PAGE of labeled NAT II. The result (Fig. 6, *right, lane 1*) indicates that Br-AAF is highly specific for NAT II, because no other protein band was labeled in the presence of [^{14}C]Br-AAF.

To obtain additional evidence that the affinity labeling process is active site directed, the effect of CoASAc on the labeling of the enzyme with [^{14}C]Br-AAF was studied. The presence of 0.5 mM CoASAc, the concentration that provided maximum protection of NAT II from inactivation, reduced the incorporation of [^{14}C]Br-AAF by approximately 50%, as indicated by densitometric analysis (Fig. 7) of the autoradiograph (Fig. 6, *right, lanes 1* and *2*). These results support the conclusion that the major site of labeling of NAT II with [^{14}C]Br-AAF is at the active site.

Identification of the amino acid residue(s) of NAT II alkylated by Br-AAF. Previous studies have shown that incubation of partially purified NAT II with NEM, a sulfhydryl reagent, resulted in the complete inactivation of the enzyme, indicating that a cysteine residue(s) may be essential for catalytic activity (27). It was anticipated that NEM might block the incorporation of [^{14}C]Br-AAF into the enzyme by covalent binding to the accessible cysteine residue(s), if labeling of NAT II by [^{14}C]Br-AAF involves alkylation of cysteine residue(s). Therefore, the effect of NEM on the labeling of partially purified NAT II with [^{14}C]Br-AAF was examined. As shown in Fig. 6 *right* (*lane 3*), the presence of 2 mM NEM completely prevented the labeling of the enzyme with [^{14}C]Br-AAF, suggesting that sulfhydryl groups may be the principal target nucleophile(s) of [^{14}C]Br-AAF.

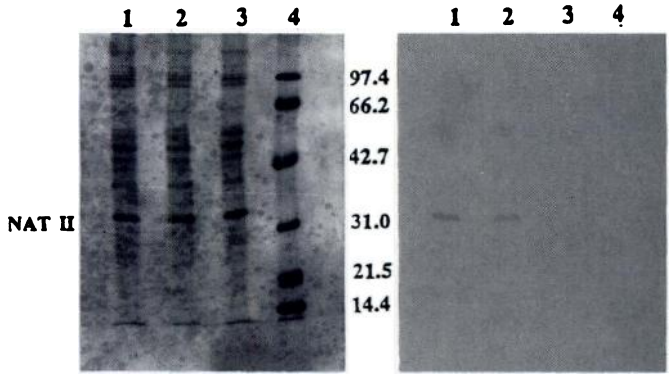

Fig. 6. Labeling of hamster hepatic NAT II with [^{14}C]Br-AAF and effect of CoASAc and NEM. *Left, lane 1*, 85 μg of partially purified NAT II labeled with [^{14}C]Br-AAF; *lane 2*, 85 μg of partially purified NAT II labeled with [^{14}C]Br-AAF in the presence of 0.5 mM CoASAc; *lane 3*, 85 μg of partially purified NAT II labeled with [^{14}C]Br-AAF in the presence of 2 mM NEM; *lane 4*, molecular weight markers. *Right*, autoradiograph obtained from the sample preparations shown on the *left*.

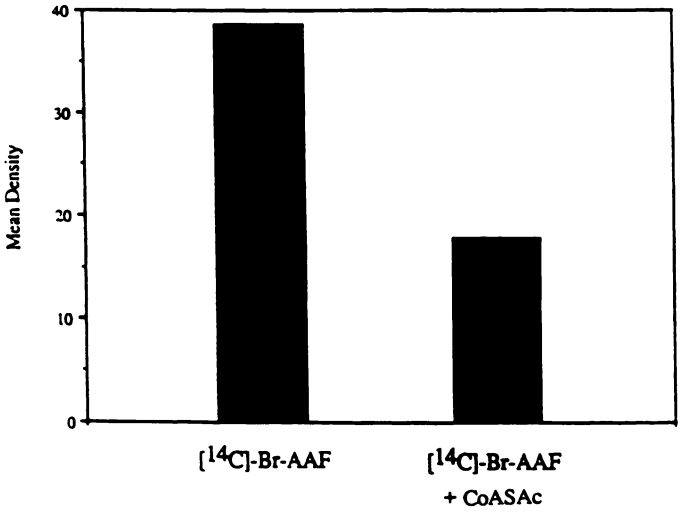

Fig. 7. Effect of CoASAc on the labeling of hamster hepatic NAT II with [^{14}C]Br-AAF. The autoradiograph shown in Fig. 6 (*right, lanes 1* and *2*) was digitized and analyzed with the Image software program, on a MacIntosh II, and is expressed as mean density per band.

To obtain direct evidence that a cysteine residue(s) is alkylated by [^{14}C]Br-AAF, the labeled NAT II protein mixture was subjected to SDS-PAGE and transferred to an Immobilon P membrane. Then, the labeled NAT II band was isolated after Coomassie Blue staining and was subjected to complete acid hydrolysis, in 6 N HCl, at 105° for 24 hr. Reverse phase HPLC analysis of the PTC-derivatized amino acid hydrolysate was carried out at room temperature. In preliminary studies in which SDS-PAGE of the labeled NAT II was carried out in the presence of DTT and 2-mercaptoethanol, the radioactive peak with a retention time of 6.5 min was barely detectable. It was reasoned that, during protein denaturation at 95°, the reactivity of the sulfhydryl groups present in DTT and 2-mercaptoethanol may be sufficient to cleave the linkage formed in the reaction between Br-AAF and the active site cysteine. Thus, the effect of 2-mercaptoethanol and DTT on the recovery and distribution of radioactivity eluted from HPLC was examined by omitting the compounds from the denaturation process. The recovery of radioactivity was increased by 1.5–2-fold when 2-mercaptoethanol and DTT were omitted. Radioactive peaks were eluted at identical retention times regardless of whether 2-mercaptoethanol and DTT were used, but the radioactive peak that eluted at 6.5 min was significantly increased in their absence.

Fig. 8 presents typical HPLC chromatograms of both the sample hydrolysate and authentic PTC-derivatized CMC. About 70% of the total radioactivity was associated with a single amino acid, with a retention time of 6.5 min (Fig. 8C). The major radiolabeled amino acid peak corresponds to CMC, which is the product that is expected to be formed from hydrolysis of the covalent conjugate produced by the reaction between Br-AAF and cysteine. These results further support the hypothesis that a cysteine residue(s) at the active site is the primary target nucleophile of Br-AAF.

Discussion

Hepatic arylamine NATs from a number of species have been purified to homogeneity. These include enzymes from pigeon

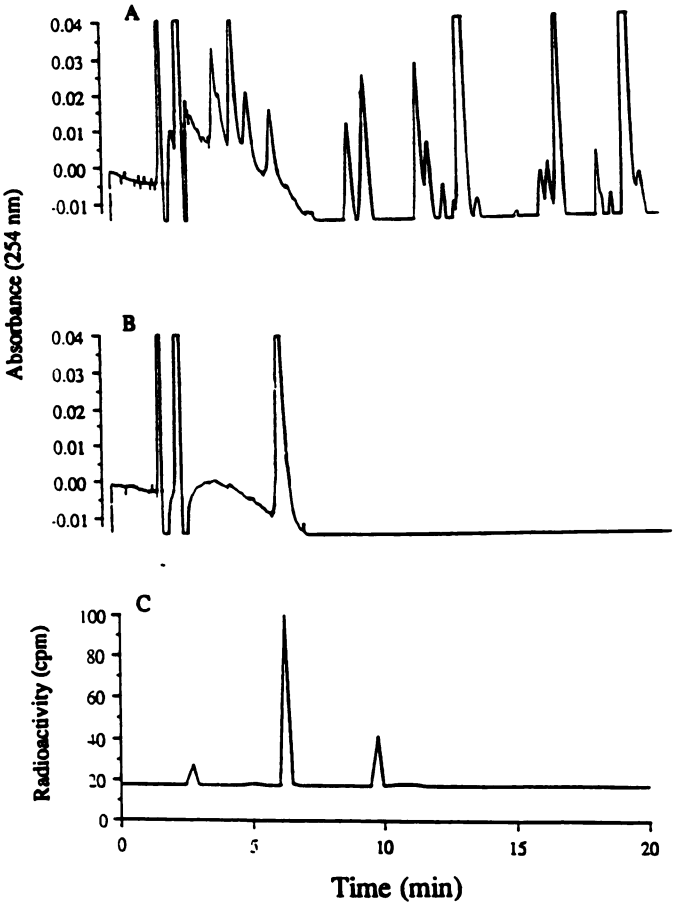

Fig. 8. Identification of the amino acid of NAT II that is alkylated by [^{14}C] Br-AAF. NAT II, partially purified by affinity chromatography, was incubated with 0.02 mM [^{14}C]Br-AAF (18 mCi/mmol). The radiolabeled NAT II was isolated by SDS-PAGE and electroblotting and was subjected to complete hydrochloric acid hydrolysis. After derivatization with PITC, the PTC-amino acid hydrolysates were subjected to reverse phase HPLC analysis. A, Absorbance profile (254 nm) of the PTC-amino acid hydrolysates. B, Absorbance profile (254 nm) of authentic PTC-CMC. C, Elution profile of the ^{14}C radioactive peaks detected in the eluents from reverse phase HPLC analysis of the PTC-amino acid hydrolysates.

(43), chicken (44), rabbit (45), and hamster (7, 19, 46). The estimated molecular masses of the NATs were similar, ranging from 30 kDa to 35 kDa. Allaben and King (47) reported a molecular mass of 38.5 kDa for a homogeneous preparation of rat liver arylhydroxamic acid *N,O*-acyltransferase. Purification procedures described in the present paper yielded nearly homogeneous preparations of hamster hepatic NAT I and NAT II, with molecular masses of 30.5 kDa and 32.6 kDa, respectively, which are comparable to those reported by Ozawa *et al.* (7). Recently, Trinidad *et al.* (19) described the purification of polymorphic hamster hepatic NAT (NAT I) to homogeneity and reported a molecular mass of 35 kDa. The reason for the discrepancy between the molecular masses reported for NAT I remains unclear.

Plapp (28) has discussed criteria for evaluation of affinity labeling reagents for enzymes. Such reagents should exhibit saturation kinetics, and preferably they are studied under conditions that permit the observation of pseudo-first-order kinetics. Protection by substrates and products against inactivation provides evidence that an affinity label is an active site-directed reagent. Finally, the most useful affinity labels cause a covalent modification of the enzyme that results in irreversible inactivation. In the present investigation, bromoacetanilide and Br-AAF were subjected to several tests of these criteria, to determine their suitability for further development as molecular probes of the active sites of hamster hepatic NAT I and NAT II.

The rationale for the design of Br-AAF was its structural relationship to N-OH-AAF, as well as the reported structure-activity relationships for substrates and inhibitors of NAT II (8, 48). Although both hamster hepatic NAT I and NAT II catalyze the CoASAc-dependent *N*-acetylation of AF, only the monomorphic isozyme, NAT II, catalyzes the N-OH-AAF-dependent acetylation of arylamines (N,NAT activity) and the N-OH-AAF-dependent *N,O*-acetyltransfer process (6, 7, 19, 46). Thus, it was anticipated that the structural similarities of VFK and Br-AAF to N-OH-AAF might lend them selectivity as inhibitors of NAT II. Indeed, VFK (Fig. 2) is a potent and selective inactivator of both rat hepatic NAT II (29) and hamster hepatic NAT II[2] but, because Br-AAF is more readily prepared in radiolabeled form than is VFK, Br-AAF was evaluated for selectivity, as described in this paper.

Br-AAF caused a very rapid and irreversible inactivation of NAT II, in a concentration-dependent manner, that appeared to follow pseudo-first-order kinetics. The inactivation was inhibited by the presence of either the cofactor CoASAc or the *N*-acetylation product 2-AAF. The latter results support the proposal that Br-AAF is an active site-directed reagent for NAT II. Br-AAF was a much less effective inactivator of NAT I than of NAT II. Whereas complete inactivation of NAT II was achieved with 0.05 mM Br-AAF, a 20-fold higher concentration was required to cause a 50% reduction in NAT I activity. The selectivity of Br-AAF as an affinity label for hamster hepatic NAT II is similar to the selectivity exhibited by VFK for the rat liver (29) and hamster liver NAT II isozymes.[2]

The effects of bromoacetanilide on NAT I and NAT II activities were examined to obtain further insight into the differences in the active site requirements of the two isozymes. In contrast to the relative ineffectiveness of Br-AAF, bromoacetanilide completely inactivated NAT I. The inactivation process followed pseudo-first-order kinetics and was saturated at 1.2 mM bromoacetanilide, which is comparable to the result reported by Andres *et al.* for rabbit hepatic NAT (K_i = 0.67 mM) (30). The irreversible nature of the inactivation and the protection by CoASAc from inactivation confirmed that bromoacetanilide acts as an active site-directed inhibitor for hamster hepatic NAT I.

In addition to its effectiveness as an inactivator of NAT I, bromoacetanilide also was an affinity labeling agent for NAT II. The inactivation of NAT II by bromoacetanilide was concentration dependent, saturable, and irreversible and followed pseudo-first-order kinetics. The cofactor CoASAc provided protection of NAT II against inactivation by bromoacetanilide, providing further evidence that the compound is an active site-directed reagent for NAT II.

The saturation concentration of bromoacetanilide and the concentration of Br-AAF that caused complete loss of activity were used to measure the time-dependent inactivation of NAT II. From these data, k_{obs} values of 1.15 min^{-1} and 2.12 min^{-1} were calculated for bromoacetanilide and Br-AAF, respectively.

[2] M. J. Wick and P. E. Hanna, unpublished observations.

A widely used parameter for comparison of the relative reactivities of enzyme inactivators is $k_{obs}/[I]$ (49, 50). The values calculated for Br-AAF and bromoacetanilide are shown in Table 3 and indicate that, under the conditions of these experiments, Br-AAF is approximately 25 times more potent than bromoacetanilide as an inactivator of NAT II.

VFK (Fig. 2), which is structurally similar to Br-AAF, in that both molecules contain a chemically reactive moiety attached to the 2-position of the fluorene ring, also selectively inactivates the rat and hamster liver NAT isozyme (NAT II) that catalyzes *N*-acetyl group transfer from *N*-arylhydroxamic acids to arylamines (N,NAT activity) and *N,O*-acyltransfer reactions. The selectivity of VFK and Br-AAF for NAT II is consistent with the structure-activity results of Mangold and Hanna (51), who found that *N*-(4-substituted phenyl)hydroxamic acids that contain large hydrophobic substituents in the 4-position are better acetyl donors in N,NAT-catalyzed reactions than are hydroxamic acids with smaller lipophilic substituents.

Based on the above-described results of the affinity labeling experiments with Br-AAF and bromoacetanilide, Br-AAF was selected for synthesis in radiolabeled form for further investigation of the molecular properties of NAT II. Due to the low recovery of enzymatic activity, it was not practical to use the most highly purified preparation of NAT II for this study. However, it was possible to identify and isolate the NAT II protein from a partially purified enzyme preparation by comparing the mobility of the proteins on SDS gels with that of nearly homogeneous NAT II. The specificity of the labeling of NAT II with [^{14}C]Br-AAF was clearly demonstrated by the results from SDS-PAGE and autoradiography of the labeled NAT II preparation. In agreement with the results of the experiments in which it was shown that CoASAc protected NAT II from inactivation by Br-AAF, the labeling of NAT II with [^{14}C]Br-AAF was substantially reduced in the presence of CoASAc (Figs. 6 and 7), indicating that the labeling was active site directed.

Chemical modification studies with NEM indicated that a cysteine residue(s) is essential to the catalytic activity of hamster hepatic NAT II and may be located at the active site (27). Although the possibility that NEM causes a conformational change of NAT II that prevents the reaction of [^{14}C]Br-AAF with the enzyme cannot be excluded, the finding that NEM completely blocked the labeling of NAT II with [^{14}C]Br-AAF (Fig. 6) suggests that a sulfhydryl group(s) is the primary target nucleophile(s) of Br-AAF. Definitive evidence that cysteine(s) is the amino acid predominantly labeled by Br-AAF was provided by the identification of a radiolabeled CMC peak in the hydrolysate of [^{14}C]Br-AAF-labeled NAT II. HPLC analysis of the hydrolysate showed that the majority of the radioactivity (70% of total) was associated with a peak that had a retention time identical to that of an authentic derivatized CMC standard. Similar to these results, Andres *et al.* (30) reported that bromoacetanilide alkylates an active site cysteine residue, resulting in the complete inactivation of a rabbit hepatic NAT. The acetyl enzyme intermediate in the rabbit and pigeon NAT-catalyzed reaction has been previously characterized as a cysteinyl thioester (43, 45). Thus, the active site cysteine residue modified by Br-AAF may be of functional importance in the binding of the acetyl moiety.

To obtain further evidence that the radioactive peak with a retention time of 6.5 min was the PTC derivative of CMC, a mixture of the radioactive peak and authentic derivatized CMC was rechromatographed, in an attempt to demonstrate coelution as a single peak. Consistent with the results previously reported (52), the derivatized CMC proved to be unstable when subjected to rechromatography. However, the decomposition patterns of the radioactive peak from the derivatized hydrolysate and the authentic derivatized CMC were identical, further confirming that the radioactive peak with a retention time 6.5 min is, indeed, CMC (results not shown).

Although most of the radioactivity was eluted in a single peak, about 30% of the radioactivity was consistently distributed into two other peaks. These findings, considered in conjunction with the results of the protection experiments, in which it was found that the presence of either CoASAc or 2-AAF provided protection of NAT II activity only up to 80% of control levels (Fig. 5), may indicate that residues not directly involved in substrate or product binding may undergo alkylation by Br-AAF. Previous studies with diethylpyrocarbonate suggested that a histidine residue(s) is essential for the catalytic activity of NAT II but may not be located in the binding site of the enzyme (27). Because bromoacetylated compounds are chemically reactive with nucleophilic functional groups such as the imidazole moiety of histidine (53), it is possible that at least part of the 30% of the eluted radioactivity not accounted for by CMC might represent an essential histidine residue(s) of NAT II that had been alkylated by Br-AAF.

In summary, the present study demonstrates that Br-AAF is an active site-directed inactivator of hamster hepatic NAT II and that this affinity label alkylates an active site cysteine residue. Br-AAF appears to offer important advantages for the study of NAT II, not only because it exhibits a high degree of selectivity in the alkylation of NAT II, compared with NAT I, but also because it specifically alkylates NAT II in the presence of other proteins. Because NAT II is difficult to purify, a reagent that will function as a specific affinity label for non-homogeneous enzyme preparations should be of considerable utility. It is anticipated that this new molecular probe will be suitable for the isolation and characterization of active site peptides from NAT II. The recent determination of the complete sequence of the monomorphic NAT (NAT II) from hamster liver (54) indicates that future investigations involving affinity labeling, site-directed mutagenesis, and, perhaps, X-ray crystallography will provide important insight into the active site architecture and catalytic mechanisms of NATs.

TABLE 3

Rate constants for inactivation of NATs by bromoacetanilide and Br-AAF

Pseudo-first-order rate constants (k_{obs}) were obtained from the slopes of the plots of log percentage of control activity versus time, with 0.05 mM Br-AAF and 0.7 mM bromoacetanilide for NAT II and with 1.2 mM bromoacetanilide for NAT I. Results are expressed as the mean of two experiments.

	$k_{obs}/[I]$	
	NAT I	NAT II
	$sec^{-1}\ M^{-1}$	
Bromoacetanilide	8.99	27.5
Br-AAF	ND[a]	707

[a] ND, not determined.

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