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Suicide Inactivation of Hamster Hepatic Arylhydroxamic Acid *N*,*O*-Acyltransferase

A Selective Probe of *N*-Acetyltransferase Multiplicity

PATRICK E. HANNA, R. BRUCE BANKS, AND VIRGINIA CREEDON MARHEVKA

Departments of Medicinal Chemistry and Pharmacology, University of Minnesota, Minneapolis, Minnesota 55455

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SUMMARY

Kinetic parameters (k_i and K_I) were determined *in vitro* for the suicide inactivation of hamster hepatic *N*-arylhydroxamic acid *N*,*O*-acyltransferase by *N*-hydroxy-2-acetamidofluorene and *N*-hydroxy-4-acetamidobiphenyl. The inhibition of hamster hepatic *N*-arylhydroxamic acid *N*,*O*-acyltransferase by *N*-hydroxy-2-acetamidofluorene was not reversed by incubation with cysteine. Partial protection of the enzyme against inactivation was observed with low molecular weight nucleophiles (e.g., cysteine). Hamster hepatic CoASAc-dependent *N*-acetyltransferases were inactivated irreversibly by incubation with *N*-hydroxy-2-acetamidofluorene. *p*-Aminobenzoic acid CoASAc-dependent *N*-acetyltransferase activity, but not sulfamethazine CoASAc-dependent *N*-acetyltransferase activity, was protected against inactivation when cysteine was included in the incubation mixtures. Therefore, although hamster hepatic CoASAc-dependent sulfamethazine *N*-acetyltransferase may be associated with *N*-arylhydroxamic acid *N*,*O*-acyltransferase, the CoASAc-dependent *p*-aminobenzoic acid N-acetyltransferase appears to be a different enzyme. The use of *N*-arylhydroxamic acids as suicide substrates is a promising technique for probing the mechanism of *N*-arylhydroxamic acid *N*,*O*-acyltransferase-mediated reactions, for exploring the relationships between *N*-arylhydroxamic acid *N*,*O*-acyltransferase and CoASAc-dependent *N*-acetyltransferases, and for selective inactivation *in vitro* of multiple forms of CoASAc-dependent *N*-acetyltransferases.

INTRODUCTION

Metabolic activation of carcinogenic arylamides (e.g., 2-acetamidofluorene) requires initial *N*-hydroxylation by a cytochrome P-450-dependent polysubstrate monooxygenase system (1). The *N*-hydroxylated metabolites, or *N*-arylhydroxamic acids (I) (Fig. 1), are proximate carcinogens which, in most cases, are more potent than the parent compounds when administered to experimental animals (2). Subsequent biochemical transformations convert the arylhydroxamic acids to reactive electrophiles containing nitrogen-centered leaving groups. These electrophiles (ultimate carcinogens) are believed to initiate neoplasia by covalent modification of DNA or other critical macromolecules (3).

Among the enzymes which catalyze the formation of electrophiles from arylhydroxamic acids, sulfotransferase and AHAT[1] have been most extensively investigated. Sulfotransferase requires 3′-phosphoadenosine 5′-phosphosulfate as a cofactor and converts arylhydroxamic acids to the corresponding sulfate esters (4). This enzyme has been found only in the hepatic cytosol (5, 6) and nuclear fractions (7) of rats and a few other species. AHAT is believed to catalyze the conversion of arylhydroxamic acids to *N*-acyloxyarylamines (II) (Fig. 1) (8, 9). This transformation leads to net isomerization of the arylhydroxamic acid, and a two-step mechanism involving an acyl-enzyme intermediate has been proposed (Fig. 1) (9). Heterolytic cleavage of the N—O bond of II (Fig. 1) leads to the formation of resonance-stabilized aryl nitrenium ions (III) which react readily with a variety of biological nucleophiles. AHAT activity has been demonstrated in the cytosol of several tissues of a variety of species (10, 11); multiple forms of the enzyme have been partially characterized from liver of hamsters and from intestine of rats and hamsters (11, 12).

Thus far, the reactivity of *N*-acetoxyarylamines has precluded their isolation and characterization. However, the existence of these transient species has been inferred from the adducts formed when nucleophiles (e.g., *N*-acetylmethionine) are added to incubation mixtures containing arylhydroxamic acids and AHAT (8, 13). Identical

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[1] The abbreviations used are: AHAT, *N*-arylhydroxamic acid *N*,*O*-acyltransferase; NAT, *N*-acetyltransferase; N-OH-AAF, *N*-hydroxy-2-acetamidofluorene; N-OH-AABP, *N*-hydroxy-4-acetamidobiphenyl; AAB, 4-aminoazobenzene; PABA, *p*-aminobenzoic acid; SMZ, sulfamethazine.

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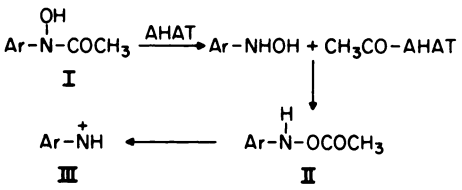

FIG. 1. *Activation of N-arylhydroxamic acids by AHAT*

adducts with nucleophiles are formed during the chemical reaction of arylhydroxylamines with acetic anhydride (8). In contrast to aryl amide *N*-sulfates which bind covalently to macromolecules with retention of the aryl amide moiety, *N*-acyloxyarylamines (II) form adducts containing only the arylamine portion of the original arylhydroxamic acid (14).

In addition to activation of arylhydroxamic acids, AHAT catalyzes the transfer of acyl groups from arylhydroxamic acids to arylamines (e.g., 4-aminoazobenzene) (15). It has been postulated that arylamines compete with arylhydroxylamines for the interception of the acyl-enzyme complex (9).

Recent reports by Glowinski *et al.* (16, 17) provide evidence that the AHAT of rabbit liver is possibly identical with the CoASAc-dependent NAT (EC 2.3.1.5), which polymorphically acetylates isoniazid, sulfamethazine, 2-aminofluorene, and other arylamines. Biochemical analogies between AHAT and NAT of other species suggest that similar relationships between these enzymes may be established.

We previously reported that certain carcinogenic arylhydroxamic acids act as apparent suicide substrates for rat and hamster hepatic AHAT (18). These enzymes are progressively and irreversibly inactivated *in vitro* during the conversion of arylhydroxamic acids to electrophiles. The kinetic features of this phenomenon are consistent with capture of the electrophiles by nucleophiles at the active site of AHAT (18). In this paper additional information is provided regarding the mechanism of suicide inactivation of AHAT. The irreversible inhibition of hamster hepatic NAT activities during inactivation of AHAT is also described.

MATERIALS AND METHODS

Chemicals

The following reagents were obtained from the commercial sources listed: 4-nitrobiphenyl and 2-nitrofluorene (Aldrich Chemical Company, Milwaukee, Wisc.) [9-^{14}C]2-nitrofluorene (Pathfinder Laboratories, St. Louis, Mo.); 4-aminoazobenzene (Eastman Kodak, Rochester, N. Y.); sodium pyrophosphate, potassium acetate, phenol and acetic anhydride (Mallinckrodt Inc., St. Louis, Mo.); trichloroacetic acid and SMZ (MCB Reagents, Cincinnati, Ohio); ammonium sulfate (Baker, Phillipsburg, N. J.); PABA, *N*-acetylmethionine, methionine, cysteine, glutathione, guanosine 2′- and 3′-phosphate, CoASAc (trilithium salt), dithiothreitol, and Type X tRNA (Sigma Chemical Company, St. Louis, Mo.); Grade A tRNA (Calbiochem, San Diego, Calif.); NCS tissue solubilizer (Amersham, Arlington Heights, Ill.); and Econofluor (New England Nuclear Corporation, Boston, Mass.).

N-OH-AAF and N-OH-AABP were prepared by reduction of the corresponding nitroarenes and subsequent acetylation of the *N*-arylhydroxylamines with acetyl chloride as described by Smissman and Corbett (19). [9-^{14}C]N-OH-AAF was synthesized in a similar manner from [9-^{14}C]2-nitrofluorene, but the *N*-hydroxy-2-aminofluorene was acetylated with acetic anhydride rather than acetyl chloride. The radiolabeled N-OH-AAF was purified by recrystallization (acetone-hexane), m.p. 150–151°.

Other Materials

Cellulose dialysis tubing (10 inches × 0.62 inch) was purchased from Sigma Chemical Company and rinsed several times in distilled water prior to use. Glass-fiber filters (GF/A, 24 cm) were obtained from Whatman Inc. (Clifton, N. J.).

Animals and Enzyme Preparations

Male golden Syrian hamsters (70–100 g) were obtained fron Charles River Farms (Wilmington, Mass.). Arylhydroxamic acid *N,O*-acyltransferase was purified 2- to 3-fold from hamster liver cytosol by fractionation with ammonium sulfate as described by King (9). The ammonium sulfate fractions were stored at −75° and dissolved in 0.05 M PP_i/NaCl buffer (pH 7) prior to use.

Assays

Transacetylation of AAB by N-arylhydroxamic acids. Incubation flasks (25 ml) contained 0.05–0.15 ml of enzyme preparation (2.5 mg of protein from 105,000 × *g* supernatant or partially purified enzyme), pyrophosphate buffer (50 μmoles, pH 7.0), dithiothreitol (1.0 μmole), an N-arylhydroxamic acid (2.5 μmoles), AAB (0.375 μmole), and sufficient 1.15% KCl to give a final volume of 2.5 ml. The *N*-arylhydroxamic acid was omitted from incubations used for reference standards. Reactions were started by addition of substrates (*N*-arylhydroxamic acid/AAB dissolved in 0.05 ml of 95% ethanol) and were carried out at 37° in air for 2 min. After termination of the reaction by addition of 2.5 ml of cold 20% trichloroacetic acid (in ethanol-water, 1:1), the transacetylation of AAB was assayed spectrophotometrically by the method of Booth (15).

CoASAc-dependent acetylation of PABA and SMZ. Reaction mixtures contained 0.02–0.05 ml of enzyme preparation (1 mg of protein), pyrophosphate buffer (25 μmoles), dithiothreitol (0.5 μmole), CoASAc (1.0 μmole), PABA or SMZ (0.1 μmole), and sufficient 1.15% KCl to give a final volume of 1.0 ml. Incubations without CoASAc were included as controls. Substrates (PABA or SMZ), dissolved in 0.1 ml of water, were added to initiate the reactions, which were carried out at 37° in air. The incubations were terminated after 1 min (PABA) or 5 min (SMZ) by addition of 2.0 ml of cold 5% trichloroacetic acid. The acetylation rate (nanomoles of substrate acetylated per milligram of protein per minute) was determined by Weber's modification (20) of the Bratton-Marshall procedure.

Inactivation of Hamster Hepatic AHAT by N-Arylhydroxamic Acids

Preincubation mixtures contained pyrophosphate buffer (50 μmoles, pH 7), dithiothreitol (1.0 μmole), 0.05–

0.15 ml of hamster hepatic enzyme preparation (2.5 mg of protein), *N*-arylhydroxamic acid (0–2.5 μmole dissolved in 0.05 ml of 95% ethanol), and sufficient 1.15% KCl to give a final volume of 2.5 ml. Control flasks contained 95% ethanol (0.05 ml) instead of *N*-arylhydroxamic acid. Substrates (2.5 μmoles of *N*-arylhydroxamic acid and 0.375 μmole of AAB in 0.05 ml of 95% ethanol) were added at the end of the preincubation period (1–15 min, 37°) in order to measure the amount of remaining enzyme activity; the mixtures were then incubated in air at 37° for 2 min. The reactions were terminated with 2.5 ml of 20% trichloroacetic acid (in ethanol-water, 1:1), and the remaining AHAT activity was determined as the transacetylation rate of AAB (15).

Kinetic Constants

Preincubations of hamster hepatic AHAT preparations (35–50% ammonium sulfate fractions) were carried out with several concentrations of *N*-arylhydroxamic acid, and the remaining AAB transacetylation activity was determined as described above. Semilog plots of remaining activity (percentage) versus preincubation time were obtained for inactivation with N-OH-AAF (12.5–40 μM) and N-OH-AABP (20–250 μM) (three or more experiments), and double-reciprocal plots were generated from the slopes of the apparent first-order lines. The kinetic parameters, k_i and K_I, were determined from the vertical and horizontal intercepts, respectively, of the double-reciprocal plots. All plots were determined by least-squares linear regression analysis. The correlation coefficients for the fit of the points to the lines generated in the double reciprocal plots were 0.98 (N-OH-AAF) and 0.99 (*N*-OH-AABP).

Comparison of AHAT and NAT Activities after Preincubation of Hamster Hepatic AHAT Preparations with N-OH-AAF

Incubation mixtures contained the following final concentrations of reagents: N-OH-AAF (0.7 μmole, dissolved in 0.05 ml of 95% ethanol) or an equivalent volume of 95% ethanol (controls), pyrophosphate buffer (175 μmoles, pH 7.0), dithiothreitol (3.5 μmoles), hamster hepatic AHAT preparation (35 mg of protein), cysteine (70 μmoles) or an equivalent volume of water, and 1.15% KCl to give a final volume of 7.0 ml. Reactions were started by addition of enzyme and were carried out in air at 37° for 15 min. At the end of the incubation period the mixtures were dialyzed (4 hr, 0–4°) against two portions (500 ml) of 0.05 M pyrophosphate-1 mM dithiothreitol buffer (pH 7) containing 1% ethanol. AHAT and NAT activities were determined with samples of dialyzed enzyme as described under Assays.

Attempted Reactivation of Hamster Hepatic AHAT by Cysteine

AHAT preparations (35–50% ammonium sulfate fractions) were inactivated with N-OH-AAF (0.1 mM) and dialyzed as described above; no cysteine was present during the inactivation. Samples of dialyzed enzyme (2.5 mg of protein) were preincubated for 5 min (37°) with the following reagents: cysteine (250 μmoles), pyrophosphate buffer (50 μmoles, pH 7), dithiothreitol (1.0 μmole), and sufficient 1.15% KCl to give a final volume of 2.5 ml. Substrates (N-OH-AAF/AAB, 2.5/0.375 μmoles) were added after the preincubation with cysteine, and the AAB transacetylation rate was determined as described previously.

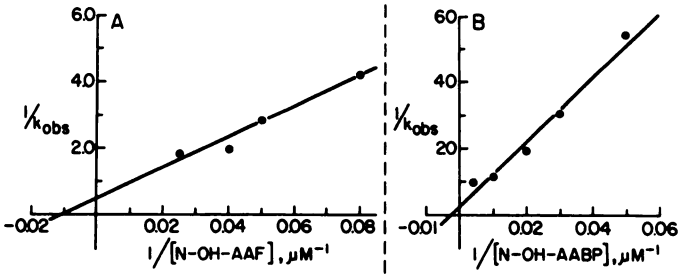

FIG. 2. *Double reciprocal plots of k_{obs} versus inactivator concentration for the inactivation of AHAT by N-OH-AAF (A) and N-OH-AABP (B)*

The kinetic plots were generated as described under Materials and Methods.

RESULTS

Kinetic parameters for inactivation of hamster hepatic AHAT by arylhydroxamic acids. N-OH-AAF and N-OH-AABP act as apparent suicide substrates of hamster hepatic AHAT. Linear semilog plots of remaining activity (percentage) versus preincubation time were obtained for several concentrations of these substrates, and apparent first-order inactivation rate constants (k_{obs}) were calculated from the slopes of the lines. Limiting rates of inactivation (k_i) and apparent dissociation constants (K_I) were obtained from plots of k_{obs}^{-1} versus (inhibitor)$^{-1}$, (Fig. 2). The values of the kinetic constants for inactivation by N-OH-AAF and N-OH-AABP are listed in Table 1. Typical semilog plots of remaining activity versus time were published previously (18). The positive k_{obs}^{-1} intercepts of the double reciprocal plots indicate that inactivation of AHAT by N-OH-AAF and N-OH-AABP involves a saturable enzyme-inhibitor complex (Fig. 2).

Effect of nucleophiles on the rate of inactivation of AHAT by arylhydroxamic acids. It was concluded in the preliminary report (18) that added nucleophiles did not protect hamster hepatic AHAT from inactivation by arylhydroxamic acids. However, additional experiments with a larger spectrum of nucleophiles, examined over a broader concentration range, revealed a relatively small but significant level of protection. The results of these protection experiments on the inactivation of AHAT with N-OH-AAF and N-OH-AABP are summarized in Tables 2 and 3, respectively. It should be noted that the

TABLE 1

Apparent kinetic parameters for inactivation of hamster hepatic AHAT by N-OH-AAF and N-OH-AABP

The apparent kinetic constants, k_i and K_I, were determined from the double-reciprocal plots of k_{obs} versus [inactivator] for several concentrations of inactivator as described under Materials and Methods. The values of k_{obs} were determined in three experiments with N-OH-AAF and in four experiments with N-OH-AABP.

Inactivator	k_i	K_I
	min^{-1}	μM
N-OH-AAF	1.95	89
N-OH-AABP	0.43	430

TABLE 2

Inactivation of hamster hepatic AHAT by N-OH-AFF: protection by nucleophiles

Activity was measured as the AAB transacetylation rate (mean ± standard error) following a 5-min preincubation of AHAT (1 mg of protein per milliliter) with N-OH-AAF (0.1 mM). The enzyme source was the 35–50% ammonium sulfate fraction. The preincubation conditions and the assay procedure are described under Materials and Methods. % Protection = the difference between the percentage of remaining AAB transacetylation activity after preincubation in the presence and absence of added nucleophile. The results obtained with tRNA represent the mean of two experiments.

Nucleophile	N-OH-AAF	Activity	% Remaining activity	% Protection	No. of experiments
		nmoles/mg protein/min			
None	−	19.3 ± 0.6			
	+	2.4 ± 0.5	12.6[a]	—	20
Cysteine (100 mM)	−	20.7 ± 1.4			
	+	8.2 ± 0.7	39.8[a]	27.2	3
Guanosine phosphate (25 mM)	−	21.3 ± 1.4			
	+	7.8 ± 1.8	36.7[a]	24.1	4
Methionine (100 mM)	−	24.1 ± 1.8			
	+	8.7 ± 2.4	36.0[a]	23.6	3
Glutathione (10 mM)	−	14.2 ± 0.9			
	+	3.7 ± 1.3	26.0[a]	13.4	3
N-Acetylmethionine (10 mM)	−	15.8 ± 1.3			
	+	5.9 ± 2.8	37.6[a]	25.1	5
tRNA (1 mg/ml)	−	19.0			
	+	2.7	14.5	1.9	2

[a] Difference from control significant at $p < 0.01$.

presence of 0.5–1.0 mM dithiothreitol in the incubation mixtures does not measurably diminish the rate of inactivation of AHAT (data not presented). Dithiothreitol enhances the stability of AHAT, and King (9) has reported that the presence of dithiothreitol in concentrations up to 1.0 mM actually increases the incorporation of AHAT generated electrophiles into nucleic acids.

Tables 2 and 3 contain the results of enzyme protection experiments with low molecular weight nucleophiles and with tRNA. The low molecular weight nucleophiles were used at either the highest concentration possible or at the concentration that produced the maximal amount of protection, whereas the concentration of tRNA was the same as that used in the electrophile trapping experiments (Table 4). The presence of low molecular weight nucleophiles diminished the rate of inactivation of AHAT by arylhydroxamic acids (Fig. 3). Moreover, first-order kinetics of inactivation were still observed in the presence of nucleophiles, as illustrated by the addition of guanosine phosphate to the preincubation of AHAT with N-OH-AABP (Fig. 3). Although cysteine (100 mM) afforded some protection of AHAT activity when the enzyme was inactivated with either N-OH-AAF or N-OH-AABP, no significant reactivation of AHAT activity was observed when AHAT was inactivated with N-OH-AAF, dialyzed to remove the inhibitor and other products, and then incubated with cysteine prior to assay (Table 4). Therefore, the protective effect of cysteine, the nucleophile which afforded the greatest degree of protection against inactivation (Tables 2 and 3), occurs during the inactivation of AHAT and is not due to subsequent restoration of the enzyme activity. Although the presence of either 10 mM glutathione or *N*-acetylmethionine appeared to depress AHAT activity somewhat, these agents also afforded some protection of AHAT activity (Tables 2 and 3).

Protection was not observed when tRNA (1 mg/ml) was included in preincubation mixtures of AHAT with

TABLE 3

Inactivation of hamster hepatic AHAT by N-OH-AABP: protection by nucleophiles

The procedure is described in Table 2 and under Materials and Methods.

Nucleophile	N-OH-AABP	Activity	% Remaining activity	% Protection	No. of experiments
		nmoles/mg protein/min			
None	−	18.1 ± 1.0			
	+	4.6 ± 0.7	25.4[a]	—	14
Cysteine (100 mM)	−	18.1 ± 0.9			
	+	10.2 ± 1.3	56.1[a]	30.7	6
Guanosine phosphate (25 mM)	−	21.5 ± 0.7			
	+	9.5 ± 2.6	44.3[a]	18.9	4
N-Acetylmethionine (10 mM)	−	12.6 ± 2.0			
	+	4.6 ± 1.5	36.8[a]	11.4	3
tRNA (1 mg/ml)	−	20.8			
	+	1.6	7.7	0	1

[a] Difference from control significant at $p < 0.01$.

TABLE 4

Effect of cysteine as a reactivator of hamster hepatic AHAT after inactivation by N-OH-AAF

Activity is expressed as the AAB transacetylation rate (mean ± standard deviation) measured after preincubation of AHAT (5 mg of protein per milliliter) with N-OH-AAF for 15 min followed by dialysis against pyrophosphate buffer (1% ethanol) containing 1 mM dithiothreitol (pH 7) and incubation with or without added cysteine as described under Materials and Methods. The enzyme source was the 35–50% ammonium sulfate fraction. Control activity: 21.4 ± 0.7 nmoles/mg of protein per minute.

Cysteine	Activity	% Activity recovered
	nmoles/mg protein/min	
None	0	0
100 mM	1.2 ± 0.8	5.4 ± 3.8

N-OH-AAF or N-OH-AABP (Tables 2 and 3). This result was somewhat surprising, since at this concentration tRNA is a relatively efficient trapping agent for certain enzymatically generated electrophiles (9). The formation of radiolabeled tRNA adducts during incubations of radiolabeled N-OH-AAF with AHAT was observed with tRNA concentrations less than or equal to those present during protection experiments (Table 5). In agreement with the observations of King (9), the use of a highly purified form of soluble yeast tRNA (Type X) provided greater incorporation of radiolabeled aninofluorene residues than the less purified form (Grade A).

Inactivation of CoASAC-dependent N-acetyltransferases. Reports of the possible identity of AHAT and the genetically polymorphic NAT of rabbit liver (16, 17) prompted the examination of the effect of arylhydroxamic acids on hamster hepatic NAT. AHAT and NAT activities were found to be present in the 35–50% ammonium sulfate fraction of hamster liver cytosol (Table 6). Partially purified preparations obtained in this manner catalyzed the CoASAc-dependent acetylation of PABA and SMZ (Table 6).

When the partially purified enzyme preparation was preincubated with N-OH-AAF, acetylation of AAB and the CoASAC-dependent acetylation of SMZ were inactivated to a similar extent, whereas the CoASAc-dependent acetylation of PABA was depressed to a lesser degree (Table 6). None of the activity was recovered when the inactivated enzyme preparations were dialyzed against pyrophosphate-dithiothreitol buffer (pH 7). In order to ensure that all organic molecules which might act as noncovalently bound inhibitors were removed by the dialysis procedures, several samples of inactivated enzyme were passed through a Sephadex G-25 gel filtration column prior to assay for activity; there was no recovery of activity after gel filtration, and the results were the same as those obtained following dialysis (data not presented). Coincubation with cysteine did not prevent the N-OH-AAF-induced depression of either AAB acetylation or the CoASAc-dependent transacetylation of SMZ, but it greatly diminished the N-OH-AAF induced depression of the CoASAc-dependent transacetylation of PABA (Table 6). These findings strongly suggest

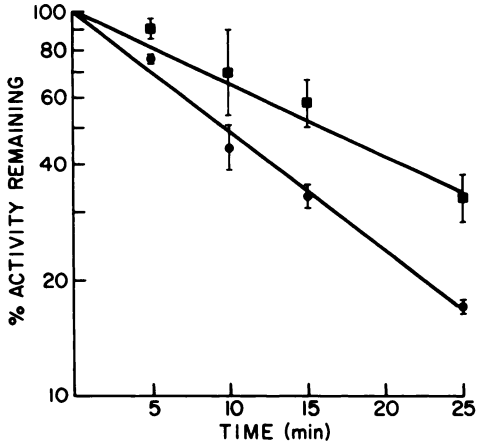

FIG. 3. *Time-dependent inactivation of hamster hepatic AHAT in the presence and absence of guanosine phosphate*

Activity is expressed as AAB transacetylation rate (percentage of control, means ± standard error of three experiments) measured after preincubation of AHAT with 1.0 mM N-OH-AABP (●) or with 1.0 mM N-OH-AABP and 25 mM guanosine phosphate (■). Activity was measured as described under Materials and Methods. Control activity: 23.3 ± 0.7 nmoles/mg of protein per min and 22.7 ± 0.9 nmoles/mg of protein per min in the absence and presence of guanosine, respectively.

TABLE 5

AHAT-mediated covalent binding of N-OH-AAF to tRNA

Assays were carried out as described by King (9) to give final concentrations of reagents as follows: tRNA, 15 A_{260} units; N-OH-AAF, 0.05 mM, 0.15 μCi/μmole; AHAT preparation (35–50% ammonium sulfate fraction), 1 mg/ml; and pyrophosphate buffer, 45 mM, containing dithiothreitol, 1 mM (pH 7.0). The final incubation volume was 0.8 ml. Activities represent the mean of two experiments carried out in triplicate, and are corrected for nonenzymatic covalent binding.

tRNA	Aminofluorene moiety bound
	nmoles/mg protein/20 min
Grade A	1.1
Type X	3.6

TABLE 6

Inactivation of hamster hepatic AHAT and NAT by N-OH-AAF

Activities are expressed as rate of acetylation (mean ± standard error of four or more experiments). The numbers in parentheses represent the percentage of control activity (± standard error). Enzyme preparations (35–50% ammonium sulfate fraction) were inactivated by preincubation with N-OH-AAF (± cysteine) for 15 min. Samples were dialyzed against two portions (500 ml) of 0.05 M PP_i/NaCl buffer containing 1 mM dithiothreitol (pH 7) and 1% ethanol for 4 hr prior to assay. Controls contained 0.05 ml of 95% ethanol instead of N-OH-AAF. Assays are described under Materials and Methods.

Preincubation system	AHAT	NAT	
	N-OH-AAF/AAB	CoASAc/SMZ	CoASAc/PABA
	nmoles/mg protein/min		
Control	18.2 ± 1.6	1.0 ± 0.3	55.8 ± 4.1
Control + cysteine (10 mM)	17.4 ± 2.4	0.9 ± 0.0	55.0 ± 1.5
N-OH-AAF (0.1 mM)	1.6 ± 0.6 (8.3 ± 2.5)	0.2 ± 0.1 (18.4 ± 7.7)	21.7 ± 3.2 (41.1 ± 7.1)
N-OH-AAF (0.1 mM) + cysteine(10 mM)	1.5 ± 0.7 (7.9 ± 3.3)	0.1 ± 0.1 (17.7 ± 10.1)	46.8 ± 0.9 (85.3 ± 1.7)

that the acetylation of SMZ may be associated with AHAT and that the acetylation of PABA is not associated with AHAT.

DISCUSSION

Arylhydroxamic acids act both as substrates and as irreversible inhibitors of hamster hepatic AHAT. The inhibition of AHAT by arylhydroxamic acids has been shown to fulfill the kinetic criteria for suicide inactivation (21–23). Although nucleophiles partially protect hamster hepatic AHAT by retarding the rate of inactivation, the irreversible inhibition is not prevented even at extremely high concentrations of nucleophile. Therefore, the rates of inactivation of hamster hepatic AHAT appear to represent contributions from a major pathway (Fig. 4) consisting of direct capture (k_1) of enzymatically generated electrophiles (I') by an active-site nucleophile and a minor pathway involving covalent modification (k_3) of the enzyme by electrophiles that have been released into solution. The latter pathway is essentially eliminated when a sufficient concentration of a low molecular weight nucleophile is present to trap the escaping electrophiles. A precise description of the inactivation mechanism requires studies of the chemical nature of the covalent modification of the active-site of AHAT by arylhydroxamic acids. However, the present experimental data for suicide inactivation of AHAT are consistent with the conversion of arylhydroxamic acids to electrophilic species that can react directly with nucleophilic groups of amino acid residues at or near the enzyme active site or, alternatively, can escape from the enzyme (k_2) to form covalent adducts with tRNA, amino acids, or other biological nucleophiles, including those present on AHAT. According to this tentative model, the overall fate of electrophiles formed from arylhydroxamic acids by AHAT would be governed by the partition ratio (k_1/k_2) for these two processes. Although this model attributes the effects of low molecular weight nucleophiles on the inactivation process to their reaction with electrophiles which have been released into the medium, the present data do not preclude the possibility that these nucleophiles exert at least part of their protective effect by reacting with active-site bound electrophiles before the electrophiles are released into solution and before they react with AHAT.

tRNA is an effective trap for the electrophiles generated from arylhydroxamic acids by hamster hepatic AHAT (9). However, in contrast to smaller nucleophiles, which partially protect AHAT against inactivation by N-OH-AAF or N-OH-AABP, tRNA does not retard the inactivation. The inability of tRNA to protect AHAT may be related to its relatively large size, which might limit its access to electrophiles near the enzyme surface,

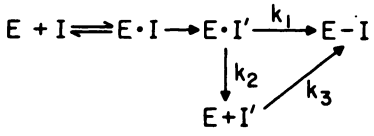

FIG. 4. *Model for the suicide inactivation of hamster hepatic AHAT by arylhydroxamic acids*

E, AHAT; *I*, arylhydroxamic acid; *I′*, Electrophile.

or for the failure of its nucleophilic groups to compete kinetically with the enzyme for those electrophiles in solution which inactivate AHAT. Although it is possible that higher concentrations of tRNA might have afforded some protection of the enzyme from inactivation, the concentration used in these experiments is adequate to trap AHAT-generated electrophiles (Table 4) (9), and it protects AHAT from inactivation by certain other arylhydroxamic acids.[2]

N-OH-AAF is a more potent suicide substrate than N-OH-AABP for both hamster and rat hepatic AHAT (18). For example, the apparent k_i for inactivation of hamster AHAT by N-OH-AAF is 4.5 times larger, whereas the apparent K_I is nearly 5 times smaller, than the corresponding values for inactivation by N-OH-AABP (Table 1). Caution must be exercised in comparisons of these kinetic parameters since they were determined with an enzyme preparation that was not highly purified. However, these results indicate that inhibitory intermediates containing the fluorene ring system appear to bind to AHAT with greater affinity and inactivate the enzyme more rapidly than analogous inhibitors containing the biphenyl moiety.

The suicide inactivation of hamster hepatic AHAT is accompanied by concurrent inactivation of NAT activities. When cysteine is added to incubations of AHAT with N-OH-AAF, PABA-NAT is selectively protected against inactivation (Table 6). Therefore, hamster hepatic PABA-NAT appears to be distinct from the NAT which acetylates SMZ, and its inactivation may result primarily from reaction with electrophiles released into the medium by AHAT. It is also possible that the small degree of inactivation of PABA-NAT which is observed in the presence of cysteine is the result of a limited amount of bioactivation of N-OH-AAF by PABA-NAT itself. The selective protection of PABA-NAT during the inhibition of AHAT and SMZ-NAT by N-OH-AAF represents the first example of the distinguishing of multiple forms of NAT by an irreversible inhibitor. It should be noted that King and Allaben (11) have stated that multiple species of AHAT, separable by gel filtration, have been identified in liver of hamsters. The results of the experiments described in the present report do not reveal whether a single species of AHAT undergoes inactivation by N-OH-AABP and N-OH-AAF or whether multiple forms of the enzyme are inactivated. If multiple species of the enzyme are inactivated, they appear to have very similar substrate specificities.

The properties of NAT isozymes have been most thoroughly studied in the hepatic and extrahepatic tissues of rabbit. In rabbit liver, SMZ and PABA appear to be acetylated by the same genetically polymorphic NAT, since it has not been possible to distinguish a PABA-specific NAT by biochemical (24) or immunochemical (25) techniques. A PABA-specific hepatic NAT has been postulated for deer mice and rats on the basis of the correlation of liver PABA-NAT activity with the PABA-specific blood NAT of these two species (26). The results presented here indicate that the hamster also contains a specific PABA-NAT which is distinct fro n SMZ-NAT.

[2] P. E. Hanna and V. C. Marhevka, unpublished data.

It has been suggested that AHAT may play a role in the carcinogenicity of aromatic amines (11). The results presented here indicate that repeated exposure to N-OH-AAF or N-OH-AABP might result in decreased levels of AHAT *in vivo*, thereby influencing tumor induction as well as the CoASAc-dependent acetylation of aromatic amines. Preliminary experiments have shown that administration of N-OH-AAF to hamsters results in lowered AHAT and SMZ-NAT activities, but has no effect on PABA-NAT activity.[3]

Suicide inactivation of hamster hepatic AHAT by N-OH-AAF and other carcinogenic arylhydroxamic acids is a useful new technique for the study of AHAT and related *N*-acyltransferases. The results presented here demonstrate the use of suicide substrates to probe the mechanism of AHAT-mediated activation of arylhydroxamic acids, to explore the relationship between AHAT and NAT, and to selectively inactivate multiple forms of NAT. Additional applications of the suicide inactivation of *N*-acyltransferases by arylhydroxamic acids are being investigated.

[3] P. E. Hanna and T. J. Smith, unpublished data.

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Send reprint requests to: Dr. Patrick E. Hanna, Department of Pharmacology, University of Minnesota, 3-260 Millard Hall, 435 Delaware Street S.E., Minneapolis, Minn. 55455.