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AN ISOZYME-SELECTIVE AFFINITY LABEL FOR RAT HEPATIC ACETYLTRANSFERASES

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Abstract—Affinity chromatography of an ammonium sulfate precipitate obtained from rat hepatic cytosol resulted in the separation of two fractions of *N*-acetyltransferase (NAT) activity. NAT I catalyzed the *S*-acetylcoenzyme A (AcCoA)-dependent acetylation of *p*-aminobenzoic acid (PABA); NAT II catalyzed the *N*-hydroxy-2-acetylaminofluorene (N-OH-AAF)-dependent acetylation of 4-aminoazobenzene (AAB) (*N,N*-acetyltransferase), the AcCoA-dependent acetylation of procainamide (PA), and the *N*-arylhydroxamic acid *N,O*-acyltransferase (AHAT) activity that results in the conversion of N-OH-AAF and related hydroxamic acids to electrophilic reactants. 1-(Fluoren-2-yl)-2-propen-1-one (vinyl fluorenyl ketone, VFK) was shown to be a potent and irreversible inactivator of NAT II activities. A 200-fold higher concentration of VFK was required to inactivate NAT I activity than was required for inactivation of NAT II activities. Similar selectivity in the inactivation of the isozymes was observed when experiments were conducted with enzyme preparations that contained both NAT I and NAT II activities. The presence of substrates and products of the NAT II-catalyzed reactions such as AcCoA, 2-acetylaminofluorene (2-AAF), and *N*-acetyl-4-aminoazobenzene (N-Ac-AAB) protected NAT II from the inactivating effects of VFK, providing evidence that VFK is an active site directed inhibitor (affinity label) of NAT II. Studies with 1-(fluoren-2-yl)-2-propan-1-one (EFK), an analogue of VFK in which the α,β-unsaturated vinyl ketone group of VFK has been replaced with an ethyl ketone group, demonstrated that the conjugated ketone of VFK is required for inactivation of enzyme activity. The results of these studies suggest that agents such as VFK should have utility as probes of acetyltransferase multiplicity and in the investigation of the active site topography of the enzymes.

N-Acetylation is a principal metabolic pathway for numerous arylamine drugs, mutagens and carcinogens (Fig. 1A) [1–4]. The *N*-acetyltransferase (NAT)§ enzymes (EC 2.3.1.5), which are responsible for catalyzing this biotransformation reaction, are cytosolic, *S*-acetylcoenzyme A (AcCoA) dependent, and widely distributed in mammalian tissues [2]. *N*-Arylhydroxamic acid *N,O*-acyltransferase (AHAT) catalyzes a process that results in the formation of reactive electrophiles which may be involved in the production of the untoward effects of toxic and carcinogenic *N*-arylhydroxamic acids (Fig. 1B) [5–7]. The results of enzyme purification experiments reported by several laboratories demonstrated that certain NAT enzymes are capable of exhibiting both the AcCoA-dependent NAT activity and the AHAT activity [8–10], as well as *N*-hydroxyarylamine *O*-acetyltransferase activity [9, 10].

In addition to the AcCoA-dependent N-acetyltransfer reaction illustrated in Fig. 1A, arylamines

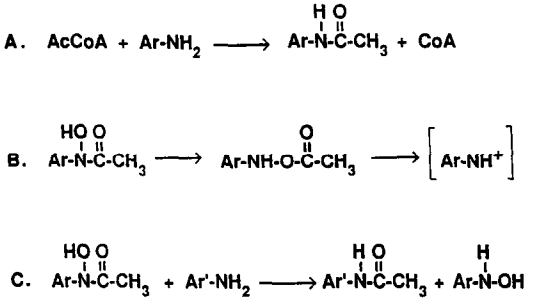

Fig. 1. Acetyltransferase-catalyzed reactions. (A) AcCoA-dependent *N*-acetyltransferase (NAT). (B) *N*-Arylhydroxamic acid *N,O*-acyltransferase (AHAT). (C) Arylhydroxamic acid-dependent *N,N*-acetyltransferase (N,N-AT).

also can be converted to arylamides in an *N*-arylhydroxamic acid-dependent *N,N*-acetyltransferase (N,N-AT) catalyzed process (Fig. 1C) [11]. Studies involving mechanism-based enzyme-inactivating agents (suicide inhibitors) and enzyme purification methods indicated that *N,N*-acetyltransferase activity is associated with AHAT activity in hamster liver and intestinal cytosols [12, 13]. More recently, Wick and Hanna [14] employed mechanism-based inactivation to reveal the existence of at least two forms of transacetylase activity in rat liver cytosol. One-thousand fold purification of the rat liver enzymes did not result in separation of the N,N-AT and AHAT activities. Thus, the results obtained with

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§ Abbreviations: AAB, 4-aminoazobenzene; AcCoA, *S*-acetylcoenzyme A; 2-AAF, 2-acetylaminofluorene; 2-AF, 2-aminofluorene; AHAT, *N*-arylhydroxamic acid *N,O*-acyltransferase; DTT, D,L-dithiothreitol; EFK, ethyl fluorenyl ketone, 1-(fluoren-2-yl)-2-propan-1-one; N-Ac-AAB, *N*-acetyl-4-aminoazobenzene; N-Ac-PA, *N*-acetylprocainamide; NAT, *N*-acetyltransferase; N,N-AT, *N,N*-acetyltransferase; N-OH-AAF, *N*-hydroxy-2-acetylaminofluorene; PA, procainamide; PABA, *p*-aminobenzoic acid; THF, tetrahydrofuran; and VFK, vinyl fluorenyl ketone, 1-(fluoren-2-yl)-2-propen-1-one.

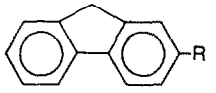

Fig. 2. Structures of VFK (R: $COCH{=}CH_2$), EFK (R: $COCH_2{-}CH_3$), and N-OH-AAF (R: $NOHCOCH_3$).

rat hepatic transacetylase activities are similar to those obtained with the hamster tissue enzymes and are consistent with earlier reports that the AHAT and N,N-AT activities coelute when rat liver cytosol is subjected to gel filtration chromatography [6, 15].

Acetyltransferase enzymes exhibit hereditary polymorphisms and exist in multiple forms in several species, including humans [1–4, 16–20]. Because of the important roles played by acetyltransferase isozymes in the toxification and detoxification of xenobiotics, it is important to develop means to detect and identify various forms of the enzymes and to provide insight into the differences in their biochemical properties and substrate specificities. Affinity labeling experiments can make important contributions to the elucidation of such information [21]. This paper reports the synthesis and enzyme-inactivating properties of 1-(fluoren-2-yl)-2-propen-1-one (vinyl fluorenyl ketone, VFK), an isozyme selective affinity label for acetyltransferases.

VFK is an analogue of the *N*-arylhydroxamic acid, *N*-hydroxy-2-acetylaminofluorene (N-OH-AAF) (Fig. 2). The latter compound is an effective acetyl donor for both the AHAT and *N,N*-acetyltransferases that are found in rat and hamster liver [15, 22]. Because of the structural similarities of N-OH-AAF and VFK (Fig. 2), it was reasoned that VFK should have a high degree of affinity for the enzymes that catalyze *N*-arylhydroxamic acid-dependent acetyltransfer reactions (Fig. 1, B and C). The propenone (vinyl ketone) functional group of VFK is an electrophilic α,β-unsaturated ketone (a Michael acceptor) and is capable of reacting with nucleophiles such as amines and thiols. Thus, the above considerations and the evidence that acetyltransferase enzymes are dependent upon nucleophilic cysteine thiol residues for acetyl group transfer [23, 24] led to the proposal that VFK would act as an affinity label by forming a covalent bond with the active site thiol of AHAT and N,N-AT.

MATERIALS AND METHODS

Materials. The following materials were obtained from the sources indicated. 2-Nitrofluorene, 2-aminofluorene (2-AF), vinyl magnesium bromide, acetyl chloride, 2-fluorene carboxaldehyde, fluorene, and *p*-dimethylaminobenzaldehyde were obtained from the Aldrich Chemical Co., Inc., Milwaukee, WI. 4-Aminoazobenzene (AAB) and *N*-1-(naphthyl)ethylenediamine dihydrochloride were purchased from the Eastman Kodak Co., Rochester, NY. *S*-Acetylcoenzyme A (AcCoA, trilithium salt), desulfocoenzyme A (desulfoCoA) D,L-dithiothreitol (DTT), grade III NAD, L-cysteine, bovine serum albumin, procainamide (PA) hydrochloride, *N*-acetylprocainamide (N-Ac-PA), *p*-aminobenzoic acid (PABA, sodium salt), tetrasodium pyrophosphate and Sephadex G-100 were purchased from the Sigma Chemical Co., St. Louis, MO. Sulfamethazine was from MCB Manufacturing Chemists, Norwood, OH. Epoxy-activated Sepharose 6B and PD-10 desalting columns (1.5 × 5 cm) pre-packed with Sephadex G-25M were purchased from the Pharmacia Chemical Co., Piscataway, NJ. Coomassie Blue G-250 Dye Reagent Concentrate was purchased from Bio-Rad Laboratories, Richmond, CA. Activated MnO_2 was purchased from Alfa, Danvers, MA.

N-OH-AAF was synthesized as previously described [25]. Coupling of 2-AF to epoxy-activated Sepharose 6B was accomplished as previously described [13, 26]. *N*-Acetyl-L-[^{14}C-CH_3]methionine (0.2 to 0.6 mCi/mmol) was prepared from L-[^{14}C-CH_3]methionine (ICN Pharmaceuticals, Irvine, CA) according to the procedure of Wheeler and Ingersoll [27].

Synthesis of 1-(fluoren-2-yl)-2-propen-1-one (VFK). The methods reported by Borchert *et al.* [28] and Wislocki *et al.* [29] for the synthesis of safrole metabolites were used. Glassware was dried for 3 hr at 125° and was cooled by flushing with a stream of dry nitrogen. Tetrahydrofuran (THF) was dried over molecular sieves. 2-Fluorene carboxaldehyde (4.15 g, 0.02 mol) was dissolved in 20 mL of THF. Vinyl magnesium bromide (6.27 g, 0.48 mol), dissolved in 48 mL of THF, was added dropwide with stirring over 1 hr. The reaction mixture was maintained at 50° under an atmosphere of dry N_2. After the addition was complete, the mixture was stirred for 1 hr at room temperature and then was cooled in an ice bath. A saturated solution of NH_4Cl was added dropwise. The aqueous layer was separated and extracted with 20 mL of ether, and the combined THF and ether fractions were dried over $MgSO_4$. The solvent was evaporated and the residue was chromatographed on a 130 g silica gel column by elution with petroleum ether (30–60°) containing increasing percentages of diethyl ether. The fractions that contained the majority of product, according to TLC, were combined and evaporated to afford 3.32 g of solid residue which, after several recrystallizations from diethyl ether:petroleum ether:carbon tetrachloride (1:1:1) yielded 1-(fluoren-2-yl)-2-propen-1-ol (1.45 g, 31%), m.p. 78–81°.

A chromatography column (4 × 40 cm) was packed with a chloroform slurry of silica (21.5 g) and activated MnO_2 (21.5 g). 1-(Fluoren-2-yl)-2-propen-1-ol (1.95 g, 0.008 mol) was dissolved in 40 mL of chloroform and was poured onto the column. The column was eluted with an additional 520 mL of chloroform; the fractions that contained the majority of the product were combined and evaporated. The residue was treated with 20 mL of ether and 30 mL of ethyl acetate, and undissolved solids were removed by filtration. The solvent was evaporated and the residue was dissolved in a minimal volume of diethyl ether and acetone and was applied to a column of silica gel (65 g). The column was eluted with petroleum ether:diethyl ether (3:1). The fractions that contained the product were combined and evaporated. The residue was recrystallized from diethyl ether:acetone (20:3) to yield 1-(fluoren-2-yl)-2-propen-1-one (1.61 g, 83%), m.p. 94–96°. MS

analysis (70 eV): *m/e* 220 $(M)^+$. Elemental analysis: Calc. ($C_{16}H_{12}O$): C, 87.24; H, 5.49, found: C, 87.29; H, 5.57.

Synthesis of 1-(fluoren-2-yl)-2-propan-1-one (EFK). Fluorene (1.66 g, 0.01 mol) was dissolved in nitrobenzene (15 mL), and aluminum chloride (2.66 g, 0.02 mol) was added in three portions with stirring. After dropwise addition of acetyl chloride (0.93 g, 0.01 mol), the mixture was stirred at 60° for 3 hr and then cooled to room temperature. The reaction mixture was poured onto a mixture of 60 g of ice and 20 mL of concentrated HCl and was stirred for 30 min. Solids were removed by filtration, and the filtrate was extracted with four 50-mL portions of chloroform which were combined, dried ($MgSO_4$) and evaporated. The residue was taken up in methanol (150 mL), solids were removed by filtration, and the methanol was evaporated. The residue was crystallized from ethanol to afford 1.43 g of solid which was chromatographed on silica gel (60 g) by elution with petroleum ether containing increasing concentrations of dichloromethane. The fractions containing the product were combined and evaporated to afford 1-(fluoren-2-yl)-2-propan-1-one (0.64 g, 30%), m.p. 124–125°. Elemental analysis: Calc. ($C_{16}H_{14}O$): C, 86.45; H, 6.34. Found: C, 86.86, H, 6.01.

Partial purification of rat hepatic acetyltransferase activities. Male Sprague–Dawley rats (170–220 g) were obtained from Bio-Lab (White Bear Lake, MN). Preparation of hepatic 105,000 *g* cytosol, ammonium sulfate fractionation of the cytosol, and Sephadex G-100 gel filtration of the rat hepatic acetyltransferase activities were performed as described [22].

Rat hepatic NAT activities were prepared for purification by affinity chromatography according to the procedures cited above (ammonium sulfate precipitation and Sephadex G-100 gel filtration chromatography) except that gel filtration was performed with degassed potassium phosphate buffer (pH 7.4, containing 1 mM DTT and 1 mM EDTA). The fractions that contained the majority of the NAT activity were combined and applied directly to a 1.5 × 14 cm column of Sepharose 6B 2-AF. The column was then eluted with 200 mL of 0.02 M potassium phosphate (1 mM EDTA, 1 mM DTT), pH 7.4, buffer. Fractions (6 mL) were colleected at a flow rate of approximately 48 mL/hr. At fraction 39, the elution buffer was changed to 0.05 M sodium pyrophosphate, 1 mM DTT, pH 7.0. The N-OH-AAF/AAB, AcCoA/PA and AcCoA/PABA transacetylation activities of each fraction were determined as described below. The fractions containing the majority of the AcCoA/PABA transacetylation activity (NAT I) were pooled, concentrated to approximately 25% of the original volume by ultrafiltration under nitrogen, and stored at −70° in 10% glycerol. The fractions containing the N-OH-AAF/AAB and AcCoA/PA activities (NAT II) were similarly pooled, concentrated, and stored at −70° in 30% glycerol. Enzyme activities stored under these conditions were stable for several months.

N-OH-AAF/AAB transacetylation assay. Incubation mixtures contained 0.02 to 0.48 mL of the enzyme preparation, 0.02 mL of substrate solution (AAB and N-OH-AAF dissolved in 95% ethanol) and sufficient 0.05 M sodium pyrophosphate buffer (1 mM DTT, pH 7.0) to bring the volume to 0.5 mL. The final concentrations of AAB and N-OH-AAF were 0.15 and 1.0 mM respectively. Reactions (37°, 4 min) were initiated by addition of substrate and terminated by the addition of 0.5 mL of cold 20% trichloroacetic acid (in ethanol:water, 1:1). The precipitated protein was removed by centrifugation. Acetylation of AAB was determined by the decrease in absorbance of the supernatant fraction at 497 nm [11].

AcCoA-dependent acetylation of PABA. Reaction mixtures contained 0.01 to 0.15 mL of enzyme preparation, 0.025 mL of 6 mM AcCoA (aqueous), 0.075 mL of 0.33 mM PABA (aqueous) and sufficient 0.05 M sodium pyrophosphate buffer (1 mM DTT, pH 7.0) to bring the incubation volume to 0.25 mL. Incubations were carried out at 37° for 10–20 min, and the supernatant fraction was analyzed by Weber's modification of the Bratton–Marshall procedure [30] as previously described [13].

AcCoA-dependent acetylation of PA. Acetylation of PA by AcCoA was determined by the method of Hein *et al.* [31]. Reaction mixtures contained 0.035 to 0.05 mL of the enzyme preparation, 0.22 mM procainamide, 2.2 mM AcCoA and enough 0.5 M sodium pyrophosphate, 1 mM DTT, pH 7.0, buffer to give a final incubation volume of 0.09 mL. The reaction was initiated by addition of PA (1 mM, aqueous) and was terminated by the addition of 0.05 mL of cold 10% trichloroacetic acid (aqueous). Protein was pelleted by centrifugation. The supernatant fraction was analyzed for unacetylated procainamide by formation of a complex of procainamide with *p*-dimethylaminobenzaldehyde [31].

Electrophile generation assay (methylthio adduct formation). The AHAT-mediated production of electrophiles from N-OH-AAF was measured by the electrophile trapping assay of Bartsch *et al.* [6], as described previously [32]. Concentrations of N-OH-AAF and *N*-acetyl-L-[^{14}C-CH_3]methionine in the incubation mixtures were 0.05 and 10 mM respectively. Reactions were carried out at 37° for 20 min.

Inactivation of rat hepatic transacetylase activity by VFK. In experiments to determine the concentration- and time-dependence of the inactivation of N-OH-AAF/AAB N,N-AT activity by VFK, incubation mixtures contained the glycerol-stabilized NAT II fractions (final protein concentration 0.3 mg/mL), 0.06 to 0.5 μM VFK, 0.16 mM DTT and 0.05 M sodium pyrophosphate buffer (pH 7.0) in a final incubation volume of 0.117 mL. Incubations (22°) were conducted in the presence of VFK [dissolved in dimethyl sulfoxide (DMSO), 4% final DMSO concentration] for 0.5 to 1.0 min; cysteine (0.363 mL) was then added (final concentration 10 mM) to stop the inactivation process. Incubation tubes were transferred immediately to a 37° shaking water bath; 40 sec later, substrate solution (N-OH-AAF/AAB) was added for measurement of remaining transacetylase activity, as described above.

Experiments to test the irreversibility of the VFK-mediated inactivation of N-OH-AAF/AAB and AcCoA/PA transacetylase activities contained in the

NAT II fractions were conducted in a fashion similar to that described above except that incubation mixtures contained 1.25 μM VFK, 5% DMSO and 0.27 mM DTT in a final incubation volume of 0.85 mL. After incubation at 22° for 0.5 min, cysteine (0.85 mL) was added (final concentration 10 mM). Aliquots (0.15 mL) of the resulting solution were removed for analysis of N-OH-AAF/AAB and AcCoA/PA transacetylase activity; 1.0 mL of the remaining solution was applied to a PD-10 column prepacked with Sephadex G-25M. The column was then eluted with two portions (2.0 and 1.5 mL) of buffer (0.05 M sodium pyrophosphate, pH 7.0, containing 1 mM DTT and 2.5% DMSO). Approximately 95% of the protein and NAT activity was eluted following application of the second portion of buffer (1.5 mL), which was collected and kept on ice. The protein concentration of this fraction was determined and adjusted for analysis of N-OH-AAF/AAB and AcCoA/PA transacetylase activity as described above.

All subsequent experiments were performed in a manner similar to that described above for the test of irreversibility of VFK-mediated inactivation of enzyme activity, with the exception that measurements of enzyme activity remaining after incubation in the presence or absence of VFK were performed only after filtration through PD-10 columns.

The effect of VFK on unresolved (5 to 6-fold purified) rat hepatic transacetylase activities was determined with 1.0-mL incubation mixtures containing 4.5 mg protein/mL of the enzyme preparation obtained by gel filtration, 6 μM VFK, 6% DMSO and 0.24 mM DTT. The ability of substrates and products of acetyltransferase-catalyzed reactions to prevent inactivation by VFK was tested by preincubating enzyme mixtures in the presence of 0.05 mM 2-AAF, N-Ac-AAB or AcCoA for 1 min at 22° prior to the addition of VFK. Following incubation in the presence of VFK for 15 sec, cysteine was added (final concentration 10 mM) and the resulting mixture was filtered through Sephadex G-25M (PD-10 columns).

The ability of substrates and products of acetyltransferase-catalyzed reactions to prevent inactivation of enzyme activity by VFK was studied further with the more highly purified NAT II fractions obtained by 2-AF affinity chromatography. Incubations were conducted in the presence of 0.3 mg protein/mL of the enzyme preparation, 5% DMSO and 0.27 mM DTT. When present, AcCoA (0.01 to 0.10 mM), 2-AAF (0.05 to 0.10 mM), 2-AF (0.4 mM) or 1.0 mM PA, N-Ac-PA or desulfo CoA were preincubated with the enzyme mixtures for 1.0 min at 22° prior to the addition of VFK (1.25 μM). After a further 0.5-min incubation in the presence of VFK, cysteine was added (final concentration 10 mM), and the mixtures were applied to a Sephadex G-25M (PD-10) column. None of the substrates or products had a significant effect on acetyltransferase activity when they were incubated with the enzymes in the absence of VFK prior to gel filtration and assay for transacetylase activities.

In separate experiments, electrophile generation (methylthio adduct formation) catalyzed by the NAT II fractions was measured after incubation with 0.27 mM VFK. The incubations were performed under conditions identical to those described above. Following filtration through Sephadex G-25M, methylthio adduct formation was measured as described above.

Experiments which tested the ability of EFK to inactivate the NAT II N-OH-AAF/AAB and AcCoA/PA transacetylase activities were conducted similarly to those described above for the irreversibility of VFK-mediated inactivation, except that incubation mixtures contained 25 μM EFK (instead of VFK) and were carried out for 5.0 min at 22°. Measurements of N-OH-AAF/AAB and AcCoA/PA transacetylation activities were made following filtration through Sephadex G-25M, as described above.

RESULTS

Partial purification of acetyltransferase activities. Acetyltransferase activities were purified 5- to 6-fold by sequential ammonium sulfate fractionation and gel filtration with Sephadex G-100. Sepharose 6B 2-AF affinity chromatography resulted in separation of the AcCoA/PABA NAT activity (NAT I) from the N-OH-AAF/AAB N,N-AT, the AcCoA/PA NAT and the AHAT (methylthio adduct forming) activities (NAT II), as previously described [14]. NAT I and NAT II transacetylase activities were purified approximately 100-fold by this procedure.

Inactivation of rat hepatic N-OH-AAF/AAB N,N-AT activity by VFK. It was anticipated that VFK might react directly with sulfhydryl groups such as those present in DTT or cysteine. Therefore, preliminary experiments were conducted which demonstrated that the presence of DTT or cysteine in concentrations of 1–10 mM prevented the inactivation of N-OH-AAF/AAB N,N-AT activity by VFK (data not presented). Because of this finding, only 0.16 to 0.27 mM DTT was present in the buffer that was used in the inactivation experiments. These concentrations of DTT were sufficient to maintain maximal levels of enzyme activity in control experiments. Additionally, advantage was taken of the ability of cysteine to prevent VFK-mediated enzyme inactivation by using 10 mM cysteine to terminate the inactivation experiments.

Preliminary experiments indicated that the N-OH-AAF/AAB transacetylase activity contained in the NAT II fractions obtained from Sepharose 6B 2-AF affinity chromatography was inhibited rapidly by low concentrations of VFK. Therefore, the concentration- and time-dependence of the inactivation process were studied. The results of these studies are illustrated in Fig. 3. Inactivation of the transacetylase activity by 0.06 to 0.5 μM VFK was dependent upon the concentration of VFK and was nearly complete after 0.5 min. Higher concentrations of VFK caused 100% loss of the activity (results not shown).

To determine whether VFK-mediated inactivation of rat hepatic NAT II N-OH-AAF/AAB N,N-AT activity was irreversible, the NAT II fractions from Sepharose 6B 2-AF affinity chromatography were incubated with VFK and were analyzed for transacetylation activity both before and after gel

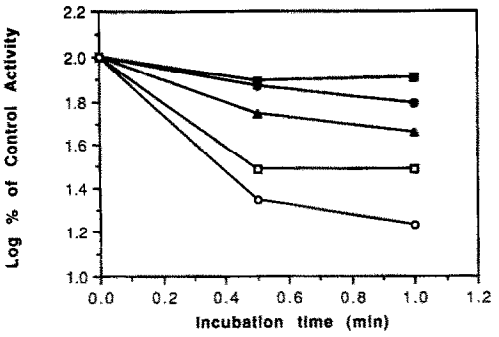

Fig. 3. Inhibition of rat hepatic N-OH-AAF/AAB N,N-AT activity by VFK. Rat hepatic NAT II fractions were incubated at 22° in the presence of various concentrations of VFK for the indicated periods of time. The remaining N-OH-AAF/AAB transacetylase activity was analyzed as described under Materials and Methods. Each point is the mean of two or three experiments, each performed in triplicate. Control N-OH-AAF/AAB N,N-AT activity was 132 ± 9 nmol/mg protein/min (mean ± SD, N = 6). Key: (■) 0.06 μM, (●) 0.125 μM, (▲) 0.25 μM, (□) 0.35 μM; and (○) 0.5 μM.

filtration. Irreversibility of inhibition of the rat hepatic NAT II AcCoA/PA transacetylase activity also was determined because of previous results [14] which indicated that the rat hepatic N-OH-AAF/AAB and AcCoA/PA transacetylase activities may be associated with a common protein. The results of these experiments are shown in Table 1. N-OH-AAF/AAB and AcCoA/PA transacetylase activities were inhibited to similar extents following incubation with VFK. Neither transacetylation activity was restored significantly following gel filtration of the incubation mixtures, demonstrating the irreversible nature of the inactivation of these activities by VFK.

Effect of EFK on rat hepatic NAT II transacetylation activities. To determine whether the mechanism whereby VFK inactivates rat hepatic N-OH-AAF/AAB and AcCoA/PA transacetylase activities requires the presence of the Michael acceptor functional group of VFK, the ability of EFK (Fig. 2), which contains an ethylketone rather than a vinyl ketone, to inactivate the transacetylase activities was evaluated. As shown in Table 1, incubation of rat hepatic NAT II fractions for 5.0 min with 25 μM EFK did not result in loss of either the N-OH-AAF/AAB N,N-AT or the AcCoA/PA NAT activities. The concentration of EFK which was used (25 μM) was 20-fold higher than that used to demonstrate irreversible inhibition by VFK (Table 1). These results establish that the Michael acceptor functional group of VFK is required for irreversible inactivation of the transacetylase activities.

Effect of VFK on unresolved rat hepatic transacetylase activities. The results of experiments which examined the ability of VFK to selectively inactivate a mixture of rat hepatic acetyltranserase activities present in the fractions obtained from Sephadex G-100 gel filtration chromatography are shown in Fig. 4. Incubation of these fractions with 6 μM VFK resulted in the irreversible reduction of the N-OH-AAF/AAB and AcCoA/PA transacetylation activities to 25–31% of control values, whereas the AcCoA/PABA NAT activity was not inhibited to a significant extent by this concentration of VFK. These results indicate that VFK is a selective inactivator of the NAT II activities.

Effects of substrates and products on the inactivation of rat hepatic acetyltransferase activities on VFK. An important criterion for determining whether irreversible inactivation of an enzyme by an affinity label is due to an active site modification is the demonstration that the presence of substrates, products, or reversible inhibitors of the enzyme can protect the enzyme from inactivation [21]. Therefore, various substrates and products of NAT II were tested for their abilities to prevent the VFK-mediated inactivation of the rat hepatic N-OH-AAF/AAB and AcCoA/PA transacetylase activities. Rat hepatic N-OH-AAF/AAB and AcCoA/PA transacetylase activities, either purified 5 to 6-fold by ammonium

Table 1. Effects of VFK and EFK on N-OH-AAF/AAB N,N-AT and AcCoA/PA NAT activities

	Transacetylation rate* (nmol/mg protein/min)					
	VFK				EFK	
Transacetylation activity	Before gel filtration		After gel filtration		After gel filtration	
	Control	Inhibited	Control	Inhibited	Control	Inhibited
N-OH-AAF/AAB	214.9 ± 17	40.2 ± 11 (19%)†	229.1 ± 24	52.9 ± 7‡ (23%)	223.2 ± 14	232.8 ± 18§
AcCoA/PA	54.5 ± 4	13.0 ± 6 (24%)	56.8 ± 6	14.2 ± 7‡ (25%)	61.1 ± 7	60.4 ± 10§

* Activities are presented as the means ± SD of three experiments.

The NAT II fractions from affinity chromatography were incubated in the absence (control) or presence (inhibited) of 1.25 μM VFK or 25 μM EFK. Following the incubation period, transacetylation activities of the fractions were measured before and after filtration through Sephadex G-25M, as indicated. The procedures are described under Materials and Methods.

† Values in parentheses represent the percent of control activity.

‡ Not significantly different from inhibited activity before gel filtration, $P > 0.05$.

§ Not significantly different from control, $P > 0.05$.

Table 2. Inactivation of rat hepatic transacetylase activities by VFK: Effects of 2-AAF, N-Ac-AAB and AcCoA

Protecting agent	% Control transacetylase activity*	
	N-OH-AAF/AAB	AcCoA/PA
None	31.0 ± 5.0†	25.4 ± 6.3†
2-AAF (0.05 mM)	74.9	75.7
N-Ac-AAB (0.05 mM)	61.1	64.8
AcCoA (0.05 mM)	78.4	66.7

* Incubations of the unresolved rat hepatic acetyltransferase fractions obtained by gel filtration were conducted in the presence of 6 μM VFK and the indicated protecting agent. Incubation mixtures were filtered through Sephadex G-25M prior to measurement of transacetylation activity as described in Materials and Methods. Control activities (incubated in the absence of VFK and expressed in units of nmol/mg protein/min, mean ± SD, N = 9) were 11.6 ± 0.7 for N-OH-AAF/AAB and 3.1 ± 0.3 for AcCoA/PA transacetylation. Each value is the mean of two experiments, except where indicated.

† Mean ± SD, N = 9.

sulfate precipitation and gel filtration, or purified approximately 100-fold by subsequent affinity chromatography of the gel filtration fractions, were incubated in the presence or absence of potential protecting agents and VFK. The results of these experiments are reported in Tables 2 and 3 and in Fig. 5. Shown in Table 2 are the results of studies performed with acetyltransferase activities that were purified approximately 5- to 6-fold by ammonium sulfate precipitation and gel filtration chromatography. The arylamide compounds 2-AAF and N-Ac-AAB, and the substrate AcCoA, at the relatively low concentration of 0.05 mM, effectively protected both the N-OH-AAF/AAB and AcCoA/PA acetyltransferase activities from inactivation by VFK. Activities were increased from 25–31% of control in the presence of 6 μM VFK alone to 65–78% of control in the presence of VFK and a 0.05 mM concentration of the protecting agents (Table 2).

The concentration-dependence of the AcCoA and 2-AAF mediated protection of the rat hepatic N-OH-AAF/AAB and AcCoA/PA transacetylation activities from inactivation by VFK was examined with the NAT II fractions obtained by affinity

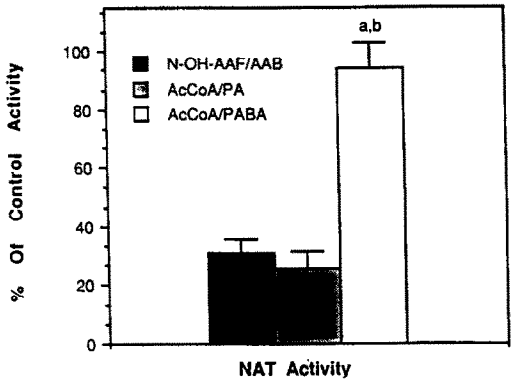

Fig. 4. Effect of VFK on unresolved rat hepatic NAT activities (purified by gel filtration). Rat hepatic acetyltransferase fractions purified by Sephadex G-100 gel filtration chromatography were incubated with 6 μM VFK and were filtered through Sephadex G-25M prior to measurement of the transacetylation activities as described under Materials and Methods. Control activities (obtained in the absence of VFK) were (in nmol/mg protein/min): 11.6 ± 0.7 for N-OH-AAF/AAB, 3.1 ± 0.3 for AcCoA/PA, and 5.6 ± 0.6 for the AcCoA/PABA transacetylation. Values are the means ± SD of nine experiments, except as indicated. (a) Mean ± SD, N = 3. (b) Not significantly different from control, P > 0.05.

Table 3. Inactivation of rat hepatic NAT II transacetylase activities by VFK: Effects of 2-AF, PA, *N*-acetylprocainamide (N-Ac-PA) and desulfoCoA*

Protecting agent	Percent of control activity	
	N-OH-AAF/AAB	AcCoA/PA
None	23.6 ± 3.7†	23.4 ± 5.3†
2-AF (0.4 mM)‡	34.5	30.7
PA (1.0 mM)	26.9	22.5
N-Ac-PA (1.0 mM)	34.9	28.5
desulfoCoA (1.0 mM)	20.9	26.8

* Rat hepatic NAT II fractions from Sepharose 6B 2-AF affinity chromatography were incubated in the presence of 1.25 μM VFK and the indicated protecting agent. Incubation mixtures were filtered through Sephadex G-25M prior to measurement of transacetylation activity as described in Materials and Methods. Control activities (incubated in the absence of VFK and expressed in units of nmol/mg protein/min, mean ± SD, N = 8) were 235.0 ± 14.1 for N-OH-AAF/AAB and 57.3 ± 3.1 for AcCoA/PA transacetylation. Each value is the mean of two experiments, except where indicated.

† Mean ± SD, N = 8.

‡ Limit of solubility of 2-AF, under the conditions of the experiments.

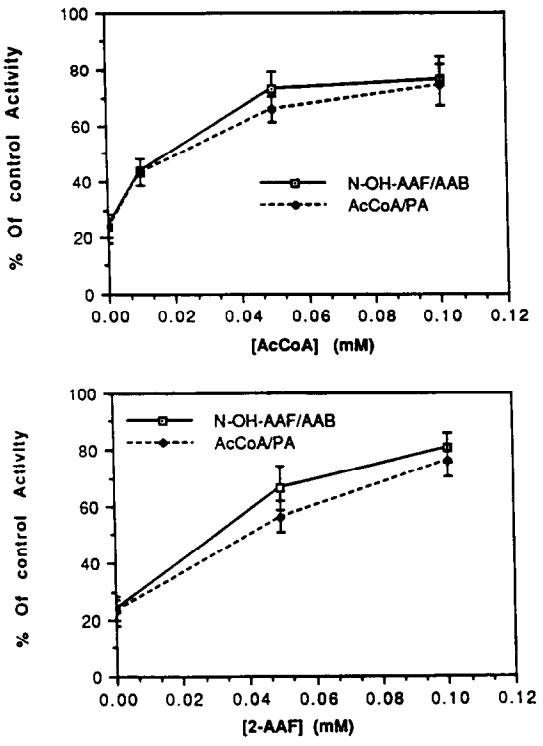

Fig. 5. Inhibition of rat hepatic NAT II transacetylase activities by VFK: effects of AcCoA and 2-AAF. Incubations of the rat hepatic NAT II fractions from Sepharose 6B 2-AF affinity chromatography were conducted in the presence of 1.25 μM VFK and the indicated concentrations of AcCoA or 2-AAF. Incubation mixtures were filtered through Sephadex G-25M prior to measurement of transacetylation activity as described under Materials and Methods. Values are the means ± SD of at least three experiments. Control activities are given under Table 3.

chromatography. As shown in Fig. 5, AcCoA and 2-AAF provided, in a concentration-dependent manner, protection of both transacetylation activities from inactivation by VFK. The levels of N-OH-AAF/AAB and AcCoA/PA transacetylation activities were very similar to each other (expressed as percent of control activity), either in the presence of VFK alone or in the presence of VFK and either AcCoA or 2-AAF.

Reported in Table 3 are the results of the experiments which tested the abilities of the arylamines 2-AF (0.4 mM) and PA (1.0 mM), the arylamide N-Ac-PA (1.0 mM) and the CoA analog desulfoCoA (1.0 mM) to protect the NAT II N-OH-AAF/AAB and AcCoA/PA transacetylase activities from inactivation by VFK. DesulfoCoA was chosen for study in lieu of CoA because it was anticipated that, due to the presence of the sulfhydryl group in CoA, it might react with the Michael acceptor functional group of VFK. None of these agents provided more than a slight amount of protection of the N-OH-AAF/AAB and AcCoA/PA transacetylase activities from VFK-mediated inactivation (Table 3). Similar studies performed on the less highly purified (5- to 6-fold) acetyltransferase gel filtration fractions

Table 4. Inactivation of rat hepatic NAT II AHAT activity by VFK*

Treatment	Methylthio adduct formation†
Control	235 ± 34
VFK	13 ± 2

* The NAT II fractions from Sepharose 6B 2-AF affinity chromatography were incubated in the presence or absence of 1.25 μM VFK and then filtered through Sephadex G-25M prior to measurement of methylthio adduct formation as described in Materials and Methods.

† Activity is expressed as nmol of methylthio adduct formed/mg protein/20 min. Results are the means ± SD of three experiments, carried out in triplicate.

indicated that the arylamine compounds AAB and sulfamethazine, at concentrations of up to 0.2 mM, also did not prevent the VFK-mediated inactivation of the N-OH-AAF/AAB and AcCoA/PA transacetylase activities (results not shown).

Inactivation of rat hepatic AHAT activity by VFK. Because VFK was designed to be an active-site-directed irreversible inhibitor of the arylhydroxamic acid-dependent acetyltransferase activities, demonstration that VFK can irreversibly inactivate arylhydroxamic acid *N,O*-acyltransferase (AHAT) activity is a critical test that it acts as anticipated. Therefore, rat hepatic NAT II fractions were incubated with VFK and then were assayed for their ability to catalyze the conversion of N-OH-AAF to an electrophile that forms methylthio adducts upon reaction with the nucleophile, *N*-acetylmethionine [6]. The results of these experiments, reported in Table 4, show that incubation of the NAT II fractions with 1.25 μM VFK resulted in the irreversible loss of 95% of the methylthio adduct-forming (*N,O*-acyltransferase) activity. This demonstrates that VFK is a potent inactivator of the rat hepatic *N,O*-acyltransferase activity.

Inactivation of rat hepatic NAT I activity by VFK. The ability of a low concentration of VFK to selectively inactivate the rat hepatic N-OH-AAF/AAB N,N-AT and AcCoA/PA NAT, but not the AcCoA/PABA NAT activity in the Sephadex G-100 fractions has been demonstrated (Fig. 4). Because VFK is an inherently reactive compound, it was believed possible that relatively high concentrations of VFK might inactivate the AcCoA/PABA NAT activity. To test this hypothesis, rat hepatic NAT I fractions, separated from the NAT II transacetylase activities by Sepharose 6B 2-AF affinity chromatography, were incubated with VFK under conditions that, except for incubation time and concentration of VFK, were identical to those used to demonstrate the concentration-dependence of VFK-mediated inactivation of rat hepatic N-OH-AAF/AAB N,N-AT activity (Fig. 3).

The results of these experiments are shown in Fig. 6. Incubation of rat hepatic NAT I fractions with high concentrations of VFK resulted in the concentration-dependent irreversible inhibition of the AcCoA/PABA NAT activity. Upon incubation with 100 μM VFK for 2 min, the AcCoA/PABA transacetylase

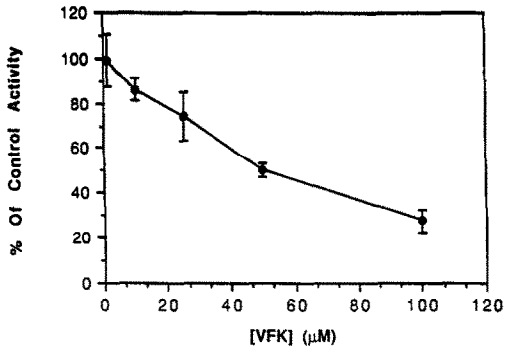

Fig. 6. Inactivation of rat hepatic NAT I transacetylase activity by VFK. Rat hepatic NAT I fractions from Sepharose 6B 2-AF affinity chromatography were incubated with the indicated concentration of VFK for 2 min and then were filtered through Sephadex G-25M. AcCoA/PABA transacetylation activity was measured after filtration, as described under Materials and Methods. Results are the means ± SD of three experiments. Control AcCoA/PABA transacetylation activity was 52.1 ± 2.2 nmol/mg protein/min.

activity was reduced to 28% of control values. In comparison, incubation of the NAT II fractions with a 200-fold lower concentration of VFK (0.5 μM) for 1 min resulted in the reduction of the N-OH-AAF/AAB transacetylase activity to 17% of control (Fig. 3). Thus, VFK is a much more potent inactivator of the NAT II transacetylase activities as compared to the NAT I transacetylase activities.

Experiments were also conducted to determine the effect of AcCoA on the VFK-mediated inactivation of the NAT I AcCoA/PABA transacetylase activity. Following incubation with 100 μM VFK for 2 min, the AcCoA/PABA transacetylation activity was inhibited to 28% of control; this was increased to 61% of control when the transacetylase preparation was incubated with 0.2 mM AcCoA prior to the addition of 100 μM VFK. Thus, even though the concentrations of VFK that are necessary to inactivate the NAT I AcCoA/PABA transacetylase activity are high in comparison to those necessary to inactivate the NAT II activities, at least a portion of the VFK-mediated inactivation of the rat hepatic NAT I transacetylase activity may be prevented by AcCoA.

DISCUSSION

The principal objective of this research was to develop an active site directed irreversible inhibitor (affinity label) for AHAT and N,N-AT activities (NAT II). The results of the enzyme inactivation experiments clearly demonstrate that VFK is a potent inactivator of NAT II activities and that much higher concentrations of VFK are required to inactivate NAT I than are required for inactivation of NAT II. The proposal that the fluorene ring system would impart selectivity for the active site of NAT II was verified by the relative effectiveness of VFK as an inactivator of NAT II compared to NAT I. Although the selectivity of affinity labels that contain ring systems other than fluorene has not been examined, it is anticipated that such structure–activity studies will provide further insight into differences in the active site topography of the isozymes. In preliminary studies, the benzene analog of VFK has been found to be unsuitable as an affinity label because of its chemical instability.

The selectivity of VFK was demonstrated further by inactivation experiments with an enzyme preparation that contained both NAT I and NAT II activities (Fig. 4). In related studies, VFK also was found to inactivate selectively the NAT II activities present in a hamster liver preparation (results not presented). The selectivity of inactivation of NAT isozymes indicates that compounds such as VFK may be useful tools for the detection and investigation of multiple forms of NAT, N,N-AT, and AHAT activities.

VFK caused extremely rapid inactivation of NAT II activity (Fig. 3). Nearly complete loss of N,N-AT activity occurred within 1 min when the enzyme was incubated with 0.5 μM VFK. The rapid rate of enzyme inactivation precluded the direct determination of kinetic constants for the inactivation process. Attempts to conduct the inactivation experiments at 0° were not successful because of the limited solubility of VFK in aqueous solution at low temperatures. The very fast rate of enzyme inactivation caused by VFK is similar to that observed for the inactivation of phosphoenolpyruvate carboxykinase by affinity labels that contain Michael acceptor functional groups [33].

The rationale for the design of VFK was based on its structural similarity to N-OH-AAF (Fig. 2). The latter compound is a highly effective acetyl donor in both the arylhydroxamic acid dependent N,N-AT reaction (Fig. 1C) and in the AHAT reaction (Fig. 1B). It was anticipated that the high degree of structural similarity between VFK and N-OH-AAF would result in VFK functioning as an active site directed inhibitor. When partially purified NAT II was preincubated with either the substrate, AcCoA, or the arylamides, 2-AAF and N-Ac-AAB, substantial protection from inactivation by VFK was achieved (Table 2). Further, the degree of the protection afforded by both AcCoA and 2-AAF was dependent on their concentration and was virtually identical for both N-OH-AAF/AAB, N,N-AT and AcCoA/PA activities (Fig. 5). The protection of the enzyme from VFK-mediated inactivation by the cofactor, AcCoA, and by the arylamide products, 2-AAF and N-Ac-AAB, strongly supports the conclusion that VFK is an active site directed reagent. Neither of the arylamines, 2-AF and PA, nor the CoA analog, desulfoCoA, afforded protection from the inactivating effects of VFK (Table 3). Also, when the arylamide N-Ac-PA was preincubated with NAT II, no protection was achieved (Table 3). Thus, the arylamides that have an extended aromatic ring system, such as 2-AAF and N-Ac-AAB, were effective protecting agents, whereas N-Ac-PA, which has only a single aromatic ring, was ineffective.

The results of the protection experiments with AcCoA, 2-AF and PA are similar to those reported by Andres *et al.*, who found that whereas preincubation of rabbit hepatic NAT with AcCoA protects the enzyme from inactivation by

bromoacetanilide, the arylamine, 4-ethylaniline, provides no protection from inactivation [23]. The ability of AcCoA to prevent inactivation of NAT II by VFK may be attributed to acetylation of an active site nucleophile, presumably a cysteine thiol residue, thereby preventing reaction of the nucleophile with the α,β-unsaturated ketone group of VFK. The protection from inactivation that was afforded by 2-AAF and N-Ac-AAB is consistent with the ping–pong reaction mechanism by which arylamine N-acetylation occurs [2]. According to the ping–pong mechanism, acetylation products, such as 2-AAF and N-Ac-AAB, would be expected to bind to the active site of the unacetylated enzyme, thus preventing access to the active site by VFK. Similarly, the inabilities of 2-AF and AAB to provide a protecting effect, even at relatively high concentrations, is consistent with the ping–pong mechanism because it would be expected that the arylamine substrates would bind preferentially to the acetylated form of the enzyme [34].

That the α,β-unsaturated carbonyl group (Michael acceptor) of VFK is required in order for the enzyme inactivation process to occur was demonstrated by determining whether EFK could cause an irreversible reduction of either N-OH-AAF/AAB N,N-AT activity or AcCoA/PA NAT activity. In EFK the conjugated double bond (vinyl group) that is present in VFK has been replaced by an ethyl group. Thus, EFK is incapable of undergoing a Michael-type conjugate addition reaction with a nucleophile. Incubation of NAT II with 25 μM EFK did not cause any irreversible loss in either the N-OH-AAF/AAB N,N-AT or the AcCoA/PA NAT activity, whereas incubation with 1.25 μM VFK caused 80% loss of enzyme activity within 30 sec (Table 1). Thus, the electrophilic vinyl ketone group is required for manifestation of the enzyme inactivating effects of VFK.

Although VFK might be expected to react by a nonenzymatic process with sulfhydryl groups that are present in the incubation medium, its very rapid enzyme inactivating effect made it possible to use a sufficient concentration of DTT in the incubation mixture to maintain maximal enzyme activity in control incubations. The lowest ratio of DTT to VFK that was used in the inactivation experiments was approximately 40:1 (80:1, based on SH equivalents). Thus, it was not possible for the DTT concentration to be reduced significantly by reaction with VFK. Other investigators also have found that active site directed affinity labels which contain Michael acceptor functional groups are effective in the presence of DTT [35].

The irreversible nature of the inhibition of the NAT II activities was demonstrated by treating the enzyme preparation with VFK and then measuring the N-OH-AAF/AAB N,N-AT and the AcCoA/PA NAT activities both before and after gel filtration. The negligible recovery of the enzyme activities following gel filtration clearly indicates that the inhibition was irreversible (Table 1). The data presented in Table 4 show that AHAT activity also was not recovered after incubation of the enzyme with VFK and gel filtration of the preparation. Similarly, extensive dialysis of a hamster hepatic NAT II preparation that had been treated with VFK did not result in recovery of enzyme activity (results not presented). The inhibition also was not reversed by the high concentration of cysteine (10 mM) that was used to terminate the VFK-mediated enzyme inactivation process or by the presence of DTT (1 mM) in the incubation mixtures in which the enzyme activities were measured.

Although definitive evidence for the identity of the active site nucleophile that is alkylated by VFK will have to await experiments with more highly purified enzyme, it is reasonable to propose that a cysteine sulfhydryl group may be involved. Studies with both rabbit and pigeon hepatic NAT indicate the importance of an active site cysteine in the catalysis of the N-acetyltransfer reaction [23, 24]. No data have been published regarding the identity of the active site nucleophile of rat liver NAT, N,N-AT or AHAT. However, both the AHAT and N,N-AT activities of rat liver are sensitive to oxygen and are inactivated by reagents that react with sulfhydryl groups, lending support to the proposed importance of one or more sulfhydryl groups for enzyme activity [15, 36, 37].

Affinity labels have found utility in the investigation of catalytic mechanisms, the identification of essential amino acid residues, and the determination of the active site topography of enzymes [21]. The findings that multiple forms of acetyltransferase activity exist in human [17], hamster [13, 16, 38], mouse [39] and rat liver [14] raise the possibility that appropriately designed affinity labels could serve as molecular probes for differentiating the properties of these isozymes. The results obtained with VFK and with bromoacetanilide [23] indicate that reagents of this type will be of value in the investigation of mammalian transacetylases.

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