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Short Communication

INACTIVATION OF HAMSTER MONOMORPHIC *N*-ACETYLTRANSFERASE BY VINYL FLUORENYL KETONE

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Abstract—Arylamine *N*-acetyltransferases (NATs) are cytosolic enzymes that play important roles in the detoxification and activation of xenobiotic arylamines and their metabolites. Vinyl fluorenyl ketone (VFK) is a selective and potent active site-directed irreversible inhibitor of rat liver monomorphic NAT. The present study demonstrated that VFK is an active site-directed affinity label for hamster liver monomorphic NAT, but is a much less effective inactivator of the polymorphic *N*-acetyltransferase isozyme. The potency, irreversibility and selectivity of VFK make it a potentially valuable tool for characterization of NATs that exhibit acetyl donor specificity similar to that of hamster monomorphic NAT.

Key words: *N*-acetyltransferase; affinity label; arylamines; hamster hepatic *N*-acetyltransferase; monomorphic *N*-acetyltransferase; polymorphic *N*-acetyltransferase

Acetyl coenzyme A:arylamine *N*-acetyltransferase (EC 2.3.1.5) catalyzes the conversion of primary arylamine xenobiotics to arylamides in mammalian tissues. Various isoforms of NAT§ also catalyze the O-acetylation of *N*-hydroxyarylamines, the transfer of acetyl groups from nitrogen to oxygen in *N*-arylhydroxamic acid substrates, and the intermolecular transfer of acetyl groups from *N*-arylhydroxamic acids to primary arylamines [1]. The versatility of NATs with regard to their capabilities for conjugating carcinogenic arylamines and arylamine metabolites, and the apparent relevance of acetylator genotype to the manifestation of the untoward effects of these agents [2], have prompted the development of chemical probes for characterization of the enzymes [3, 4]. Wick *et al.* [4] demonstrated that VFK (Fig. 1) is a selective, potent and irreversible inactivator of one of the two NAT isozymes present in rat liver cytosol. Studies of the relationship between acetylator phenotype and the expression of NAT isozymes in rat liver indicate that the isozyme which is selectively affinity labeled by VFK is monomorphic [5]. The present studies provide evidence that VFK also is a selective affinity label for hamster liver NAT1‖ and extend the potential utility of VFK as a tool for characterization of NATs.

Materials and Methods

AcCoA (trilithium salt), D,L-dithiothreitol, PA hydrochloride, PABA (sodium salt), DMSO, tetrasodium pyrophosphate, L-cysteine, bovine serum albumin, cellulose dialysis tubing and Sephadex G-100 were purchased from the Sigma Chemical Co., St. Louis, MO. Epoxy-activated Sepharose 6B was purchased from Pharmacia, Piscataway, NJ.

The syntheses of 1-(fluoren-2-yl)-2-propen-1-one (vinyl fluorenyl ketone, VFK), 1-(fluoren-2-yl)-2-propan-1-one (ethyl fluorenyl ketone, EFK) and N-OH-AAF were described previously [4, 6]. Coupling of AAB to epoxy-activated Sepharose 6B was accomplished as previously described [7].

Male Golden Syrian hamsters (50–80 g) were obtained from Harlan ARS/Sprague Dawley (Indianapolis, IN). The partial purification of hamster liver NATs was carried out as reported previously [7].

Acetyltransferase-containing fractions obtained from Sepharose 6B AAB chromatography were concentrated by ultrafiltration and stored in 10–30% glycerol at −70°. Protein concentrations were measured by the method of Lowry *et al.* [8] with bovine serum albumin as the standard.

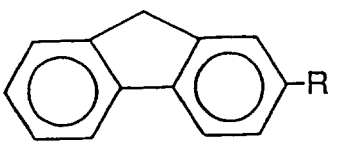

Fig. 1. Structures of VFK (R: $COCH{=}CH_2$), EFK (R: $COCH_2CH_3$) and N-OH-AAF (R: $NOHCOCH_3$).

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§ Abbreviations: NAT, *N*-acetyltransferase; NAT1, monomorphic *N*-acetyltransferase; NAT2, polymorphic *N*-acetyltransferase; N-OH-AAF, *N*-hydroxy-2-acetylaminofluorene; PA, procainamide; PABA, *p*-aminobenzoic acid; AAB, 4-aminoazobenzene; 2-AF, 2-aminofluorene; 2-AAF, 2-acetylaminofluorene; AcCoA, *S*-acetyl coenzyme A; N-Ac-AAB, *N*-acetyl-4-aminoazobenzene; SMZ, sulfamethazine; VFK, vinyl fluorenyl ketone; EFK, ethyl fluorenyl ketone; and DTT, dithiothreitol.

‖ Previous papers from this laboratory (e.g. Ref. 3 and references contained therein) have used the designation NAT II and NAT I for the monomorphic and polymorphic isozymes, respectively. To be consistent with current convention, the designations NAT1 and NAT2 are used in the present paper to indicate, respectively, the monomorphic and polymorphic isozymes.

The N-OH-AAF/AAB, AcCoA/PABA and AcCoA/PA transacetylation assays were performed as previously described [3, 4, 9]. In each assay, the concentration of unacetylated amine substrate (AAB, PABA or PA) is measured spectrophotometrically after the reaction is terminated. Protein concentrations varied with the state of purification of the protein and were chosen from the linear portion of the plots of amine acetylation versus protein concentration. Incubations were performed at 37° for a time period determined from the linear portion of the graphs of amine acetylation versus time.

Inactivation of hamster hepatic NAT1 by VFK. Incubation mixtures contained the glycerol-stabilized hamster hepatic NAT1 fractions from Sepharose 6B AAB affinity chromatography (final protein concentration 0.06 mg/mL), 0.5 μM VFK, 2% DMSO, 1 mM DTT, and 0.05 M sodium pyrophosphate buffer (pH 7.0) in a final volume of 2.6 mL. The incubation was conducted at 37° for 0.5 min. At the end of the incubation period, 1.2 mL of a solution of cysteine was added, which brought the cysteine concentration to 10 mM. Aliquots (0.48 mL) of the resulting solution were removed for analysis of N-OH-AAF/AAB transacetylation activity. The remainder (2.1 mL) was subjected to 3 hr of dialysis against three 200-mL portions of 0.05 M sodium pyrophosphate buffer (pH 7.0, 1 mM DTT, 2% DMSO). The buffer was purged with nitrogen throughout the dialysis period. At the end of the dialysis period, 0.48-mL aliquots of the dialysate were assayed for N-OH-AAF/AAB transacetylation activity. In all of the experiments described herein, enzyme preparations that had not been treated with VFK were subjected to dialysis and were used as controls.

Effect of VFK on unresolved hamster hepatic NAT1 and NAT2 activities: Protection from inactivation by substrates and products. Incubation mixtures contained the glycerol-stabilized unresolved NAT activities obtained from Sephadex G-100 gel filtration chromatography (final protein concentration 1.0 mg/mL), 1.0 μM VFK, 2% DMSO, 0.5 mM DTT and 0.05 M sodium pyrophosphate buffer (pH 7.0) in a final incubation volume of 1.5 mL. Reactions were conducted for 0.5 min in the presence or absence of the potential protecting agents, which were incubated with the enzyme at 37° for 1 min prior to the addition of VFK. After the addition of cysteine to a concentration of 10 mM, the mixtures were dialyzed for 15 hr against 3 L of 0.05 M sodium pyrophosphate buffer (pH 7.0, 1 mM DTT, 2–5% DMSO), and then for 3 hr against two 500-mL portions of fresh buffer. The buffer was purged with nitrogen throughout the dialysis period. At the end of the dialysis period, aliquots of the dialysate were analyzed for N-OH-AAF/AAB, AcCoA/PA and AcCoA/PABA transacetylation activities.

Inactivation of hamster hepatic NAT2 by VFK. The glycerol-stabilized NAT2 fractions from Sepharose 6B AAB affinity chromatography (final protein concentration 0.06 mg/mL) were incubated at 37° for 2 min in the presence of 5–25 μM VFK, 2% DMSO, 1 mM DTT and 0.05 mM sodium pyrophosphate buffer (pH 7.0) in a final incubation volume of 1.3 mL. The entire contents of the incubation flasks were subjected to dialysis (3 hr) as described above for the irreversible inhibition of hamster hepatic NAT1. The dialysate was analyzed for AcCoA/PABA transacetylation activity.

Results and Discussion

Catalysis of the transfer of the acetyl group from N-OH-AAF to AAB (N-OH-AAF/AAB transacetylation) and catalysis of the AcCoA-dependent acetylation of PA (AcCoA/PA transacetylation) are activities that are associated almost exclusively with the monomorphic NAT (NAT1) in hamster liver. The AcCoA-dependent acetylation of PABA (AcCoA/PABA transacetylation) is catalyzed principally by the polymorphic isozyme (NAT2) [7, 10–12]. In the present study, previously published methods were used to achieve a 7- to 9-fold purification of the NAT activities through ammonium sulfate precipitation and Sephadex G-100 gel filtration chromatography [7]. Further purification by Sepharose 6B AAB affinity chromatography [7] resulted in separation of NAT1 and NAT2 activities and afforded a cumulative 40- to 50-fold purification of the activities (data not shown).

The NAT1 fractions, obtained by affinity chromatography, were incubated with VFK and were subjected to dialysis to remove any portion of the affinity label that

Table 1. Inactivation of resolved hamster hepatic NAT1 and NAT2 by VFK

	% Control transacetylase activity	
VFK (μM)	NAT1*	NAT2†
0	100	100
0.5	7.0 ± 6	
5.0		75 ± 6
25.0		48 ± 2

* Hamster hepatic NAT1 fractions from Sepharose 6B AAB affinity chromatography were incubated with VFK for 0.5 min. N-OH-AAF/AAB transacetylation activity was measured after dialysis, as described in Materials and Methods. Results are expressed as the mean ± range of two experiments. Control activity was 422 ± 17 nmol/mg protein/min.

† Hamster hepatic NAT2 fractions from Sepharose 6B AAB affinity chromatography were incubated in the presence of the indicated concentration of VFK for 2 min, and then subjected to dialysis. AcCoA/PABA transacetylation activity was measured after dialysis, as described in Materials and Methods. Results represent the mean ± range of two experiments. Control activity was 327 ± 16 nmol/mg protein/min.

Table 2. Inactivation of hamster hepatic NAT1 activities by VFK: protection by arylamines, arylamides and AcCoA*

	% Control transacetylase activity	
Protecting agent	N-OH-AAF/AAB	AcCoA/PA
None	39 ± 5†	37 ± 4†
2-AF (0.1 mM)	57 ± 3	61 ± 4
2-AAF (0.1 mM)	74 ± 1	80 ± 7
AAB (0.1 mM)	78 ± 1	77 ± 4
N-Ac-AAB (0.1 mM)	93 ± 3	92 ± 1
PA (0.25 mM)	31 ± 2	32 ± 2
SMZ (0.25 mM)	34 ± 1	33 ± 4
AcCoA (0.05 mM)	88 ± 0	93 ± 1

* Hamster hepatic NAT activities, purified by Sephadex G-100 gel filtration chromatography, were incubated with 1 μM VFK and the indicated protecting agent. Following dialysis, transacetylase activities were measured as described in Materials and Methods. Results represent the mean ± range of two experiments, except where indicated. Control activities were (in units of nmol/mg protein/min) 82 ± 7 (N = 7) for N-OH-AAF/AAB and 6.3 ± 0.4 (N = 7) for AcCoA/PA transacetylation.

† Mean ± SD, N = 7.

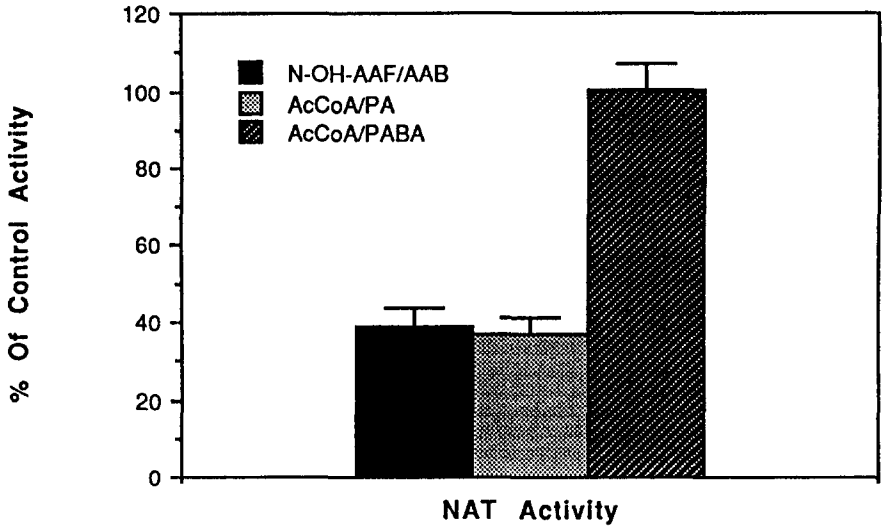

Fig. 2. Effect of VFK on unresolved hamster hepatic NAT activities. Hamster hepatic NATs, purified by Sephadex G-100 gel filtration chromatography, were incubated with 1 μM VFK. Following dialysis of the incubation mixtures, transacetylase activities were determined as described under Materials and Methods. Results represent the means ± SD of seven experiments, except for AcCoA/PABA (N = 3). Control activities were (in units of nmol/mg protein/min) 82 for N-OH-AAF/AAB, 6.3 for AcCoA/PA, and 235 for AcCoA/PABA.

was not covalently bound to the enzyme. As shown in Table 1, a low concentration of VFK (0.5 μM) caused extensive and irreversible inactivation of N-OH-AAF/AAB transacetylation activity after a 0.5-min incubation period. In contrast, treatment of NAT2 fractions from affinity chromatography for 2 min with 10- and 50-fold greater concentrations of VFK caused only 25 and 52% reductions, respectively, in AcCoA/PABA transacetylation activity (Table 1). The inactivation of both NATs was irreversible, as indicated by the lack of recovery of activity after extensive dialysis.

The previously reported studies of the effect of VFK on rat NATs established that the agent inactivated the enzymes by an active site-directed mechanism [4]. To determine whether the VFK-mediated inactivation involves the active site of hamster liver NAT1, the abilities of substrates and products of NAT-catalyzed reactions to protect the enzyme from inactivation were evaluated. The NAT cofactor, AcCoA, and the *N*-acetylated compounds 2-AAF and N-Ac-AAB, afforded the greatest protection against inactivation by VFK (Table 2). The arylamine substrates, PA and SMZ, did not reduce the extent of inactivation. Two other arylamines, 2-AF and AAB, attenuated the inhibitory action of VFK, but neither compound was as effective as N-Ac-AAB or AcCoA. The results are similar to those obtained from analogous experiments with rat NAT, in that AcCoA and *N*-acetylated arylamines tend to be better protecting agents than arylamines. The data support the conclusion that VFK is an affinity label for the acetyl donor (N-OH-AAF) binding site of hamster liver NAT1.

The isozyme selectivity of VFK was evaluated further by incubating it with a preparation of partially purified, but unresolved, NAT1 and NAT2. The results are illustrated in Fig. 2. The NAT1 transacetylation activities were irreversibly lowered to approximately 40% of control values under the conditions of these experiments, whereas there was no loss of the NAT2-mediated AcCoA/PABA transacetylation activity. These results, together with those presented in Table 1, demonstrate that VFK is a highly selective irreversible inhibitor of hamster hepatic NAT1.

Experiments with the VFK analogue, EFK, which is an ethyl ketone rather than a vinyl ketone (Fig. 1), were conducted to determine whether or not the conjugated carbonyl is essential to the inhibitory effectiveness of VFK. Concentrations of EFK as high as 10 μM did not inactivate hamster NAT1 (data not presented). This result is identical to that obtained with rat NAT, and supports the proposal that VFK inactivates NATs by reaction of the conjugated ketone with an active site nucleophile [4].

In summary, the data presented in this report demonstrated that VFK, which contains an electrophilic conjugated ketone in place of the *N*-hydroxy-*N*-acetyl group of N-OH-AAF (Fig. 1), is a potent and highly isozyme-selective affinity label for hamster hepatic NAT1. VFK can be expected to be a useful tool for characterization of NAT isozymes that exhibit acetyl donor specificities similar to those of the rat and hamster monomorphic NATs.

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