Formation of Difluorothionoacetyl-Protein Adducts by *S*-(1,1,2,2-Tetrafluoroethyl)-L-cysteine Metabolites: Nucleophilic Catalysis of Stable Lysyl Adduct Formation by Histidine and Tyrosine†

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ABSTRACT: ^{19}F NMR spectroscopy was used in conjunction with isotopic labeling to demonstrate that difluorothionoacetyl-protein adducts are formed by metabolites of the nephrotoxic cysteine conjugate *S*-(1,1,2,2-tetrafluoroethyl)-L-cysteine (TFEC). To determine which amino acid residues can be involved in adduct formation, the reactivity of TFEC metabolites with a variety of *N*-acetyl amino acids was also investigated. An N^{α}-acetyl-N^{ϵ}-(difluorothionoacetyl)lysine (DFTAL) adduct was isolated and characterized by ^{19}F and ^{13}C NMR spectroscopy and mass spectrometry. N^{α}-Acetylhistidine and *N*-acetyltyrosine were found to act as nucleophilic catalysts to facilitate the formation of both the protein and DFTAL adducts. Adduct formation was greatly reduced when lysyl-modified protein was used as the substrate, indicating that lysyl residues are primary sites of adduct formation. However, N^{α}-acetyllysine, at concentrations of >100-fold in excess compared to protein lysyl residues, was not effective in preventing binding of metabolites to protein. Therefore, nucleophilic catalysis at the surface of the protein may be an important mechanism for the binding of TFEC metabolites to specific lysyl residues in protein. TFEC metabolites were very reactive with the thiol nucleophiles glutathione and *N*-acetylcysteine. However, the predicted difluorodithioesters could not be isolated. Both stable difluorothioacetamide and less stable difluorodithioester protein adducts may play a role in TFEC-mediated nephrotoxicity.

Cysteine conjugates derived from a variety of halogenated ethylenes are nephrotoxic in rats (Terricini & Parker, 1965; Bonhaus & Gandolfi, 1981; Nash et al., 1984; Odum & Green, 1984) and mice (Darnerud et al., 1988; Cojocel et al., 1989) and are also toxic to primary cultures of renal proximal tubule cells from several species, including human (Schnellmann et al., 1987; Boogaard et al., 1989; Chen et al., 1990). The enzymatic conjugation of halogenated ethylenes with glutathione, subsequent enzymatic processing to the corresponding cysteine conjugates and mercapturates, and β-lyase-catalyzed elimination of toxic sulfur-containing metabolites from the cysteine conjugates are well-documented events (Figure 1). For recent reviews see Dekant et al. (1989), Stevens and Jones (1989), and Commandeur and Vermeulen (1990).

Covalent binding of the sulfur-containing metabolites to cellular macromolecules is also well documented, and this binding is presumed to initiate a cascade of events that eventually leads to cell death and in some cases mutagenesis (Dekant et al., 1989; Chen, Q., et al., 1990). However, the chemical nature of the metabolites ultimately responsible for binding and toxicity is not completely clear. Likewise, the chemical nature of the cellular molecules susceptible to attack by cysteine conjugate metabolites is poorly understood. With the nephrotoxic gas tetrafluoroethylene as an example, Figure 1 summarizes the metabolic transformations that halogenated ethylenes are known to undergo in vivo, the proposed subsequent chemical transformations of the metabolites, and the unknown events following covalent binding of metabolites that lead to cell death.

Mechanisms have been proposed whereby thiols (Green & Odum, 1985), thiiranes (Commandeur et al., 1988, 1989; Boogaard et al., 1989), thioketenes (Veltman et al., 1988), or thionoacyl halides (Green & Odum, 1985; Dekant et al., 1987, 1988a,b; Commandeur et al., 1988, 1989; Boogaard et al., 1989) are the ultimate species responsible for the binding of metabolites of various cysteine conjugates to cellular macromolecules. Thioamides, presumably resulting from the reaction of thionoacyl and/or thioketene metabolites with nucleophilic amines (diethylamine, benzylamine, and aniline) have been reported (Dekant et al., 1987, 1988a,b; Commandeur et al., 1988, 1989; Boogaard et al., 1989), suggesting protein or nucleic acid amine nucleophiles as probable candidates for in vivo reaction with cysteine conjugate metabolites.

For saturated, fluorinated conjugates, thioketene formation is not envisioned. Rather, thiirane or thionoacyl fluoride intermediates might be expected, difluorothionoacyl fluoride formation being confirmed for TFEC as mentioned above (Commandeur et al., 1988, 1989). However, no structural information is available to verify that these proposed reaction mechanisms actually occur with nucleophiles commonly found in the cellular environment. Therefore, we employed ^{19}F NMR[1] spectroscopy in conjunction with ^{35}S isotopic labeling

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[1] Abbreviations: BSA, bovine serum albumin; DFAA, difluoroacetic acid; DFTAA, difluorothionoacetic acid; DFTAL, N^{α}-acetyl-N^{ϵ}-(difluorothionoacetyl)-L-lysine; DFTA-TFEC, *N*-(difluorothionoacetyl)-*S*-(1,1,2,2-tetrafluoroethyl)-L-cysteine; DMAP, 4-(*N*,*N*-dimethylamino)pyridine; HPLC, high-pressure liquid chromatography; γMTB, α-keto-γ-methiolbutyric acid; NMR, nuclear magnetic resonance; PLP, pyridoxal 5′-phosphate; TCA, trichloroacetic acid; TFA, trifluoroacetic acid; TFEC, *S*-(1,1,2,2-tetrafluoroethyl)-L-cysteine; TLC, thin-layer chromatography; TMS, tetramethylsilane.

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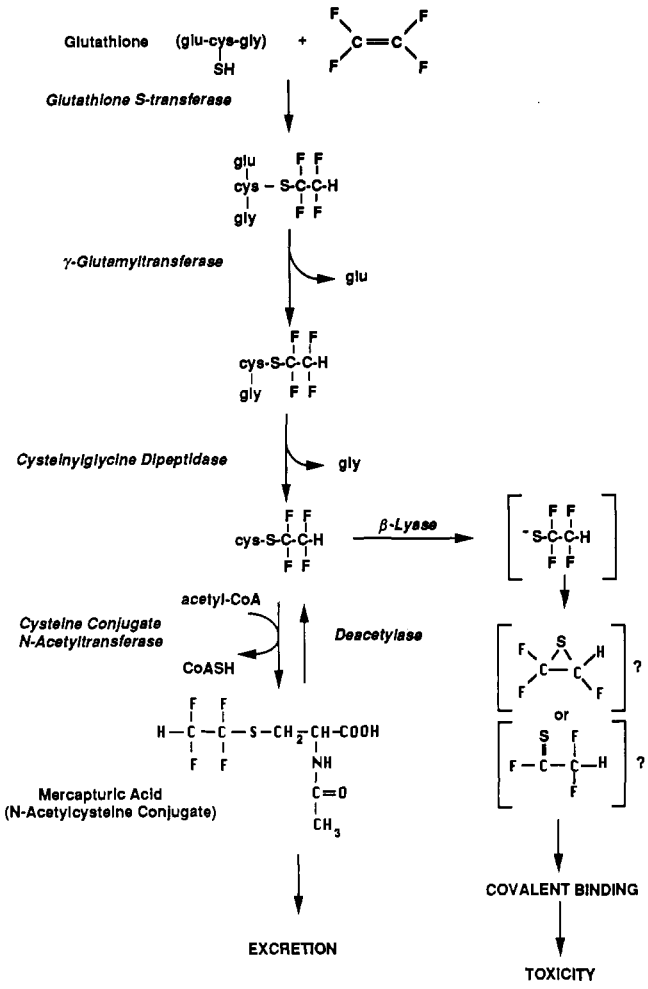

FIGURE 1: The involvement of the mercapturic acid and β-lyase pathways in halogenated ethylene induced nephrotoxicity. Italics indicate enzymes involved in metabolic transformations. Brackets indicate proposed structures of reactive intermediates that have not been isolated.

to determine the structure of the sulfur-containing adducts formed between metabolites of the nephrotoxic cysteine conjugate *S*-(1,1,2,2-tetrafluoroethyl)-L-cysteine (TFEC) and proteins.

In addition, to gain an understanding of TFEC metabolite reactivity with nucleophiles other than amines that are commonly found in cellular environments, we examined the ability of TFEC metabolites to react with the ubiquitous intracellular nucleophile glutathione and the N^{α}-acetyl derivatives of a variety of amino acids possessing nucleophilic moieties. A stable N^{α}-acetyl-N^{ϵ}-(difluorothionoacetyl)lysine (DFTAL) adduct was isolated and characterized. The thiol compounds glutathione and *N*-acetylcysteine were found to be considerably better nucleophiles toward TFEC metabolites than amines. However, no stable adducts of these compounds could be isolated. N^{α}-Acetylhistidine and *N*-acetyltyrosine were found to facilitate the formation of both protein and DFTAL adducts. A nucleophilic catalysis mechanism is proposed to account for this phenomenon.

Materials and Methods

Instrumentation. ^{19}F and ^{13}C NMR spectra were recorded with an IBM NR/250 FTNMR spectrometer at 235.33 and 62.90 MHz, respectively. Samples were dissolved in 50 mM potassium phosphate buffer (pH 7.4). Concentrations, based on ^{35}S incorporation, were 340 μM for the BSA adduct, 300 μM for the tubule protein adducts, and in the range of 5–20 mM for the DFTA-TFEC and DFTAL adducts. References [trifluoroacetic acid (TFA) and tetramethylsilane (TMS)] were assigned 0 ppm.

Positive-ion thermospray mass spectra were obtained on a Finnigan 4600 single-quadrupole instrument using a Finnigan thermospray ion source. Conditions: Brownlee RP-300 column (100 × 4.6 mm), gradient mobile phase (0.1 M ammonium acetate, pH 5.0, vs acetonitrile; 5–70% B over 30 min; flow rate 1.4 mL/min). The ion source block temperature was 220 °C; the vaporizer temperature was 120 °C initially and was decreased with increasing acetonitrile concentration. Spectra were recorded in 1.95-s scans over the mass range 150–800 amu. Electron multiplier voltage was 1400 eV.

Continuous-flow FAB mass spectra were recorded with a Finnigan TSQ70 triple-quadrupole mass spectrometer using a BioProbe flow FAB ion source and FAB11NF saddle field ion gun. The mobile phase was 5% glycerol in 86:24 water/acetonitrile. Mobile phase was introduced at 6 μL/min with use of a Brownlee microgradient pump; probe temperature was 25 °C. The sample was dissolved in 10 μL of DMSO + 20 μL of mobile phase and introduced via a Rheodyne Model 7410 injection valve (1 μL). Spectra were acquired in alternating positive/negative ion scan mode using 2.0-s scans over the mass range 150–800 amu. Samples were bombarded with a xenon atom beam at 8 kV of kinetic energy (1.0 mA).

Analytical and preparative high-pressure liquid chromatography (HPLC) was performed with a Rainin Rabbit HP dual-pump system equipped with a Gilson model 116 dual-wavelength UV detector and Gilson system controller software. Mobile phase was 0.1% trifluoroacetic acid (TFA) in water vs 0.09% TFA in 84% acetonitrile or 1.0% ammonium acetate (pH 6.7) in water vs acetonitrile. Typically, gradients were 0–75% B over 75 min. Analytical samples were assayed with a Waters μBondapak C18 column (300 × 3.9 mm) at a flow rate of 1.0 mL/min. Detection was at 210 and 280 nm, 0.1 AUFS. Preparative samples were purified on a Waters μBondapak C18 semiprep column (300 × 7.8 mm) with the TFA system at a flow rate of 3.0 mL/min. Detection was at 210 and 280 nm, 2.0 AUFS.

Synthesis of S-(1,1,2,2-Tetrafluoroethyl)-L-cysteine (TFEC). TFEC, [^{14}C]TFEC, and [^{35}S]TFEC were synthesized from L-cysteine, [^{14}C]-L-cystine, or [^{35}S]-L-cystine, respectively, and tetrafluoroethylene (Columbia Organic Chemical Co., Cassatt, SC) by a modification of the method of van Bladeren (1980) reported previously (Hayden et al., 1987, 1990).

Enzyme Purification. Cysteine conjugate β-lyase (glutamine transaminase K) was purified from kidney cortex of male Sprague-Dawley rats (200–250 g) to a specific activity of 6.01 μmol/10 min·mg [10 mM L-phenylalanine and 5 mM α-keto-γ-methiobutyrate (γMTB) as substrates] according to the procedure of Cooper and Meister (1981) as modified by Stevens et al. (1986a).

Metabolism Assay. Metabolism of TFEC was measured by quantification of [^{14}C]pyruvate produced from [^{14}C]TFEC as reported previously (Stevens et al., 1986b). Incubations were carried out for 1 h at 37 °C and contained 50 nmol of [^{14}C]TFEC (0.5 μCi/μmol), 50 nmol of γMTB, 43 μg of β-lyase, and 100 μg of BSA (~1.5 nmol), 5 μmol of GSH, or N-acetyl amino acid as indicated in a total volume of 500 μL of 50 mM potassium phosphate buffer (pH 7.5).

Binding Assay. Covalent binding of [^{35}S]TFEC metabolites to BSA was measured under conditions identical with those described for metabolism except that [^{14}C]TFEC was replaced with [^{35}S]TFEC (3.22 μCi/μmol). After 1 h at 37 °C, protein

was precipitated by addition of 2.0 mL of 10% trichloroacetic acid (TCA). After 1 h at 4 °C, precipitated protein was collected by vacuum filtration onto Whatman GF/C glass fiber filters and washed twice with 5.0 mL of cold 5% TCA and four times with 5 mL of ice-cold 95% ethanol. ^{35}S label incorporated into protein was quantified by scintillation counting.

Modification of BSA. BSA was acetylated and succinylated with the respective anhydrides by a procedure similar to that described by Habeeb et al. (1958). BSA was dissolved in 50 mM $NaHCO_3$ (pH 8.6), and a 500-fold molar excess of the anhydride was added slowly over 1 h, the pH being maintained above 8.0 by the addition of 2.0 N NaOH. The solution was then dialyzed extensively against water and the protein recovered by lyophilization. The extent of modification was estimated by reaction with 2,4,6-trinitrobenzenesulfonic acid (TNBS) according to the procedure of Okuyama et al. (1960) as modified by Endo (1978). Greater than 97% reduction in free amino groups was achieved. *S*-Cysteinyl-BSA was purchased from Sigma Chemical Co., St. Louis, MO.

Nonenzymatic Generation of β-Elimination Products from TFEC. β-Elimination from TFEC was achieved by employing a pyridoxal 5′-phosphate (PLP) and copper(II) catalysis system similar to those described by Metzler et al. (1954) and Thomas et al. (1968).

N-Difluorothionoacetyl-S-(1,1,2,2-tetrafluoroethyl)-L-cysteine (DFTA-TFEC). DFTA-TFEC was prepared by incubating 10 mM TFEC, 2 mM PLP, and 1 mM copper sulfate in 50 mM potassium phosphate (pH 7.4) (total volume 100 mL). After 4 h at 37 °C, the mixture was acidified to pH 1.5 with 2.0 N HCl and immediately extracted three times with 50 mL of ethyl acetate. The ethyl acetate fractions were pooled and reduced to dryness under vacuum. The dark-brown residue was suspended in 0.1% TFA and filtered, and the soluble product was purified by preparative HPLC. After evaporation of the eluting solvent, a light-yellow oil was obtained (typically 3–10 mg).

N^{α}-Acetyl-N^{ϵ}-(difluorothionoacetyl)-L-lysine (DFTAL). DFTAL was prepared by slowly adding 100 mL of a 10 mM solution of TFEC in 50 mM potassium phosphate (pH 7.4) over 6 h to 100 mL of a solution containing 10 mM N^{α}-acetyl-L-lysine, 2 mM PLP, and 1 mM $CuSO_4$ at 37 °C. Both DFTAL and DFTA-TFEC were produced by this procedure. The products were extracted into ethyl acetate, and DFTAL was purified by preparative HPLC as described above, again producing a light-yellow oil.

Preparations and Incubations of Rat Kidney Proximal Tubules. Suspensions of rat kidney proximal tubules were prepared according to the procedure of Hatzinger and Stevens (1989) as modified by Zhang and Stevens (1989). To a suspension of tubules (20 mg) in 5.0 mL of isolation buffer was added 3 mL of buffer containing 30 μmol of [^{35}S]TFEC (3.22 μCi/μmol). The mixture was shaken gently for 3 h at 37 °C. Protein was then precipitated by the addition of 20 mL of 10% TCA. After centrifugation, the pellet was washed by resuspension in 30 mL of 5% TCA, centrifuged again, and then washed with 30 mL of 95% ethanol. Finally, the pellet was resuspended in 10 mL of 50 mM potassium phosphate (pH 7.4), and 12.0 mg of proteinase K was added. Digestion was continued for 12 h at 37 °C with an additional 12 mg of proteinase K added at 6 h. Insoluble protein was removed by centrifugation, and the supernatant was analyzed by ^{19}F NMR. The resultant solution contained 300 μM ^{35}S-adduct (1.5×10^{-7} mol of adduct/mg of protein).

Labeling of BSA for NMR Analysis. BSA was labeled with [^{35}S]TFEC metabolites by incubating a solution containing 4.8 μmol of [^{35}S]TFEC (3.22 μCi/μmol), 2.4 μmol of γMTB, 4 mg of BSA (60.6 nmol), and 175 μg of β-lyase in a total volume of 1.2 mL of 50 mM potassium phosphate buffer (pH 7.4) for 6 h at 37 °C, with additional β-lyase (175 μg) added at 2 and 4 h. Protein was precipitated by the addition of 2.4 mL of acetone and 20 μL of glacial acetic acid. After centrifugation, the pellet was redissolved and precipitated twice to ensure removal of unbound metabolites. Finally the protein was dissolved in 2.0 mL of 50 mM potassium phosphate buffer (pH 7.4) and digested with 6 mg of proteinase K for 10 h. The final concentration of ^{35}S-adduct was 340 μM (based on incorporation of ^{35}S) or approximately 1.7×10^{-7} mol of adduct/mg of BSA. This corresponds to 11.2 mol of adduct/mol of BSA or ~19% of the 59 lysine residues.

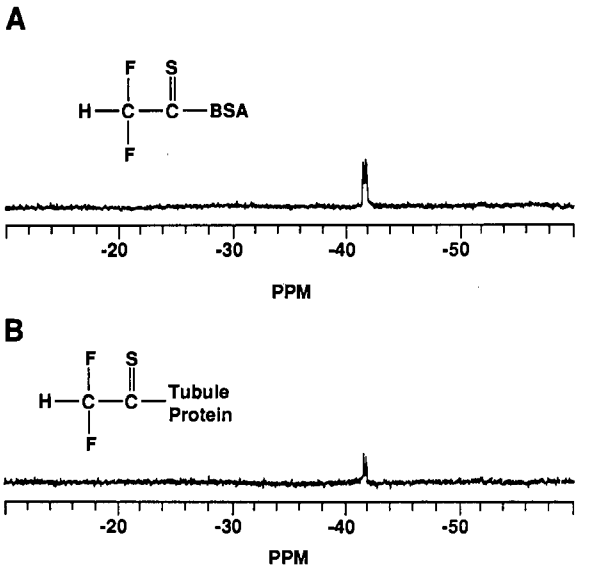

FIGURE 2: ^{19}F NMR spectra of [^{35}S]TFEC metabolites bound to BSA and rat kidney proximal tubule cell protein. Proteins were labeled with TFEC metabolites and digested with proteinase K as described in Materials and Methods. Spectra were acquired at 235.33 MHz. Chemical shifts are referenced to TFA. (A) BSA adduct. (B) Tubule protein adduct.

Statistics. Student's *t* test was used to determine significant differences ($p < 0.05$) between control and treated samples.

Results

A partially purified β-lyase from rat kidney cytosol was employed to generate ^{35}S-labeled TFEC metabolites in the presence of BSA. [^{35}S]TFEC metabolites were also generated in primary suspensions of rat renal proximal tubules by endogenous β-lyase. The [^{35}S]TFEC metabolite–protein adducts thus formed were analyzed by ^{19}F NMR spectroscopy.

Initially, no signals were observed. After enzymatic digestion with proteinase K, however, doublets with chemical shifts of −41.38 and −41.60 ppm, relative to trifluoroacetic acid (TFA), were observed for the BSA and tubule adducts, respectively (Figure 2). Coupling constants of 55.2 and 56.0 Hz for BSA and tubule adducts, respectively, indicate geminal HF bonding and are consistent with the formation of difluorothionoacetyl adducts with protein nucleophiles. However, because any differences in structure of the adducts (e.g., dithioester, thioamide, thionoester) due to reaction with various protein nucleophiles (e.g., cysteine, lysine, histidine, tyrosine, etc.) would be three atomic nuclei distant from the fluorine atoms, no information concerning the specific nucleophiles was provided by these experiments. Therefore, to determine which protein nucleophiles are involved in the binding, several experimental approaches were pursued.

Table I: Binding of [^{35}S]TFEC Metabolites to Modified Bovine Serum Albumins[a]

protein	nmol of ^{35}S	% of control
BSA (control)	1.36 ± 0.09	100.0 ± 6.6
S-cysteinyl-BSA	1.29 ± 0.15	94.8 ± 5.7
acetylated BSA	0.73 ± 0.03*	53.7 ± 4.4*
succinylated BSA	0.55 ± 0.01*	40.4 ± 1.6*

[a] Incubation mixtures containing 100 μg of protein (~1.5 nmol), 50 nmol of [^{35}S]TFEC, 50 nmol of γMTB, and 43 μg of β-lyase in a total volume of 500 μL of 50 mM potassium phosphate buffer (pH 7.4) were maintained at 37 °C for 1 h before quantitation of binding as described in Materials and Methods. Results are reported as the mean ± standard deviation of $n = 3$ experiments. The asterisks mark values significantly different from control $p < 0.05$.

BSA contains one free cysteinyl residue and 59 lysines (Peters, 1975). To obtain evidence to indicate if these nucleophilic residues are involved in covalent binding, experiments were performed with *S*-cysteinyl- or *N*-acetyl- and *N*-succinyl-modified BSA. Under conditions where approximately 1 mol of adduct/mol of BSA was obtained, modifying the sulfhydryl function had no significant effect on ^{35}S-metabolite binding to BSA. Modifying the lysyl residues, however, significantly reduced the binding to 40% of control (Table I).

To further examine the nucleophile specificity for TFEC metabolite reaction with nucleophilic groups present in proteins, the N^{α}-acetyl derivatives of a variety of amino acids containing nucleophilic side chains were tested for their ability to compete with BSA for reaction with metabolites. As glutathione is a major intracellular nucleophile, it was also included in these binding inhibition experiments.

Results summarized in Table II show that, in contrast to the insignificant effect of modifying the BSA sulfhydryl group on binding of metabolites to BSA as described above, the sulfhydryl nucleophiles glutathione and *N*-acetylcysteine were very efficient at preventing the incorporation of ^{35}S label into BSA, reducing relative binding (binding/metabolism) to approximately 8 and 20% compared to controls, respectively. N^{α}-Acetyllysine, *N*-acetylserine, and *N*-acetylglutamate also caused a statistically significant, but less dramatic, reduction in the amount of relative ^{35}S binding to BSA. Surprisingly, N^{α}-acetylhistidine and *N*-acetyltyrosine produced a significant increase in the amount of label incorporated (185 and 159% of control, respectively). *N*-Acetylmethionine, *N*-acetylarginine, and *N*-acetylglutamine did not cause significant changes in the amount of relative binding to BSA. With the exception of glutathione and *N*-acetylcysteine, which reduced metabolism to approximately 92 and 71% of control, respectively (an amount insufficient to account for the decreased binding), the presence of the various nucleophiles in the incubation mixtures did not significantly affect TFEC metabolism by the β-lyase (Table II).

N^{α}-Acetyllysine proved to be a relatively poor nucleophile toward TFEC metabolites, reducing relative binding to BSA in competition experiments by only 13%, even at concentrations of >100-fold in excess of protein lysyl residues (Table II). Yet, lysyl residues in BSA seem to be particularly reactive with the metabolites. This was at first glance puzzling. The facilitation of binding of TFEC metabolites to BSA in the presence of N^{α}-acetylhistidine and *N*-acetyltyrosine was also puzzling. Therefore, a closer look at these phenomena was undertaken.

Difficult acylations in synthetic organic chemistry are often achieved through the use of nucleophilic catalysis, in which an acyl halide or acid anhydride is first reacted with a suitable nucleophilic reagent to form an intermediate that can easily transfer the acyl group to another nucleophile, thus forming the final product. Acylations catalyzed by pyridine are perhaps the best known examples of this phenomenon, 4-(*N*,*N*-dimethylamino)pyridine being a particularly good catalyst (Steglich & Hofle, 1969). However, imidazoles are also very efficient in this type of nucleophilic catalysis (Bender, 1971). In fact, *N*-acetylimidazole can be prepared under nonaqueous conditions (Boyer, 1952). It has been used as a mild acylating agent for proteins (Simpson et al., 1963) and in organic synthesis (Staab & Rohr, 1968). Phenols can also function as nucleophilic catalysts. For example, 2,4,5-trichlorophenol is used as a peptide-coupling reagent by virtue of its ability to form activated phenolic esters (Pless & Boissonnas, 1963). It is therefore probable that the reaction with difluorothionoacetyl fluoride occurs first at tyrosine or histidine and the difluorothionoacetyl group is then transferred to lysine to form the final stable thioamide adduct.

To further investigate the hypothesis that stable binding of TFEC metabolites to lysyl residues in BSA could be facilitated by nucleophilic catalysis, binding experiments were repeated in the presence of a variety of reagents that would be predicted to either inhibit binding by competing for reactive metabolites (primary or secondary amines) or facilitate binding through nucleophilic catalysis (aromatic amines or phenols). The results are shown in Table III. With the exception of diethylamine, which did not significantly reduce binding as expected, the results were as predicted: primary amines (N^{α}-acetyllysine, methylamine, benzylamine) decreased the relative binding of metabolites to BSA; aromatic amines [pyridine, 4-(*N*,*N*-dimethylamino)pyridine, N^{α}-acetylhistidine, imidazole] or phenols (*N*-acetyltyrosine, phenol, 2,4-dinitrophenol, 2,4,5-trichlorophenol) all increased relative binding compared to controls. These results provide strong supportive evidence that a nucleophilic catalysis mechanism, such as that

Table II: Effect of Various Amino Acid Nucleophiles on TFEC Metabolism and Binding of Metabolites to Bovine Serum Albumin[a]

	metabolism		binding		relative binding
nucleophile	nmol of ^{14}C	% of control	nmol of ^{35}S	% of control	(nmol of ^{35}S/ nmol of ^{14}C) × 10^2
none (control)[b]	33.85 ± 1.49	100.0 ± 4.4	1.34 ± 0.08	100.0 ± 6.0	3.9 ± 0.3
glutathione[c]	31.11 ± 0.73*	91.9 ± 4.6*	0.10 ± 0.01*	7.5 ± 0.9*	0.3 ± 0.1*
N-acetylcysteine[c]	24.03 ± 1.28*	71.0 ± 4.9*	0.18 ± 0.02*	13.4 ± 1.7*	0.8 ± 0.1*
N-acetylmethionine[c]	32.80 ± 2.63	96.9 ± 8.8	1.39 ± 0.10	103.7 ± 9.7	4.3 ± 0.1
N-acetylserine[c]	34.44 ± 0.75	101.7 ± 5.0	1.20 ± 0.02*	89.6 ± 5.6*	3.5 ± 0.1*
N-acetyltyrosine[b]	32.92 ± 3.37	97.3 ± 10.8	2.01 ± 0.07*	150.7 ± 10.8*	6.2 ± 0.9*
N-acetylhistidine[b]	34.33 ± 1.90	101.4 ± 7.2	2.47 ± 0.38*	185.2 ± 30.4*	7.2 ± 0.9*
N^{α}-acetyllysine[b]	35.16 ± 2.48	103.8 ± 8.6	1.19 ± 0.09*	89.1 ± 8.5*	3.4 ± 0.3*
N-acetylarginine[c]	33.45 ± 2.22	98.8 ± 7.8	1.24 ± 0.05	92.5 ± 6.7	3.7 ± 0.3
N-acetylglutamine[c]	33.40 ± 1.93	98.7 ± 7.2	1.36 ± 0.10	101.5 ± 9.6	4.1 ± 0.5
N-acetylglutamate[c]	35.65 ± 1.77	105.3 ± 7.0	1.21 ± 0.06	90.3 ± 7.0	3.4 ± 0.3*

[a] See Materials and Methods for experimental conditions. The asterisks mark values significantly different from control $p < 0.05$. [b] $n = 6$. [c] $n = 3$.

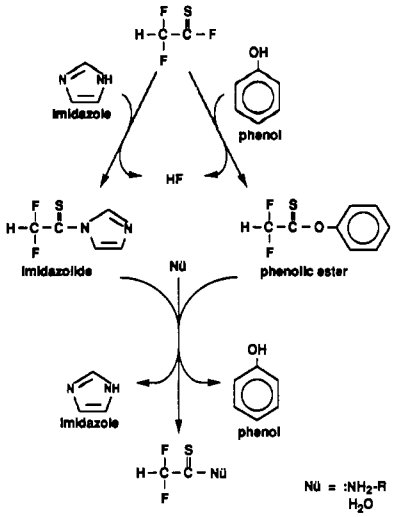

FIGURE 3: Proposed nucleophilic catalysis mechanism for facilitation of stable primary difluorothioacetamide adduct formation by imidazoles and phenols.

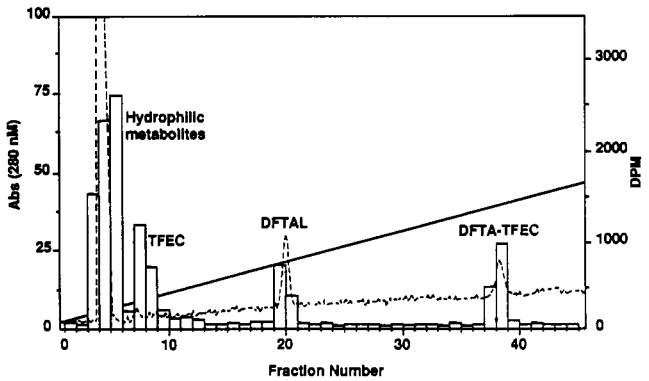

FIGURE 4: HPLC analysis of ^{35}S-products resulting from β-lyase metabolism of [^{35}S]TFEC in the presence of N^{α}-acetyllysine. Reaction mixtures contained 50 nmol of [^{35}S]TFEC (sp. act. = 3.22 μCi/μmol), 50 nmol of γMTB, 5 μmol of N^{α}-acetyllysine, and 43 μg of β-lyase in a total volume of 500 μL of 50 mM potassium phosphate (pH 7.4). After 30 min at 37 °C, 50 μL was injected directly onto a RP-HPLC column as described in Materials and Methods. One-minute fractions were collected and assayed for ^{35}S by scintillation counting. DPM (bars); Abs 280 nm (---); gradient (—).

outlined in Figure 3, can operate to facilitate the binding of TFEC metabolites to protein.

Having gained indirect evidence for the ability of GSH, *N*-acetylcysteine, N^{α}-acetyllysine, *N*-acetylserine, and *N*-acetylglutamate to react with TFEC metabolites, we next sought to isolate and characterize the resultant adducts. Analysis by HPLC of incubation mixtures containing the model nucleophiles and TFEC metabolites revealed the presence of the same stable ^{35}S-containing product in all samples. An additional stable ^{35}S-containing product was found only in samples incubated with N^{α}-acetyllysine (Figure 4). As will be described in detail below, these peaks represented the difluorothionoacetyl-TFEC adduct (DFTA-TFEC) reported previously by Commandeur et al. (1989) and the N^{ϵ}-difluorothioacetamide adduct of N^{α}-acetyllysine (DFTAL). Despite the efficiency of the sulfhydryl nucleophiles in preventing the binding of TFEC metabolites to BSA, no evidence was obtained (both the acidic and neutral HPLC systems) to indicate the formation of stable adducts with GSH or *N*-acetylcysteine.

It was not practical to produce enzymatically enough of the stable adducts for structural analysis. Therefore, a chemical system utilizing PLP, the catalytic cofactor of β-lyase enzymes,

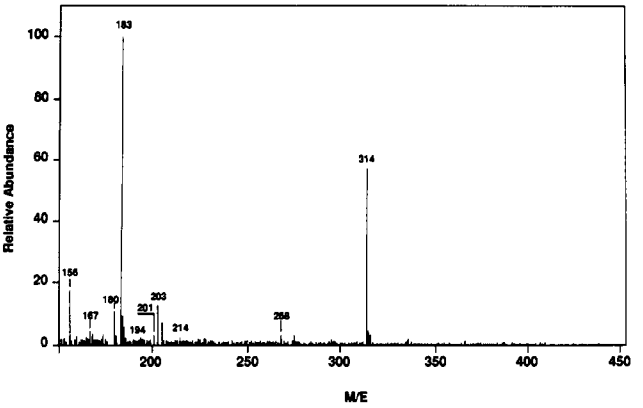

FIGURE 5: Continuous-flow FAB mass spectrum (negative ion) of *N*-difluorothionoacetyl-*S*-tetrafluoroethyl-L-cysteine (DFTA-TFEC). DFTA-TFEC was prepared with the PLP/Cu^{2+} system and purified as described in Methods and Materials. Detailed FAB-MS conditions are also described in Materials and Methods. The peak at m/z 314 corresponds to $(M - H)^-$. The base peak ion at m/z 183 is due to the glycerol matrix.

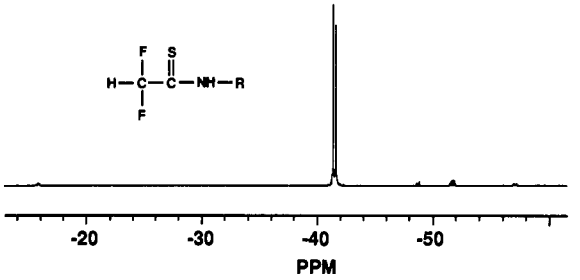

FIGURE 6: ^{19}F NMR spectrum of N^{α}-acetyl-N^{ϵ}-difluorothionoacetyl-L-lysine (DFTAL). DFTAL was prepared by the PLP/Cu^{2+} system and purified as described in Materials and Methods. Spectra were acquired at 235.33 MHz. Chemical shifts are referenced to TFA.

and copper(II) cations was used to generate β-elimination products from TFEC in larger quantities (see Materials and Methods). With this system, ^{35}S-products that coeluted in two HPLC solvent systems with the ^{35}S-products of the enzymatic system were obtained (data not shown). After preparative HPLC purification, these ^{35}S-adducts were analyzed by ^{19}F and ^{13}C NMR spectroscopy and mass spectrometry.

The adduct eluting in fractions 38 and 39, Figure 4, was shown to be the DFTA-TFEC adduct reported earlier by Commandeur et al. (1989). NMR data are reported in Table IV. The continuous-flow FAB mass spectrum (negative ion) of DFTA-TFEC showed an $(M - H)^-$ ion at m/z 314 with no fragmentation. The base peak ion in this spectrum at m/z 183 is due to the glycerol matrix (Figure 5).

The expected structure of the adduct resulting from reaction of difluorothionoacetyl fluoride with N^{α}-acetyllysine is the N^{ϵ}-difluorothioacetamide. The ^{19}F and ^{13}C NMR and mass spectral analyses of the adduct eluting in fractions 20 and 21 (Figure 4) demonstrate that this was indeed the product obtained. As shown in Figure 6, the ^{19}F NMR spectrum consists of a doublet centered at $\delta = -41.64$ ppm (similar in chemical shift to the DFTA-TFEC, but not an AB pattern) with $^2J_{HF} = 56.0$ Hz.

The proton-decoupled ^{13}C spectrum of DFTAL displayed 10 signals as shown in the top spectrum in Figure 7. Alternate population transfer editing (APT), which results in quaternary and methylene carbons appearing as negative peaks and methyl and methine carbons appearing as positive peaks, assisted in making assignments as shown in the bottom spectrum in Figure 7. Carbon A was not visible in the APT experiment because the sample was not fluorine decoupled. Chemical

Table III: Effect of Various Amine and Phenolic Nucleophiles on TFEC Metabolism and Binding of Metabolites to Bovine Serum Albumin[a]

nucleophile	metabolism		binding		relative binding (nmol of ^{35}S/ nmol of ^{14}C) × 10^2
	nmol of ^{14}C	% of control	nmol of ^{35}S	% of control	
none (control)[b]	33.85 ± 1.49	100.0 ± 4.4	1.34 ± 0.08	100.0 ± 6.0	3.9 ± 0.3
N-acetyllysine[b]	35.16 ± 2.48	103.8 ± 8.6	1.19 ± 0.09*	89.1 ± 8.5*	3.4 ± 0.3*
methylamine[c]	33.32 ± 2.58	98.9 ± 8.8	1.12 ± 0.06*	83.9 ± 7.0*	3.2 ± 0.3*
diethylamine[c]	36.25 ± 1.56	107.1 ± 6.6	1.33 ± 0.13	99.4 ± 11.4	3.7 ± 0.5
benzylamine[c]	34.22 ± 1.04	101.1 ± 5.4	0.74 ± 0.03*	55.2 ± 4.2*	2.2 ± 0.1*
pyridine[c]	33.38 ± 3.09	98.6 ± 10.1	3.13 ± 0.56*	234.7 ± 44.9*	9.5 ± 2.2*
DMAP[c]	35.83 ± 2.64	105.8 ± 8.4	2.59 ± 0.21*	193.8 ± 20.1*	7.2 ± 0.9*
N-acetyltyrosine[b]	32.92 ± 3.37	97.3 ± 10.8	2.01 ± 0.07*	150.7 ± 10.8*	6.2 ± 0.9*
phenol[c]	35.99 ± 2.14	106.3 ± 7.9	2.84 ± 0.16*	213.0 ± 18.1*	7.9 ± 0.9*
2,4-dinitrophenol[c]	7.77 ± 1.04*	22.9 ± 3.2*	0.93 ± 0.07*	69.7 ± 6.8*	12.1 ± 1.5*
2,4,5-trichlorophenol[c]	10.29 ± 7.45*	30.4 ± 22.0*	2.44 ± 0.47*	182.6 ± 37.2*	32.8 ± 23.1*
N-acetylhistidine[b]	34.33 ± 1.90	101.4 ± 7.2	2.47 ± 0.38*	185.2 ± 30.4*	7.2 ± 0.9*
imidazole[c]	37.41 ± 0.52	110.5 ± 5.1	2.64 ± 0.14*	197.5 ± 16.1*	7.0 ± 0.5*

[a] See Material and Methods for experimental conditions. The asterisks mark values significantly different from control $p < 0.05$. [b] $n = 6$. [c] $n = 3$.

Table IV: Summary of NMR Data[a]

compound	δ ppm (intensity, multiplicity)	
	^{19}F	^{13}C
TFEC	AB system: $\delta_A = -14.83$ ppm (dt, $^2J_{FF} = 233.0$ Hz, $^3J_{FF} = 8.5$ Hz, 1 F); $\delta_B = -15.30$ ppm (dt, $^2J_{FF} = 232.5$ Hz, $^3J_{FF} = 8.5$ Hz, 1 F); −56.34 ppm (dt, $^2J_{HF} = 54.1$ Hz, $^3J_{FF} = 8.4$ Hz, 2 F)	28.48 ppm (s); 54.73 ppm (s); 110.03 ppm (tt, $^1J_{CF} = 250.8$ Hz, $^2J_{CF} = 37.3$ Hz); 123.99 ppm (tt, $^1J_{CF} = 284.2$ Hz, $^2J_{CF} = 31.3$ Hz); 171.97 ppm (s)
DFTA-TFEC	AB system: $\delta_A = -15.72$ ppm (d, $^2J_{FF} = 232.5$ Hz, 1 F); $\delta_B = -16.54$ ppm (d, $^2J_{FF} = 224.5$ Hz, 1 F). AB system: $\delta_A = -41.11$ ppm (dd, $^2J_{FF} = 290.9$ Hz, $^2J_{HF} = 56.3$ Hz, 1 F); $\delta_B = -41.92$ ppm (dd, $^2J_{FF} = 291.1$ Hz, $^2J_{HF} = 54.3$ Hz, 1 F); −51.11 ppm (dt, $^2J_{HF} = 53.3$ Hz, $^3J_{FF} = 8.4$ Hz, 2 F)	28.56 ppm (s); 60.19 ppm (s); 109.99 ppm (tt, $^1J_{CF} = 250.8$ Hz, $^2J_{CF} = 37.4$ Hz); 111.37 ppm (t, $^1J_{CF} = 249.0$ Hz); 123.95 ppm (tt, $^1J_{CF} = 313.6$ Hz, $^2J_{CF} = 29.7$ Hz); 173.70 ppm (s); 191.42 ppm (t, $^2J_{CF} = 24.0$ Hz)
DFTAL	−41.64 ppm (d, $^2J_{HF} = 56.0$ Hz)	22.98 ppm (s); 23.56 ppm (s); 27.08 ppm (s); 32.16 ppm (s); 46.29 ppm (s); 56.06 ppm (s); 112.28 ppm (t, $^1J_{CF} = 249.0$ Hz); 174.64 ppm (s); 180.22 ppm (s); 190.76 ppm (t, $^2J_{CF} = 24.0$ Hz)

[a] ^{19}F spectra were recorded at 235.33 MHz and referenced to trifluoroacetic acid. ^{13}C spectra were recorded at 62.90 MHz and referenced to tetramethylsilane.

Table V: Facilitation of *N*-Acetyllysine Adduct Formation by Imidazole and Phenol[a]

catalyst	products			
	DFTA-TFEC		DFTAL	
	nmol	% of control	nmol	% of control
none (control)	0.50 ± 0.10	100.0 ± 20.0	0.88 ± 0.12	100.0 ± 13.6
imidazole	0.82 ± 0.18	164.0 ± 48.7	4.01 ± 0.63*	455.7 ± 94.8*
phenol	0.53 ± 0.12	106.0 ± 32.0	2.83 ± 0.41*	321.6 ± 64.0*

[a] Incubation mixtures containing 50 nmol of [^{35}S]TFEC, 50 nmol of γMTB, 5 μmol of N^{α}-acetyllysine, 43 μg of β-lyase, and 4 μmol of catalyst as indicated in a total volume of 250 μL of 50 mM potassium phosphate buffer (pH 7.4) were maintained at 37 °C for 1 h. Reactions were stopped by addition of 250 μL of 2.0 N HCl. Products were extracted into ethyl acetate, dried, and analyzed by HPLC as described in Materials and Methods. Results are reported as the mean ± standard deviation of $n = 3$ experiments. The asterisks mark values significantly different from control $p < 0.05$.

shifts and coupling constants are reported in Table IV.

The positive-ion thermospray mass spectrum of DFTAL consisted of an $(M + H)^+$ ion at m/z 283 and an ammonium adduct at m/z 300, also in agreement with the proposed structure (Figure 8).

Other products produced by the PLP/Cu^{2+} system were identified by ^{19}F NMR spectroscopy as fluoride ($\delta = -44.15$ ppm), difluoroacetic acid ($\delta = -48.74$ ppm, $^2J_{HF} = 55.2$ Hz), and an unstable product ($\delta = -42.15$ ppm, $^2J_{HF} = 58.16$ Hz) that decomposed with a concomitant increase in difluoroacetic acid (DFAA) (Figure 9). This unidentified peak, also reported by Commandeur et al. (1989), is presumably difluorothionoacetic acid (DFTAA). Although its chemical shift and coupling constant are very similar to those of the stable difluorothioacetamides, its hydrolysis to DFAA, even at neutral pH, ensures that no interference with the spectra of protein samples occurred.

The identification and characterization of DFTA-TFEC and DFTAL by HPLC provided a convenient method to test the nucleophilic catalysis hypothesis by directly measuring the stable end products formed. Results summarized in Table V show that imidazole and phenol increased the yield of DFTAL by approximately 446 and 322%, respectively. This conclusively demonstrates that nucleophilic catalysis can operate to facilitate stable adduct formation between TFEC metabolites and primary amines.

Discussion

Many chemical species have been proposed to account for binding of cysteine conjugate metabolites to cellular macromolecules. In particular, Green and Odum (1985) suggested that thionoacylating metabolites may be responsible. Subsequent identification of thionoacylating metabolites after trapping with nucleophilic amines (Dekant et al., 1988a,b;

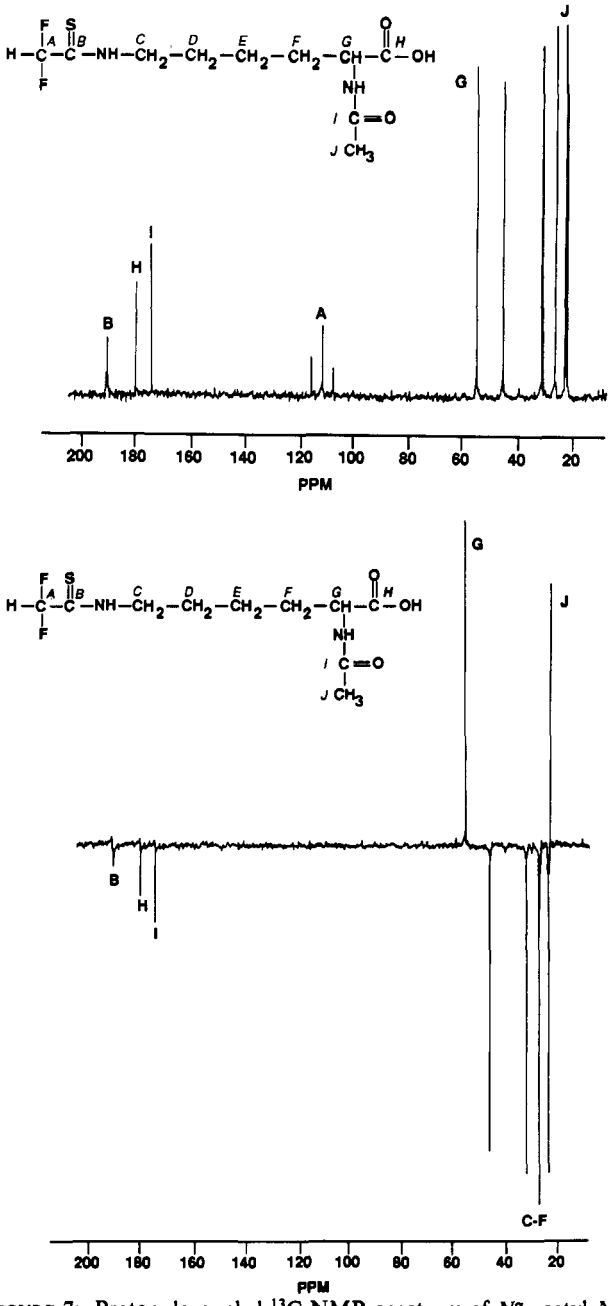

FIGURE 7: Proton-decoupled ^{13}C NMR spectrum of N^{α}-acetyl-N^{ϵ}-difluorothionoacetyl-L-lysine (DFTAL). DFTAL was prepared and purified as described in Materials and Methods. (Top) Spectra were recorded at 62.90 MHz. Chemical shifts are referenced to TMS. (Bottom) Alternate population transfer spectral editing shows quaternary and methylene carbons as negative signals and methyl and methine carbons as positive signals.

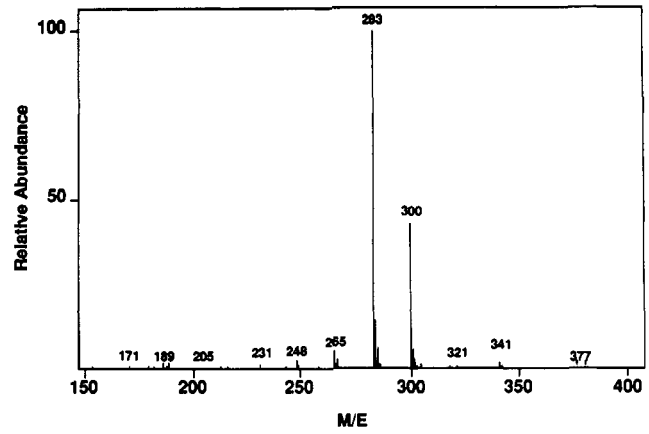

FIGURE 8: Postive-ion thermospray mass spectrum of N^{α}-acetyl-N^{ϵ}-difluorothionoacetyl-L-lysine (DFTAL). DFTAL was prepared with the PLP/Cu^{2+} system. The peak at m/z 283 corresponds to $(M + H)^+$ and m/z 300 to $(M + NH_4)^+$. Detailed conditions are described in Materials and Methods.

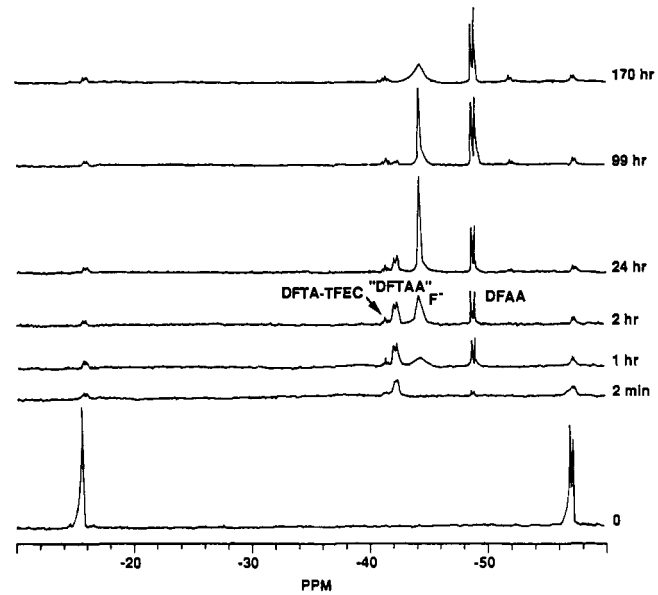

FIGURE 9: Time course of TFEC metabolite production by ^{19}F NMR. Reaction mixtures containing 50 μmol of TFEC, 50 μmol of PLP, and 25 μmol of $CuSO_4$ in a total volume of 3.0 mL of 50 mM potassium phosphate buffer (pH 7.4) were incubated in the NMR tube. Spectra were acquired at 235.33 MHz at the time points indicated. Chemical shifts are relative to TFA.

Commandeur et al., 1988, 1989) has provided evidence in support of this hypothesis. However, direct structural determination of protein or DNA adducts has been lacking. A primary purpose of these investigations was to utilize ^{19}F NMR spectroscopy to obtain such structural information. The data presented here demonstrate that TFEC metabolites form stable difluorothionoacetyl adducts with protein nucleophiles. No evidence was found to indicate formation of adducts derived from thiirane intermediates.

Results of binding experiments utilizing modified bovine serum albumins as model nucleophiles indicate that the stable protein adducts are most probably difluorothioacetamide adducts of lysyl residues. In support of this hypothesis, stable difluorothioacetamide adducts of N^{α}-acetyllysine have been isolated and characterized. The fact that only 60% of the binding to succinylated BSA was prevented while >97% of the free amino groups were succinylated indicates that the metabolites could displace the modifying group, bind to other residues, or otherwise bind nonspecifically to the protein. The formation of a DFTA-TFEC adduct (also a difluorothioacetamide), reported previously by Commandeur et al. (1989), was also confirmed. The protein adducts and both thioacetamides all have nearly identical ^{19}F NMR chemical shifts, further suggesting the thioacetamide structure for the protein adducts.

The toxic consequences of difluorothionoacetylation of cellular proteins is difficult to assess. Halothane and other inhalation anesthetics produce a similar metabolite, trifluoroacetyl chloride, which is known to trifluoroacetylate lysyl residues in specific proteins in vivo (Satoh et al., 1989). Both a mild and a severe form of hepatotoxicity are caused by these agents. However, the severe hepatotoxicity is thought to be associated with with an immune response to the modified protein, rather than interference with critical cellular enzymes

(Pohl et al., 1989). Other drugs are also known to acetylate (e.g., aspirin; Pinckard et al., 1968) or acylate (e.g., penicillins; Schwartz, 1969) proteins in vivo. However, again, allergic reactions seem to be the primary toxic consequence of protein lysyl acylation in humans (Schwartz, 1969). Furthermore, at least five deacetylases, including an N^{ϵ}-acyllysine deacylase (EC 3.5.1.17), are known to exist in the kidney (Paik et al., 1957). Thus, further studies are warranted to ascertain the toxic consequences of the protein-difluorothioacetamide formation by TFEC metabolites reported here.

Competitive binding experiments indicate that the ϵ-amino group of N^{α}-acetyllysine is not a particularly good nucleophile for TFEC metabolites. However, a previously unsuspected phenomenon, nucleophilic catalysis of lysine adduct formation by histidine or tyrosine side chains, may operate at the surface of the protein to account for the apparently good nucleophilicity of lysyl residues in protein. Although this catalytic effect is widely known in synthetic organic chemistry, to our knowledge, this is the first report of this phenomenon operating to facilitate the binding of reactive xenobiotic metabolites to specific sites in the protein. The existence of this effect is further confirmation of the thionoacyl nature of the TFEC metabolites. It is conceivable that this effect may be a common mechanism for binding of other acylating agents to protein as well and may predispose specific proteins (that contain lysyl residues in proximity to catalytic tyrosyl or histidyl residues) to reaction with acylating agents. Additional experimentation will be required to prove or disprove this hypothesis.

Binding inhibition studies with various *N*-acetylamino acids reveal that the thiol compounds GSH and *N*-acetylcysteine (soft nucleophiles) are most reactive toward TFEC metabolites. Nucleophilicity under physiological conditions typically follows the order S > N > O (Swain & Scott, 1953; Osterman-Golkar & Ehrenberg, 1970), at least for reaction with soft electrophiles (Coles, 1984–85). However, very hard electrophiles, such as the methylcarbonium ion formed from the decomposition of methylnitrosourea or *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine that typically react with the harder N and O nucleophiles found in DNA and RNA, are reportedly unreactive with cysteine (Jenson & Magee, 1981). This indicates a relatively soft electrophilic character for the thionoacylating metabolite of TFEC.

Attempts to isolate ^{35}S-adducts of TFEC metabolites from incubations with thiol compounds in sufficient quantities for structural analysis have so far been unsuccessful. The expected products of thiol reaction with the difluorothionoacetylating metabolite are the corresponding dithioesters. While these compounds are reportedly more stable than their oxygen analogues (Duus, 1979), they would be expected to be labile to hydrolysis in aqueous solution, especially under acidic conditions (Janssen, 1969; Duus, 1979), as were employed in protein precipitation (TCA) or the acidic HPLC solvent system. Thus, despite the fact that the thiol compounds were superior nucleophiles for thionoacylating metabolites compared to primary amines, the adducts are apparently labile under in vitro conditions. Hydrolysis of thiol adducts would also explain the apparent lack of effect of modifying the free cysteinyl group of BSA on TFEC metabolite binding. Any adducts formed would be hydrolyzed during the TCA precipitation of the protein. Thus, no difference would be seen compared to controls.

Although stable thioacetamides were isolated in this study and may eventually be shown to have toxic consequences, a component of toxicity due to modification of sulfhydryl groups cannot yet be discounted. The reactivity of thiols reported here is consistent with previous proposals by Stonard and Parker (1971) and more recently by Lock and Schnellmann (1990) that cysteine conjugate nephrotoxicity may be the result of metabolite interaction with sensitive sulfhydryls on critical enzymes. However, the isolation of stable thioamides and the instability of the presumed dithioesters complicates this hypothesis. Whether sulfhydryl modification plays a significant role in toxicity will depend on the stability of the thiol adducts under in vivo conditions, which may include relatively hydrophobic regions of intermembrane proteins where they may be protected from hydrolysis. For example, the inner membrane of the mitochondrian, a primary target organelle of cysteine conjugate toxicity, reportedly contains critical hydrophobic sulfhydryl groups present in membrane proteins (Lê-Quôc & Lê-Quôc, 1982, 1985). Thus, determination of the relative contributions to toxicity of protein-amine and protein-thiol modification by TFEC metabolites will require further investigation.

In summary, stable difluorothionoacetyl-protein adducts have been shown to result from interaction of TFEC metabolites with BSA and rat kidney proximal tubule proteins. N^{α}-Acetyl-N^{ϵ}-(difluorothionoacetyl)lysine and *N*-(difluorothionoacetyl)-TFEC were also isolated and characterized. By comparison of the ^{19}F NMR spectra of the protein adducts DFTA-TFEC and DFTAL and results of binding experiments with modified BSAs, the protein adducts appear to be difluorothioacetamide adducts of lysyl residues. An unsuspected nucleophilic catalysis mechanism has been shown to facilitate stable adduct formation with both BSA and N^{α}-acetyllysine, providing further support that the protein adduct is also a difluorothioacetamide. This nucleophilic catalysis mechanism may be important for binding of other acylating agents to protein as well. The sulfhydryl nucleophiles GSH and *N*-acetylcysteine were shown to be considerably better nucleophiles than primary amines, indicating a soft electrophilic nature for the thionoacylating TFEC metabolite. However, the predicted dithioester adducts could not be isolated and are presumed to be too unstable under the experimental conditions employed. Further details regarding how protein-difluorothioacetamide adducts or protein-sulfhydryl modification by TFEC metabolites may be coupled to cell death remain to be investigated.

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