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Sulphydryl Group Modification of Aspartate Aminotransferase with 3-Bromo-1,1,1-trifluoropropanone during Catalysis†

W. J. Critz‡ and M. Martinez-Carrion*,§

ABSTRACT: After protection of cysteine-45 and -82 with iodoacetamide or *N*-ethylmaleimide, and in the presence of saturating concentrations of substrates, the supernatant isozyme of pig heart aspartate transaminase has been covalently modified at cysteine-390 with 3-bromo-1,1,1-trifluoropropanone. The modified enzyme retains 60–70% of the initial specific activity and is similar to native enzyme in pH and temperature stability. After tagging cysteine-390 with the fluorinated compound, the enzyme retains substrate and inhibitor binding abilities, as shown by direct spectrophotometric titration of the active-site chromophores. The ^{19}F NMR spectrum of the modified enzyme has been obtained by a Fourier transform NMR method. Although the transaminase is a dimeric enzyme, ^{19}F bound at each subunit's cysteine-390 gives rise to only a single ^{19}F resonance upfield from that of trifluoroacetic acid. The fact that the chemical shifts of the ^{19}F probe differ in native and guanidine hydrochloride (Gdn-HCl) denatured enzyme is interpreted as the effect of the native protein groups on the probe. The discordance between the changes induced by varying concentrations of Gdn-HCl on the ^{19}F resonance parameters, on the one hand, and the changes in enzyme activity and prosthetic group absorbance, on the other, suggests that, in aspartate transaminase, cysteine-390 lies in an environment dissimilar from that of the active-site components.

Chemical modification of enzymes is used as a tool to determine amino acid residues essential for activity, and for the introduction of various probes into macromolecules to obtain structural and/or functional information. Supernatant aspartate aminotransferase (EC 2.6.1.1) has five cysteine residues per subunit, but only three react with alkylating agents unless the enzyme is completely denatured. The inaccessible cysteine residues have been identified as cysteine-191 and -252 (Birchmeier et al., 1973; Wilson et al., 1974; Zufarova et al., 1973). Of the three remaining cysteines residues, cysteine-45 and -82 react 10^5 times faster than Cys-390 (Birchmeier et al., 1973; Stankewicz et al., 1971).

The relationship of the chemically accessible sulphydryl groups to enzymatic activity has been studied in detail. Cysteine residues 45 and 82 can be blocked with a variety of al-

† From the Department of Chemistry, Biochemistry and Biophysics Program, University of Notre Dame, Notre Dame, Indiana 46556. *Received September 8, 1976.* This work was supported by Grants HL-11448 and GM-20727 from the National Institutes of Health.

‡ Present Address: Department of Biochemistry, University of Iowa, Iowa City, Iowa 52240.
§ NIH Career Development Awardee.

kylating agents without loss of enzymatic activity (Torchinsky and Sinitsina, 1970; Torchinsky et al., 1972; Polyanovsky et al., 1974). Cys-390 can be selectively modified in the presence of a 35:1 ratio (syncatalytic modification) of amino acid to α-keto acid substrates (Birchmeier et al., 1973). Direct modification of this cysteine has always produced inactivation of the enzyme; however, after modification with Nbs_2,[1] the Nbs moiety can be displaced by cyanide with regeneration of catalytic activity (Birchmeier et al., 1973; Boettcher and Martinez-Carrion, 1975).

Cys-390 is not an amino acid residue essential for catalysis and its increased accessibility to chemical modification in the presence of substrates can be explained on the basis of a steric proximity to the active site. This residue can become exposed in the enzyme–substrate complex either through a direct conformational change of the protein region containing Cys-390 or by an indirect removal of steric hindrance from the active-site conformation in the formation of a covalent enzyme–substrate complex.

In this work, we aim at the introduction, with retention of catalytic activity, of a fluorine label into a strategic region of aspartate transaminase. In the following paper of this issue, we show how the greater sensitivity and ease of detection of the ^{19}F nuclei by NMR techniques proves advantageous to an inquiry into the properties of the protein environment of Cys-390 in free enzyme and in the presence of physiologically significant ligands.

Materials and Methods

Materials. The α form of aspartate transaminase was prepared and assayed as previously reported (Martinez-Carrion et al., 1965). Trifluorobromopropanone was from PCR, Inc. α-Ketoglutarate, α-methylaspartate, malate dehydrogenase, NEM, iodoacetamide, and Ellman's reagent were from Sigma Chemical Co. Cyanogen bromide was from Aldrich Chemical Co. pMB and Gdn-HCl were purchased from Mann Research Laboratories, and [^{3}H]$NaBH_4$ (304 mCi/mmol) was from Amersham-Searle. *S*-Trifluoroacetonylmercaptoethanol was synthesized by the method of Huestis and Raftery (1972).

Sulphydryl Protection. The two easily modified thiol groups (Cys-45 and -82) were protected using enzyme (10 mg/mL) in 0.1 M phosphate buffer. Two millimolar NEM for 1 h at 25 °C, pH 7.5, and 0.4 M iodoacetamide, for 24 h at 4 °C, pH 6.0, were the modifying agents.

Borohydride Reductions. [^{3}H]$NaBH_4$ (1 mg, 2.5 mCi/mmol) reduction of the ^{19}F-labeled enzyme (200 mg) took place at 25 °C in 0.2 M Tris–acetate buffer, pH 8.5, for 2 h.

Cyanogen Bromide Treatment and Peptide Characterization. The cyanogen bromide cleavage of the modified enzyme was carried out in 70% (v/v) formic acid for 24 h at 25 °C using equal weights of CNBr and lyophilized protein. All other steps of the procedure for fractionation and analysis of the peptide were as reported by Birchmeier et al. (1973).

NMR Analyses. The ^{19}F NMR spectra were recorded on a Varian XL-100-15 NMR spectrometer equipped with a Nicolet Technology Corp. Model TT-100 pulsed Fourier transform apparatus. The ^{19}F resonance was observed at 94.1 MHz using a 20-μs pulse width at 30 °C and locked in the ^{1}H resonance of water. All solutions were in 12-mm tubes and contained 1 mM EDTA. The enzyme subunit concentration was 2 mM (100 mg/mL).

Results

Effect of Sulphydryl Modification upon Activity and Thiol Content. The results of the assays for activity and sulphydryl content of aspartate transaminase after modification by NEM and trifluorobromopropanone show that NEM reduces the number of free sulphydryl groups to 2.8 (from 4.6 in native monomer) and the activity is 383 units/mg vs. 366 units/mg in native enzyme. Subsequent treatment with trifluorobromopropanone lowered the sulphydryl value to 1.8/monomer and the activity to 277 units/mg. Similar results were obtained using iodoacetamide as the alkylating agent. Unlike other reagents (Birchmeier et al., 1973), modification of a third sulphydryl group with trifluorobromopropanone under syncatalytic conditions does not eliminate enzymatic activity.

Three of the five thiol groups per enzyme monomer are titrated with pMB in the native enzyme, but none react when the enzyme has first been treated with NEM or iodacetamide followed by trifluorobromopropanone. However, addition of 1% sodium dodecyl sulfate to the reaction mixture exposes two additional cysteine residues for reaction with pMB in both native enzyme and, after its reaction, with NEM or iodacetamide, and trifluorobromopropanone.

During the reaction of the transaminase (NEM protected) with trifluorobromopropanone at 22 °C and in the presence of 70 mM glutamate and 2 mM α-ketoglutamate at pH 7.0, the activity diminishes slightly and after 90 min reaches a plateau value of about 70% of the initial activity. Concomitant sulphydryl group determinations show that the amount of cysteine remaining decreased from 3 to 2. For a similar reaction in the absence of substrates, after 2 h there is a 5% decrease in the specific activity, and only about a 10% decrease in total cysteine content. The optimum pH range for the trifluorobromopropanone modification is 7.0–7.5 (phosphate buffers); at lower pH values it is slower and at higher pH values there is denaturation.

Reaction of ^{19}F-Labeled Enzyme with Nbs_2. After modification of the two accessible thiols, Cys-45 and -82, with alkylating agents, the partially inaccessible Cys-390 is known to be more reactive toward Nbs_2 in the presence of substrates (Birchmeier et al., 1973). However, after the same modifications of cysteine-45 and -82 with iodoacetamide or *N*-ethylmaleimide and the subsequent treatment with the trifluorobromopropanone no reaction with Nbs_2 takes place. Denaturation with sodium dodecyl sulfate exposes only two thiols for reaction with Nbs_2. Without exposure to trifluoropropanone, enzyme treated under the same experimental condition showed three thiol groups exposed to Nbs_2.

Peptide Isolation. The gel-filtration profiles of CNBr-cleaved aspartate transaminase are very distinctive and the fractions containing the different cysteinyl residues have been pinpointed (Birchmeier et al., 1973; Polyanovsky et al., 1974). Those fractions with Cys-390 are not contaminated with peptides containing any of the other four cysteinyl residues (Zufarova et al., 1973; Polyanovsky et al., 1974; Boettcher and Martinez-Carrion, 1975). The gel-filtration elution profile of CNBr fragments of trifluoropropanone-labeled enzyme is shown in Figure 1. The fractions containing the ^{19}F resonance (300-Hz upfield from trifluoroacetic acid) were only those known to contain Cys-390.

[1] Abbreviations used are: NEM, *N*-ethylmaleimide; Nbs_2, 5,5′-dithiobis(2-nitrobenzoate); pMB, *p*-mercuribenzoate; trifluorobromopropanone, 3-bromo-1,1,1-trifluoropropanone; Gdn-HCl, guanidine hydrochloride; EDTA, ethylenediaminetetraacetic acid; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; NMR, nuclear magnetic resonance.

TABLE I: Ligand Affinities and Extinction-Coefficient Comparisons between Native and Modified Aspartate Transaminase.

Modifying Agent	α-Methylaspartate[a] Chloride[a] K_d (mM)	K_d (mM)	Extinction Coefficient (M^{-1} cm^{-1})	Pyruvate[b] K_m (mM)
None	10[c]	0.7	3700	0.4[c]
Iodoacetamide	22	0.8	3600	0.1
Iodoacetamide, then trifluorobromopropanone	22	2.0	3800	0.1

[a] The values for the dissociation constants were determined by UV/Visible spectrophotometric titrations using a Cary 15 recording spectrometer at 25 °C, using the methods of Jenkins and Taylor (1965), Michuda and Martinez-Carrion (1969), and Cheng et al. (1971). [b] Determined with a Durrum stopped-flow spectrometer at 25 °C as described by Cheng et al. (1971). Pyruvate converted all the enzyme from the pyridoxamine to the pyridoxal form. [c] From Cheng et al. (1971).

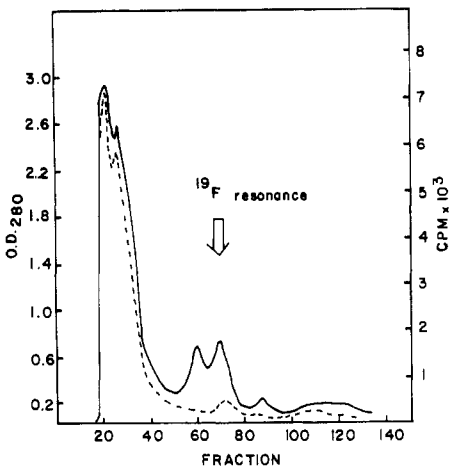

FIGURE 1: Elution pattern of cyanogen bromide cleaved peptide fragments from [^{3}H]$NaBH_4$-reduced aspartate transaminase using a Bio-Gel P-30 column (130 × 1 cm) equilibrated with 20% formic acid. Absorbance at 280 nm (—) and radioactivity (- - -) are displayed.

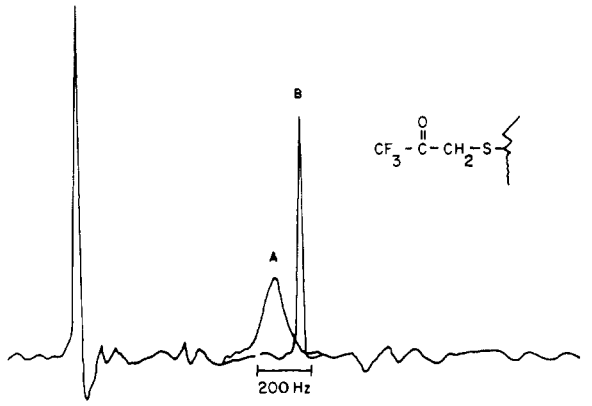

FIGURE 2: ^{19}F NMR spectra (26 000 scans) of aspartate transaminase (2 mM) after syncatalytic modification of Cys-390 with trifluoropropanone. (A) Modified enzyme alone in Tris-Cl buffer, pH 8.3. (B) After addition of 6 M Gdn-HCl, Tris-Cl buffer, pH 8.3. The sharp downfield resonance is that of 1.5 mM trifluoroacetic acid as an external reference.

[^{3}H]$NaBH_4$ was added to radioactively label those cysteines modified with the trifluoroketone as an aid in identifying the modified peptides. Borohydride reduces carbonyl groups and, thus, radioactivity is introduced into trifluoropropanone-labeled Cys-390, the two carbonyl groups on each of the *N*-ethylmaleimide protected cysteines (45 and 83), and the Schiff's base of the pyridoxal phosphate. Therefore, most radioactivity appears in the earlier fractions which do not contain the ^{19}F NMR label. After syncatalytic modification with trifluorobromopropanone and reduction with [^{3}H]$NaBH_4$ no half-cystine appears in amino acid analyses of the peptide fraction containing both the fluorine and tritium labels. Blanks treated under identical conditions, except for the syncatalytic modification with the fluorinated compound, produce a similar elution profile with both fluorine and radioactivity absent from these peptides (fraction 70) and containing half-cystine.

Properties of the Modified Enzyme. The fluorine-labeled enzyme retains the stability of native transaminase with respect to sensitivity to heat and pH. No activity was lost in modified enzyme incubated for 2 h in 0.05 M Tris–acetate buffers at pH values between 5.3 and 9.0. In the presence of 0.06 M glutarate, pH 6.0, the modified enzyme, as for native enzyme, is also stable at 80 °C.

In this transaminase, it is possible to spectroscopically probe the formation of binary complexes between substrates or inhibitors with the active-site chromophore or even between a modified active site and active-site-directed ligands (Jenkins and Taylor, 1965; Peterson and Martinez-Carrion, 1970). Furthermore, one enzyme turnovers are an intrinsic property of this "ping-pong" type enzyme and easily detectable as a conversion of the two forms of the enzyme (pyridoxal ⇌ pyridoxamine). We monitor the formation of the covalent aldimine and ketimine complexes of either form of the holoenzyme with α-methylaspartate or pyruvate, respectively. The well known (Jenkins and D'Ari, 1966; Cheng et al., 1971) competition of chloride for the formation of such binary complexes was also tested. The results of the direct probing of the interaction of the above substrate analogues with the enzyme-bound chromophores are shown in Table I. The ligand binding affinities of the modified enzyme do not vary significantly from those of native transaminase (Table I). Furthermore, the extinction coefficients for the complexes of α-methylaspartate with both native and modified enzymes were essentially equal and there is complete conversion with pyruvate of a pyridoxamine form of the enzyme (330 nm) to a pyridoxal-absorbing species (360 nm).

^{19}F NMR of Labeled Aspartate Transaminase. The ^{19}F resonance from enzyme specifically labeled with the trifluoropropanone at Cys-390 is observed about 750-Hz upfield from the fluorine signal of external trifluoroacetic acid (Figure 2). The resonance is near the chemical shift value of 1025 Hz (upfield from trifluoroacetic acid) obtained for a model compound, *S*-trifluoroacetonyl-β-mercaptoethanol. On the other hand, the half-height line widths of the model compound (2–3

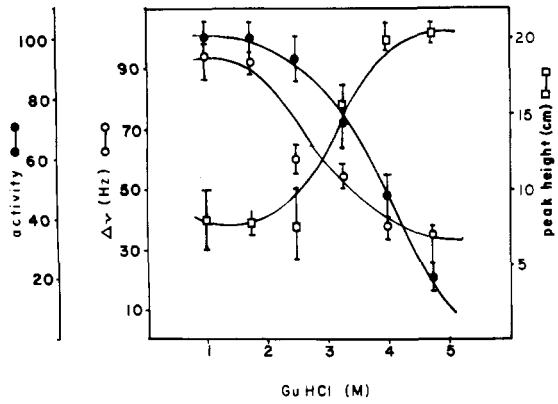

FIGURE 3: Effect of Gdn-HCl on the activity, line width, and peak height of the ^{19}F probe in aspartate transaminase.

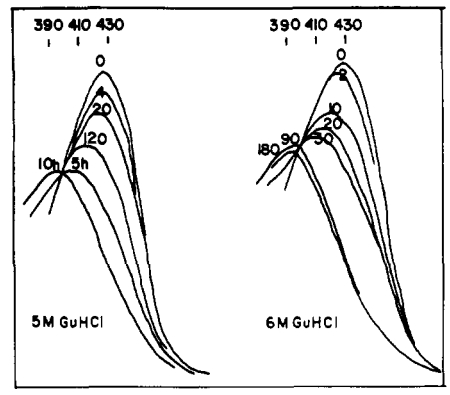

FIGURE 4: Time dependence of the effect of Gdn-HCl on the absorption spectrum of the active-site chromophore of aspartate transaminase at 31 °C. Unless otherwise noted, numbers represent minutes after the addition of a given concentration of Gdn-HCl. Values over the notch marks refer to wavelength.

Hz) and the enzyme-bound fluorine (100 Hz) are markedly different. The NMR spectrum of the CNBr peptide fragments isolated after the gel-filtration procedure (Figure 1) is indistinguishable in both chemical shift and line width at half-height from that of a sample (0.1 mM) of $NaBH_4$-reduced trifluoroacetonyl-β-mercaptoethanol in the same solvent (20% formic acid).

Trifluoroacetonyl alkyl sulfides have pK values in the pH region near 10 (Bendall and Low, 1976); at the pH range tested they should exist mostly in the ionized form. Titrations in the pH range 6–9 result in no change in the chemical shift or line width of either the model compound or the enzyme-bound trifluoropropanone.

Guanidine Hydrochloride Denaturation. Addition of denaturing agents like Gdn-HCl can provide information regarding the topographical relationships of the fluorine atoms and the active-site chromophore. The NMR resonance of the trifluoromethyl group is susceptible to changes in its mobility or protein environment in the transition from native protein to denatured random-coil state of the fully denatured enzyme. The aldimine between pyridoxal phosphate and the lysine has absorption properties which differ from free pyridoxal phosphate, and enzyme activity provides a criterion for the relative array of the components of the active site at different stages of denaturation. As seen in Figure 3, both the resonance line width and signal height of the trifluoropropanone probe are affected by Gdn-HCl. There is also an upfield change in chemical shift which now approaches the 950-Hz, upfield from trifluoroacetic acid, resonance of model S-trifluoroacetonyl-mercaptoethanol in 6 M Gdn-HCl. The changes in the NMR parameters terminated after a 2-h exposure to 4 M Gdn-HCl. On the other hand, the activity loss with incubation at increasing concentrations of Gdn-HCl is less dramatic and even at 5 M Gdn-HCl there is considerable activity remaining. Only after incubation for over 3 h in 6 M Gdn-HCl is there loss of 93% of the activity. The absorbance changes of the active-site chromophore, under the same experimental conditions, parallel the activity changes. As shown in Figure 4, it is clear that the aldimine form of the enzyme (430 nm) shifts toward a new form with lower absorbance and a maximum approaching that of free pyridoxal phosphate (390 nm). After 2-h exposure to 5 M Gdn-HCl, there is considerable structure left in the active-site region of the holoenzyme which disappeared after a similar exposure time to 6 M Gdn-HCl. The resilience of the active-site region is evident in the 5 M Gdn-HCl treatment, which shows that even after 10-h exposure to this denaturant at 31 °C it does not completely free pyridoxal phosphate (Figure 4).

Discussion

Part of the evidence for the identification of Cys-390 as the site of trifluorobromopropanone modification comes from the variation in lability of the cysteines of aspartate transaminase towards alkylating agents. Cys-390 has rates of alkylation which are much slower than those of the readily accessible cysteine-45 and -82 (Birchmeier et al., 1973; Stankewicz et al., 1971). Cysteine-191 and -252, on the other hand, must be exposed to strong denaturants before they become accessible to alkylation (Birchmeier et al., 1973; Stankewicz et al., 1971). Since after alkylation of cysteine-45 and -82 with iodoacetamide the subsequent treatment with trifluorobromopropanone blocks the accessibility of a third cysteine residue to pMB, Cys-390 is the most likely site of modification. Corroborating evidence that Cys-390 is the site of the ^{19}F label is provided by the well-known syncatalytic reaction with Ellman's reagent after blocking of cysteine-45 and -82 (Birchmeier et al., 1973; Boettcher and Martinez-Carrion, 1975). This reagent, after the syncatalytic modification with trifluorobromopropanone, fails to react with any cysteine residue.

The loss of thiol content due to the reaction of the enzyme with trifluorobromopropanone was slower in the absence of substrates. An increase in the rate of reaction with alkylating agents in the presence of the 35:1 ratio of amino acid to α-keto acid is a property that has been associated with modifications of a cysteine by a variety of alkylating agents (Birchmeier et al., 1973; Zufarova et al., 1973; Polyanovsky et al., 1974). Moreover, after the CNBr cleavage only those fractions known to contain Cys-390 show a ^{19}F nuclear resonance, while peptide fractions known to include the other cysteine residues (Birchmeier et al., 1973; Wilson et al., 1974) have no peptide(s) with ^{19}F NMR signal. The disappearance of cysteine (after syncatalytic modification) from the CNBr peptide containing the fluorine also shows that a cysteinyl residue has been modified with trifluorobromopropanone.

It is of interest that ^{19}F labeling of Cys-390 does not greatly alter the transaminase's specific activity and the substrate and inhibitor binding properties. These observations as well as the retention of stability to pH and temperature changes argue for a major conservation of structure and little steric hindrance subsequent to trifluoropropanone modification. The possibility that the reduced level of activity in the modified enzyme is due to a combination of native, fully active, enzyme and modified, nearly inactive, enzyme seems unlikely, since both native and modified enzymes bind α-methylaspartate to form complexes with equal extinction coefficients, and *all* of the pyridoxamine

form of the modified enzyme is transaminated to pyridoxal-absorbing species.

The ^{19}F NMR signal of the fluorine-tagged Cys-390 differs in chemical shift from that of the free-model compound *S*-trifluoroacetonylmercaptoethanol. The chemical shifts of both the enzyme and model compound are, however, identical in the presence of Gdn-HCl, after the enzyme has been denatured. The ^{19}F chemical shift of a fluorine atom attached to a protein can be affected by (a) van der Waals interactions with neighboring residues, (b) ring-current effects caused by aromatic groups, (c) electric-field effects caused by charged groups, and (d) specific bonding interactions (including hydrogen bonds) (Millet and Raftery, 1972). Each of these factors can contribute independently in as yet unknown amounts and their contribution is related to the distance of the interacting groups from the ^{19}F probe. Thus, a difference in chemical shift in the native state from that of the random coil can be interpreted as a contribution from the tertiary structure of the protein into the probe's chemical shift. After denaturation of the protein or after removal of the probe from its native protein environment, the above effect on the resonance signal disappears.

The Gdn-HCl results also provide some clues about the relationship of Cys-390 to the active site. That the NMR parameters cease to change above 4 M Gdn-HCl, when both enzyme activity and the active-center aldimine are still much in evidence, suggests that the particular region of the protein affecting Cys-390 becomes disordered in 4 M Gdn-HCl. These findings agree with the fact that this particular cysteinyl residue is not an intrinsic part of the active site (Birchmeier et al., 1973) and may lie in a region of the protein more susceptible to denaturing agents than the active site itself.

In view of the known hydrated states of trifluoroacetonyl compounds in aqueous solution (Middleton and Lindsey, 1964), the carbonyl compound could be hydrated when attached to the enzyme and thus subject to extensive hydrogen bonding with other groups in the protein. Thus, hydrogen bonding perturbation, along with other protein structural changes in the region neighboring the fluorine probe and transmitted through space, constitute factors that would provide sensitivity in detecting ligand-induced environmental change in a strategic region of the protein. The detection of such changes by substrates and/or inhibitors of the enzyme is the subject of the following paper in this issue.

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