Fluorine-19 Nuclear Magnetic Resonance Studies of Effects of Ligands on Trifluoroacetonylated Supernatant Aspartate Transaminase†

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ABSTRACT: The selective reaction of Cys-45 and -82, on the one hand, and Cys-390, on the other, with 3-bromo-1,1,1-trifluoropropanone allows for the probing of these regions of aspartate transaminase in the absence and in the presence of enzymatic ligands by ^{19}F nuclear magnetic resonance (NMR). The ^{19}F chemical shifts of the resonance lines differ for the three cysteines and so does their behavior with pH changes. The resonance signals with chemical shifts at 615 and 800 Hz upfield from trifluoroacetic acid correspond to modified cysteine-82 and -45 and have tentatively been assigned in this order. The 615-Hz resonance is affected by pH changes that fit best the influence of a single ionizing residue. On the 800-Hz line, the pH changes appear to be the influence of a minimum of two ionizing residues. The ^{19}F resonance from modified Cys-390 is pH independent in the pH range 5–9 for the pyridoxal phosphate, pyridoxamine phosphate, and apoenzyme forms of the enzyme. Occupation of the active site by a quasi-enzyme–substrate complex, trifluoromethionine pyridoxyl phosphate, affects the ^{19}F chemical shift of modified Cys-390, making it pH dependent with a pK value of 8.4. The ^{19}F NMR properties of the pyridoxal form of Cys-390-modified enzyme can be used to monitor some ligand interactions with the active-center region. Addition of α-ketoglutarate or succinate to the ketone labeled enzyme causes a decrease in the resonance line width, and titrations show that this procedure is a good method with which to study the affinity of the enzyme for these ligands. The interpretation of the chemical shift and line-width characteristics of the ^{19}F resonance arising from Cys-390 are most consistent with a model in which the region around this residue seems to be affected by conformational changes arising from substrate binding to the active-center subsites in productive (covalent) manner. Nonproductive complexes which possess fast ligand-protein exchange, such as those between α-ketoglutarate or succinate with the pyridoxal phosphate form of the enzyme, may result only in a greater degree of freedom for Cys-390.

Nuclear magnetic resonance techniques are extremely valuable in gaining insight into the structure to function relationships of proteins (Roberts and Jardetzky, 1970). A goal in using NMR[1] techniques is the resolution and assignment of signals arising from individual atoms, and the utilization of these signals as probes of the dynamic properties (motion and environment) of a given atom in the enzyme alone or in the presence of ligands. The use of ^{1}H or ^{13}C NMR is at present limited by the current state of the instrumentation, given the vastness of the task, where large proteins are concerned. Thus, several laboratories have directed their efforts to the use of other magnetic nuclei probes.

As an alternative approach, selective magnetic nuclei can be introduced into proteins by either chemical (Huestis and Raftery, 1972; Chaiken et al., 1973; Hunkapiller et al., 1973; Bode et al., 1975; Boettcher and Martinez-Carrion, 1975; Martinez-Carrion et al., 1976; Staudenmayer et al., 1976) or biological manipulations (Sykes et al., 1974). Essential criteria for this pursuit are (a) that the protein activity remain unaltered as proof of retention of its native structure, and (b) that the magnetic resonance of the probe be detectable and assignable to a given residue in the protein.

In aspartate transaminase, we have exploited the use of artificially induced reporter groups with mixed success. The ^{19}F NMR of the enzyme-bound coenzyme derivatives revealed the existence of a group with a pK of 8.4 at the active site (Martinez-Carrion et al., 1976). On the other hand, the introduction of a ^{13}C probe in Cys-390 was uninformative regarding new insight on the properties of the protein environment of this thiol (Boettcher and Martinez-Carrion, 1975).

Since substrates influence the susceptibilities of Cys-390 to chemical attack (Birchmeier et al., 1973), the possible correlation between the active-site occupancy by specific ligands and a modification of the properties of the strategic thiol appeared to be a reasonable assumption. In the preceding paper of this issue, we present evidence for the introduction of a ^{19}F probe into Cys-390 (Critz and Martinez-Carrion, 1977). In this work, we show how the chemical insertion of this ^{19}F probe provides information regarding the effected active-site ligands on the protein's environment and some aspects of the relationship of Cys-390 to other thiols in the protein.

Materials and Methods

Materials. The α form of aspartate aminotransferase was prepared as described earlier (Martinez-Carrion et al., 1965). Tris base, cacodylic acid, α-methylaspartic acid and α-ketoglutaric acid were purchased from Sigma Chemical Co. 3-Bromo-1,1,1-trifluoropropanone was from PCR, Inc. The fluorinated methionine pyridoxyl phosphate derivative was prepared as described by Relimpio et al. (1975).

pH Adjustments. For the NMR experiments, changes in pH were made by addition of microliter amounts of 2 M solutions of either Tris base or cacodylic acid directly to the en-

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[1] Abbreviations used are: NMR, nuclear magnetic resonance; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.

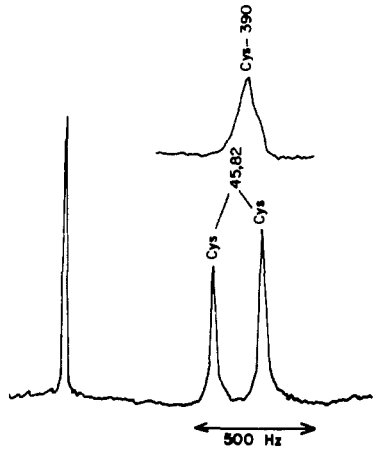

FIGURE 1: The ^{19}F NMR spectra of the modifiable cysteine residues of aspartate transaminase. The lower spectrum shows the two signals arising after reaction with bromotrifluoropropanone in the absence of substrates. *Upper spectrum* is enzyme modified syncatalytically after protection of the easily modified thiol (Cys-45 and -82) residues with *N*-ethylmaleimide. Left sharp resonance is the trifluoroacetic acid reference.

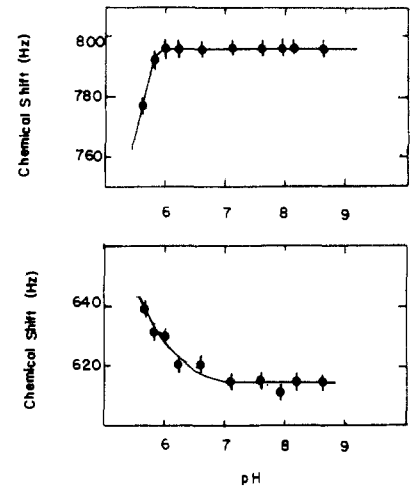

FIGURE 2: The effect of pH on the ^{19}F NMR spectra of modified Cys-45 and -82. The chemical-shift changes are relative to external trifluoroacetic acid.

zyme solution with rapid stirring. Cacodylic acid was chosen because it binds only weakly to the transaminase (Cheng et al., 1971).

Modifications with 3-Bromo-1,1,1-trifluoropropanone. Aspartate aminotransferase was modified at Cys-390 by the procedure described in the previous paper of this issue. Cysteine-45 and -83 were ^{19}F labeled by addition of four 10-μL aliquots containing 0.1 mmol of the bromotrifluoropropanone over a 1-h period to a solution of 10 mg/mL (2×10^{-4} M) enzyme, and 0.1 M sodium phosphate buffer at pH 7.5 in a Sargent S-30240 recording pH stat. Isolation of the modified enzyme was achieved by gel filtration through Sephadex G-25.

Absorption Spectra. Absorption spectra were taken on a Cary 15 spectrometer using 2-mm path-length cuvettes. The spectra were recorded over the range 380–550 nm. The measurement of the dissociation constants (K_d) for α-ketoglutarate and succinate were based on a molar extinction coefficient of 3850 M^{-1} cm^{-1} (α-ketoglutarate) and 3670 M^{-1} cm^{-1} (succinate) for the enzyme–ligand complex with absorbance at 430 nm (Michuda and Martinez-Carrion, 1970). Absorption spectra were obtained from the same samples of modified enzyme used in the ^{19}F NMR experiments without dilution.

^{19}F NMR. The NMR spectra were recorded at 94.1 MHz on a Varian XL-100-15 spectrometer as described by Critz and Martinez-Carrion (preceding paper in this issue).

Preparation of the ^{19}F Enzyme–Substrate Complex. The sodium borohydride-reduced Schiff base between 2 amino-4-(trifluoromethylthio)butanoic acid (i.e., trifluoromethionine) and pyridoxal phosphate was prepared and incorporated into the apo form of the enzyme as described by Relimpio et al. (1975).

Results

Resonances of the Three Modified Cysteine Residues. Figure 1 shows the positions of the ^{19}F labels of cysteine-45, -82, and -390 relative to internal trifluoroacetic acid. The environments, based on inequality of chemical shifts, of all three thiols differ. The cysteines can also be distinguished by their line widths, with the Cys-390 signal being broader (~100 Hz) than either of the other two resonances (~35 Hz). When cysteine-45 and -82 were protected with iodoacetamide, the resonance of the ^{19}F probe in Cys-390 was 815 Hz upfield from trifluoroacetic acid. When cysteine-45 and -82 were modified with *N*-ethylmaleimide or with bromotrifluoropropanone, the resonance of the ^{19}F probe in Cys-390 was 750 Hz upfield from trifluoroacetic acid.

The pH Dependence of the Chemical Shift Positions of Cysteine-45 and -82. Figure 2 shows the variation of the chemical shift of the trifluoroacetonyl group on cysteine-45 and -82. The data indicate that the peak which appears farther upfield (~820 Hz from the trifluoroacetic acid) has a more abrupt chemical-shift change as the solution becomes acidic than does the resonance at 615 Hz from trifluoroacetic acid. The slope of the lower plot in Figure 2 matches a theoretical plot for a chemical-shift change, due to a single ionization (Shrager et al., 1972), much better than the slope of the upper plot in Figure 2 does. Multiple ionizations are known to affect the slope of titration curves, and the apparent steepness of the partial titration curve shown in the upper plot of the figure could indicate that more than one amino acid ionization is observed.

At the end of the titration, the pH was increased with Tris base to pH 7.8, and the NMR spectrum was recorded again. This spectrum matched the other spectra at the higher pH region, indicating that the observed chemical-shift change is due to a reversible change in the enzyme.

The pH Dependence of the Resonance of Cys-390. There is no significant change in chemical shift of the ^{19}F nuclei resonance of the modified Cys-390 in the pH range in which the enzyme is stable (pH 5.5–9.0) for the pyridoxal, pyridoxamine, and apo forms of the enzyme (Figure 3). On the other hand, there is a chemical-shift change for the modified enzyme when it is in the form of an enzyme–substrate complex and an amino acid, trifluoromethionine, occupies the substrate site(s) at the active center (Relimpio et al., 1975). The specific activity of the enzyme recovered at the end of all these titrations was not different from its initial value of 60–70% of the specific activity of the native enzyme. In the case of the apo and the enzyme–substrate forms of the enzyme, the activity was checked after conversion of the enzyme to the pyridoxal form by addition of pyridoxal phosphate to the apoenzyme directly or after the removal of the trifluoromethione pyridoxyl phosphate (Relimpio et al., 1975).

Thus, the chemical shift of the ^{19}F probe on Cys-390 is susceptible to pH changes only when a substrate occupies the

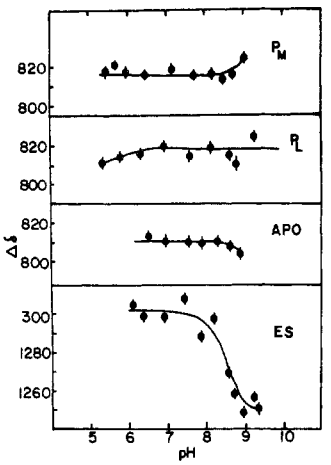

FIGURE 3: Dependence of the ^{19}F NMR signal upon pH after modification of Cys-390 with bromotrifluoropropanone. The enzyme forms are: P_M, pyridoxamine form; P_L, pyridoxal form; APO, apoenzyme form; and ES, the complex formed with *S*-trifluoromethylmethionine pyridoxyl phosphate. Numbers on the ordinate axis represent the chemical shift upfield from an internal reference of trifluoroacetic acid, or free ES analogue. The samples were in 100 mM NaCl.

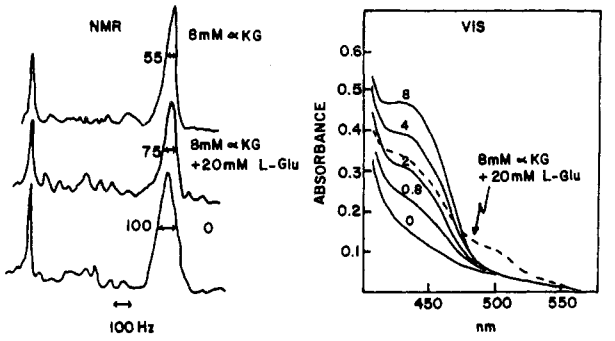

FIGURE 4: ^{19}F NMR (*left*) and visible absorption (*right*) spectra of Cys-390 labeled transaminase in the presence of substrates. Spectra in the absence of substrate (O) and in the presence of α-ketoglutarate (α-KG) and glutamate (L-Glu). The numbers at half height (26 000 scans) indicate line width of the resonance line; the peak at left is due to trifluoroacetate. In the absorption spectra, the numbers correspond to the concentration of α-ketoglutarate. Buffer: 100 mM Tris-Cl, pH 8.1.

active-site region which is the condition favoring accessibility of Cys-390 to modifying agents.

The p*K* of the group which is responsible for this chemical shift is approximately 8.4 at the 100-mM concentration of chloride. At 0 mM NaCl concentration, with the pH adjusted with dry Tris, the pK is 7.7.

Effect of α-Ketoglutarate on the Resonance of Modified Cys-390. Addition of α-ketoglutarate to the pyridoxal form of the enzyme results in an increased absorbance at 430 nm (Jenkins and D'Ari, 1966) and in decreases in the line width of the resonance of the fluorocarbon probe in Cys-390 (Figure 4). Both the effects of α-ketoglutarate on the absorption at 430 nm and the line width of the fluorine resonance are affected by the addition of the amino acid substrate. In the presence of L-glutamate there is an absorbance decrease at 430 nm and the formation of a well-known enzyme-substrate complex with absorbance at 490 nm, while in the NMR spectrum the only detectable change is a reversal of the α-ketoglutarate-induced narrowing of the resonance line width (Figure 4). Treatment of the absorption or NMR spectral changes by a modified Scatchard plot (Figure 5) shows agreement between the two methods used for arriving at the dissociation constant for the

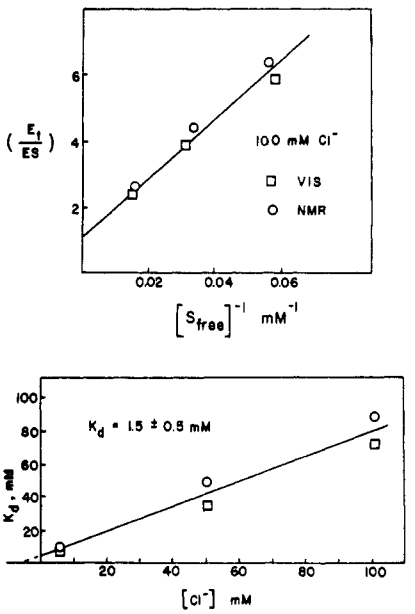

FIGURE 5: *Top:* Effect of α-ketoglutarate on the fraction of substrate-bound enzyme after modification with NEM and bromotrifluoropropanone. Scatchard plot comparing absorption (VIS) with ^{19}F NMR methods of determining the apparent dissociation constant. *Bottom:* Dependence of the apparent dissociation constant (K_d) of α-ketoglutarate upon [Cl$^-$].

keto acid from its complex with the modified enzyme. The Scatchard plot is based on the following equation, which assumes one binding site per monomer:

$$\frac{[E_{total}]}{[ES]} = K_d[S_{free}]^{-1} + 1$$

where $[E_{total}]$ is the enzyme concentration, [ES] is the α-ketoglutarate-enzyme complex concentration, K_d is the dissociation constant, and $[S_{free}]$ is the substrate concentration.

The dissociation constant of the α-ketoglutarate from its complex with the pyridoxal form of the enzyme is given by the slope of the plot of the ratio $[E_{total}]/[ES]$ vs. the inverse of the amount of free substrate in solution.

From the NMR data, the total half-height line-width change between 0 and infinite substrate concentrations is calculated separately. The vertical coordinate, $[E_{total}]/[ES]$, is equal to the ratio of the calculated maximum line width narrowing to the amount that is observed. Under our experimental conditions, an observed decrease in the half-height line width of 20 Hz indicates that about two-thirds of the enzyme is bound.

Throughout this experiment, there was no change in the line width (18 Hz) of the internal reference, trifluoroacetic acid. The pH of the enzyme solution and the α-ketoglutarate solution were kept constant at pH 8.2, and the chloride ion concentration for both solutions was 100 mM. The line-width narrowing effect seems to be a specific effect of the substrates, since the dilution caused by addition to the α-ketoglutarate was corrected for, and was less than 5%. Furthermore, the high chloride ion concentration used should eliminate any ionic-strength effects.

Effect of Chloride Ion on the Affinity of the Enzyme for α-Ketoglutarate. The dissociation constants of α-ketoglutarate from the dead-end keto acid–enzyme complex are anion dependent. Chloride is the best competitor for α-ketoglutarate (Jenkins and D'Ari, 1966; Cheng et al., 1971). This chloride ion concentration dependence of the dissociation constant for α-ketoglutarate can also be measured by the dependence of the

line width of the ^{19}F on α-ketoglutarate concentrations. When several titrations similar to those in Figure 5 were carried out at varying chloride concentrations, a variation of the K_d values in the amount of chloride present was detectable. A plot of the apparent K_d vs. chloride concentration should, in case of pure competitive behavior, produce a graphical method for the determination of both the enzymes affinity for α-ketoglutarate and chloride (Cheng et al., 1971). By such treatment, as shown in Figure 5, a value of 1.5 ± 0.5 mM can be obtained for the anion-independent dissociation constant (ordinate intercept) for α-ketoglutarate and about 10 mM for the chloride dissociation constant (abcissa intercept), and good agreement between the light absorption and NMR methods.

Effect of Succinate and Other Specific Ligands on the ^{19}F Resonance of Modified Cys-390. Dicarboxylic acids are good specific ligands of aspartate transaminase and they form binary complexes with either the pyridoxal or pyridoxamine forms of the enzyme. The complexes with the pyridoxal form of the enzyme are detectable by their characteristic absorption spectra (Jenkins and Taylor, 1965; Jenkins and D'Ari, 1966) and those with the pyridoxamine enzyme only by NMR techniques (Martinez-Carrion et al., 1976). Succinate when added to the ^{19}F-modified pyridoxal form of the enzyme produces a line narrowing of the resonance signal arising from modified Cys-390. The line narrowing cannot be produced by addition of D-amino acids. Graphical treatment of the data of succinate-induced resonance sharpening, as outlined above for α-ketoglutarate, shows that, at pH 8.1 in the presence of 100 mM NaCl, a dissociation constant value of 42 mM can be detected for succinate. This value compares well to 45 mM obtained by the absorbance method with this same enzyme preparation.

Other well-known enzyme substrates, α-methylaspartate, L-glutamate, or *erythro*-β-hydroxyaspartate, produce no significant changes in either the chemical shift or line width of the ^{19}F resonance arising from modified Cys-390.

No ligand produced detectable changes in the resonances arising from trifluoropropanone-modified cysteine-45 and -82.

Discussion

The introduction of the ^{19}F probe at selected sites of aspartate transaminase has allowed the study of structural and functional features of the protein in solution.

The environments about the three modified cysteine residues differ. The primary sequence about Cys-45 is Asp-Asp-Cys-Gln-Pro, and the sequence about Cys-82 is Arg-Thr-Cys-Ala-Ser (Doonan et al., 1975). The primary sequence about Cys-390 is Asn-Met-Cys-Gly-Leu (Doonan et al., 1975). In addition to the amino acid residues which are close to Cys-390 in the primary sequence, the aromatic residues tyrosine (Bocharov et al., 1973) and tryptophan (Wilson et al., 1974) have both been reported to be in the vicinity of Cys-390.

The chemical-shift changes observed for the ^{19}F nuclei on aspartate transaminase were induced by adjustments in pH. Charge alterations in amino acid residues could directly affect the electron density about a neighboring nucleus, or it could affect the magnetic field of a distant nucleus by inducing a change in the conformation of the environment about that nucleus.

The NMR signals of the three modified cysteines can be distinguished easily from each other, an indication of the sensitivity of ^{19}F NMR to environmental differences within the protein. The narrower line width of the fluorine labels on cysteine-45 and -82 compared to that of Cys-390 is probably a consequence of their proposed location on the exterior of the enzyme (Zufarova et al., 1973, Wilson et al., 1974; Polyanovsky et al., 1973), and hence their longer NMR relaxation times.

It is interesting that there is a 65-Hz difference between the chemical shift of the ^{19}F NMR signal of the modified Cys-390 when cysteine-45 and -82 are protected with iodoacetamide rather than *N*-ethylmaleimide. Birchmeier et al. (1973) report that modification of cysteine-45 and -82 with *N*-ethylmaleimide increases the specific activity of the enzyme by 25%, while modification with Ellman's reagent does not change the activity, and tetranitromethane decreases the activity to 65%. Since cysteine-45 and -82 are on the exterior of the enzyme, the activity changes are unexpected. However, the ^{19}F NMR data suggest that modifications of cysteine-45 and -82 affect the region near Cys-390. The effect is probably due to a conformational change which depends on the type of substituent on cysteine-45 and -82. Because Cys-45 has two neighboring aspartate residues in the primary structure, the upfield resonance has been tentatively assigned to Cys-45. In support of this, the negatively charged aspartate residues 43 and 44 are expected to shield the ^{19}F nuclei of the modified Cys-45 and cause its ^{19}F NMR signal to appear upfield of that of Cys-82. Furthermore, protonation of the carboxyl groups of aspartate residues 43 and 44 would probably decrease the shielding of the ^{19}F nuclei on Cys-45 and, as a result, cause the observed downfield chemical shift.

In the fluorine probe attached to Cys-390, the fact that no chemical-shift changes are observed with variation in pH unless the enzyme is in the form of the ES complex probably means that there is a difference between the environment about Cys-390 when the enzyme is binding substrates and when it is not. That is, when the enzyme binds substrates the region about Cys-390 changes, becoming sensitive to pH changes. The data also imply that the region about Cys-390 is more greatly affected by addition of substrates to the holoenzyme than the apoenzyme is by addition of coenzyme.

It is interesting that the pK value obtained by the probe at Cys-390 matches the pK value obtained by the ^{19}F probe on the methionine analogue in the covalent enzyme–substrate complex (Martinez-Carrion et al., 1975). Furthermore, the titration curve fits the theoretical shape of a single rather than multiple ionizable sites. While there are many ionizable groups in this protein, it is tempting to speculate that both events are the result of deprotonation of the same amino acid residue. It should be pointed out that trifluoromethionine pyridoxyl phosphate behaves as an enzyme–substrate complex with regard to the facilitation of reactivity of Cys-390 to alkylating agents.[2] Because the region about Cys-390 is known to contain tyrosine and tryptophan (Wilson et al., 1974), magnetic anisotropy due to ring currents from neighboring aromatic residues could be a major contribution to magnetic environment of the ^{19}F nuclei on Cys-390. If the chemical-shift changes in the resonance of the ^{19}F labels are due to the effect of the same amino acid residue, then the best explanation of the chemical shift is that protonation of the active-site ionizing group, most likely Lys-258 (Martinez-Carrion et al., 1976), causes a conformational change in the enzyme which alters the environment about Cys-390.

Why the pyridoxamine form of the enzyme, which also has a free Lys-258, fails to show a chemical-shift pH dependency remains a clouded issue. However, the reactivity or degree of

[2] Boettcher, B., Relimpio, A. M., Slebe, J. C., and Martinez-Carrion, M., unpublished observations.

exposure of this lysyl residue does not depend only on being free from the internal aldimine with pyridoxal phosphate. This particular lysyl residue does not react with amino reagents in the pyridoxamine form, even though in the apoenzyme it is very reactive toward those reagents (Fasella and Turano, 1970). Moreover, the syncatalytic modification of Cys-390 cannot occur in the pyridoxamine form of the enzyme (Birchmeier et al., 1973). These observations agree with the existence of variances in the relationships of the active site components and Cys-390 in the different forms of the enzyme.

The fact that the NMR results agree with those measured by absorption spectroscopy shows that this NMR technique measures ligand interaction with the enzyme. The simplest explanation for the decrease in line width of the probe on Cys-390 as α-ketoglutarate is added is that binding of this substrate by the pyridoxal form of the enzyme induces a conformational change which allows more segmental motion in that cysteinyl-residue region. By way of analogy, we know that solutions which cause unfolding of proteins cause their NMR line widths to become narrower (Critz and Martinez-Carrion, preceding paper in this issue).

An alternative explanation of the line-width decrease is a chemical change of the ^{19}F group between two environments or, most likely, an interchangeable conformation difference between the subunits of the free enzyme.

If a ^{19}F nucleus is slowly exchanging between two different chemical environments, two NMR signals result. As exchange rates increase, these two signals merge and eventually become narrow. Thus, narrowing of the ^{19}F NMR line width observed could be the result of an α-ketoglutarate-stimulated increase in the rate of exchange of the ^{19}F nuclei between two environments.

For a nucleus exchanging between two environments, the two NMR signals coalesce due to exchange phenomena when (Becker, 1966):

$$\tau = [(\sqrt{2\pi})(\nu_A - \nu_B)]^{-1}$$

where τ is the lifetime in either environment and $(\nu_A - \nu_B)$ is the difference between resonance frequencies. For a difference of 100 Hz between NMR signals, the lifetime of the nucleus in one of the environments is calculated to be 2×10^{-3} s. Since conformational changes have been reported in the range of 10^{-4} to 10^{2} s, a conformation difference between subunits cannot be ruled out on the basis of this rough approximation.

However, we do not favor the notion of a conformational difference between the subunits, which would imply predetermined asymmetry in the dimeric enzyme for four reasons. (a) If there is predetermined asymmetry, it should have been detectable in the active-site region, and no such phenomenon was observed with active-site ^{19}F NMR probes (Martinez-Carrion et al., 1976). (b) Stopped-flow or temperature-jump experiments of the interaction of substrates or other ligands with the transaminase reveal a simultaneous and stoichiometric disappearance of free enzyme and formation of enzyme–ligand complexes from both subunits (Hammes and Fasella, 1963; Jenkins and D'Ari, 1966). (c) Preparation of hybrid dimers of the enzyme, one subunit active and the other inactive, shows independence of the subunits for enzymatic activity or ligand binding (Boettcher and Martinez-Carrion, 1975a, b). (d) Since Cys-390 is modifiable with greater ease in the presence of substrates, an increase in mobility is most likely to occur due to the environment or even an opening of a pocket where this residue lies.

That different ligands interacting with the active-site chromophore, pyridoxal phosphate, cause different effects on the ^{19}F resonance of the Cys-390 probe is not surprising, since the ligands occupy different subsites in the enzyme (Jenkins and D'Ari, 1966; Martinez-Carrion et al., 1973).

It is concluded that the strategic attachment of the ^{19}F probe is useful in detecting ligand–enzyme interactions, which are of use when the absorption spectra of these complexes are obscured by other chromophores. Such an approach would be the only practical way to detect selective interaction of ligands with aspartate transaminase in multienzyme complexes. Finally, the method, although low in sensitivity, has the advantage that the experiments have to be carried out at concentrations close to those estimated for aspartate transaminase in intracellular compartments.[3]

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[3] Estimated from the number of milligrams of enzyme extracted from a given volume of wet mitochondria and the knowledge that the enzyme is located in the mitochondrial matrix (Marco et al., 1969) gives a value of $\sim 10^{-4}$ M for the concentration of aspartate transaminase in mitochondria. Further compartmentalization within the organelle would result in increased molar concentration values.

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Evidence for a Critical Glutamyl and an Aspartyl Residue in the Function of Pig Heart Diphosphopyridine Nucleotide Dependent Isocitrate Dehydrogenase†

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ABSTRACT: The pH dependence of the maximum velocity of the reaction catalyzed by diphosphopyridine nucleotide (DPN) dependent isocitrate dehydrogenase indicates the requirement for the basic form of an ionizable group in the enzyme–substrate complex with a pK of 6.6. This pK is unaltered from 10 to 33 °C, suggesting the ionization of a carboxyl rather than an imidazolium ion. The enzyme is inactivated upon incubation with 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide in the presence of glycinamide or glycine ethyl ester. This inactivation is dependent on pH and the rate constant (k) increases as the pH is decreased in the range 7.3 to 6.25. A plot of $1/(H^+)$ vs. $1/k$ suggests that the enzyme is inactivated as a result of the modification of a single ionizable group in this pH range. The coenzyme DPN and substrate α-ketoglutarate do not affect the rate of inactivation. In contrast, manganous ion (2 mM) and isocitrate (60 mM) produce a sevenfold decrease in the rate constant. The allosteric activator ADP (1 mM) does not itself influence the rate of inactivation; however, it reduces the concentration of Mn^{2+} (1 mM) and isocitrate (20 mM) required to produce the same decrease in the inactivation constant. These observations imply that the modification occurs at the substrate-binding site. Experiments employing [1-^{14}C]glycine ethyl ester show a net incorporation of 2 mol of glycine ethyl ester per subunit (40 000), concomitant with the complete inactivation of the enzyme. The radioactive modified enzyme, after removal of excess reagent by dialysis, was exhaustively digested with proteolytic enzymes. High voltage electrophoretic analyses of the hydrolysate at pH 6.4 and 3.5 yield two major radioactive spots with approximately equal intensity, which correspond to γ-glutamylglycine and β-aspartylglycine, the ultimate products of reaction with glutamic and aspartic acids, respectively. Modification in the presence of manganous ion and isocitrate results in significant reduction in the incorporation of radioactivity into the two dipeptides. These results suggest that carbodiimide attacks one glutamyl and one aspartyl residue per subunit of the enzyme and that the integrity of these residues is crucial for the enzymatic activity.

As with most mammalian tissues, the pig heart muscle contains two distinct isocitrate dehydrogenases: a TPN[1]-dependent enzyme (*threo*-D$_S$-isocitrate:NADP$^+$ oxidoreductase (decarboxylating), EC 1.1.1.42), found in both mitochondria and cytoplasm, and a DPN-dependent enzyme (*threo*-D$_S$-isocitrate:NAD$^+$ oxidoreductase (decarboxylating), EC 1.1.1.41) located in the mitochondria (Plaut, 1963). Though both enzymes catalyze the oxidative decarboxylation of isocitrate to α-ketoglutarate in the presence of a divalent cation and the respective coenzyme, considerable differences exist in their physicochemical properties. Thus the TPN-dependent enzyme is relatively small and consists of a single polypeptide chain with a molecular weight of 58 000 (Colman, 1972), while the DPN-dependent enzyme is considerably larger, having a molecular weight of 340 000 and is composed of multiple subunits (Cohen and Colman, 1971). Only the

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[1] Abbreviations used are: CMC, 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluenesulfonate; DTNB, 5,5-dithiobis(2-nitrobenzoic acid); Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); GlyOEt, glycine ethyl ester; DPN and TPN, diphosphopyridine and triphosphopyridine nucleotides, respectively; Tris, tris(hydroxymethyl)aminomethane.