Identification of the Active-Site Residue of γ-Cystathionase Labeled by the Suicide Inactivator β,β,β-Trifluoroalanine†

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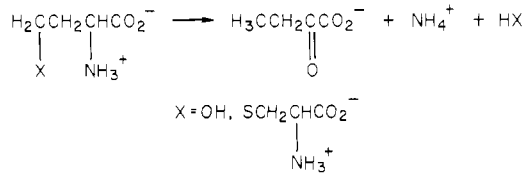
ABSTRACT: Inactivation of γ-cystathionase by β,β,β-trifluoroalanine, a suicide inactivator of the enzyme, results in covalent labeling of an amino group of the protein [Silverman, R. B., & Abeles, R. H. (1977) *Biochemistry 16*, 5515–5520]. We have established that this modified amino function is the ε-NH_2 group of a lysine residue. A heptapeptide which includes this modified lysine residue was isolated, and its sequence was found to be Cys-Ser-Ala-Thr-Lys-Tyr-Met. The amino acid sequence was the same as that determined for peptides containing the active-site lysine residue which forms a Schiff base with pyridoxal phosphate. Therefore the ε-NH_2 group of the active-site lysine which binds pyridoxal phosphate is capable of interacting with the β carbon of trifluoroalanine, and presumably the β carbon of normal substrates. We therefore propose that this lysine residue may function as a proton-transfer agent in the reactions catalyzed by γ-cystathionase.

γ-Cystathionase catalyzes β- and γ-elimination reactions of a number of amino acid substrates. The physiologically important reactions are the following (Matsuo & Greenberg, 1958):



The rat liver enzyme consists of four subunits of molecular weight 40 000 (Deme et al., 1972) and contains four pyridoxal 5′-phosphates (Churchich et al., 1975). The enzyme is irreversibly inactivated by β,β,β-trifluoroalanine, a suicide inactivator of γ-cystathionase (Silverman & Abeles, 1976, 1977). Inactivation is the result of covalent modification of the enzyme. Two moles of inactivator are bound per mole of γ-cystathionase. The structure proposed for the adduct formed between enzyme and trifluoroalanine is shown in Figure 1. In the inactivation process, the trifluoromethyl group is hydrolyzed, and an aminomalonyl residue is formed. The adduct readily decarboxylates upon denaturation of the protein. Studies on the chemical stability of the covalent bond between the inactivator and the enzyme indicated that the inactivator is bound to an active-site amino function, probably the ε-amino group of a lysine residue. The work reported here was carried out to confirm that the ε-amino group of lysine interacts with the inactivator and, if a lysine residue is modified, to determine whether it is the same active-site lysine residue which forms a Schiff base with the cofactor. Toward this end, we isolated a peptide which is labeled by trifluoroalanine as well as peptides containing the lysine which binds pyridoxal phosphate.

Experimental Procedures

Materials

γ-Cystathionase was isolated from rat liver by the procedure of Greenberg (1962), but included additional purification by hydroxylapatite chromatography. Conditions for the stepwise elution from hydroxylapatite were essentially as described by Mushahwar & Koeppe (1973), except that the molarity of the initial phosphate buffer was 50 mM. An enzyme preparation with a specific activity of 347 units/mg displayed a single protein band on $NaDodSO_4$[1]–polyacrylamide gel electrophoresis.

β,β,β-Trifluoroalanine was kindly donated by Dr. B. Lapinskas. Sodium [^{3}H]borohydride (250 Ci/mmol) and ^3H_2O (1 Ci/mL) were obtained from New England Nuclear. Pyridoxal 5′-phosphate, iodoacetic acid, Sephadex (fine), α-chymotrypsin (bovine pancreas), alkaline phosphatase (*Escherichia coli*), and Dowex-50 were purchased from Sigma Chemical Co. Trypsin (TPCK treated) was obtained from Worthington Biochemicals. Cyanogen bromide and urea (recrystallized from 95% ethanol) were from Fisher Chemicals; guanidine hydrochloride (recrystallized from methanol) was from Aldrich Chemical Co.; DE-52 cellulose was from Whatman; dansyl chloride was bought from Pierce Chemicals; polyamide chromatography sheets were obtained from Gallard-Schlesinger.

Methods

Sodium Borohydride Reduction and Carboxymethylation of γ-Cystathionase. Sodium [^{3}H]borohydride (40–90 mCi/mmol) was dissolved at 0 °C in 0.1 N NaOH (1.5 mg/mL). An amount equal to 0.015 mg of $NaBH_4$/mg of protein was quickly added to 6–9 mg/mL γ-cystathionase in 0.2 M potassium phosphate. The yellow color of the enzyme solution rapidly disappeared. After a 20-min incubation at room temperature, the reaction was quenched with acetone. Following dialysis vs. 0.005 M Tris-HCl, pH 8.6, the reduced enzyme was denatured and carboxymethylated by the procedure of Crestfield et al. (1963). The carboxymethylated protein was dialyzed vs. 0.002 M HCl and lyophilized.

Trypsin Digestion. The lyophilized carboxymethylated protein was suspended in 1% $NaHCO_3$ containing 0.002 M $CaCl_2$ to a final protein concentration of 10 mg/mL. Trypsin was added in two additions to a final ratio to protein (or peptide) of 1:60 (w/w), and incubated at 37 °C for 15 h.

Chymotrypsin Digestion of Tryptic Peptides. The Sephadex G-50 pool of the tryptic digest was lyophilized and resuspended in 1.3 mL of 0.025 M $NaHCO_3$ containing 0.01 M $CaCl_2$. A total of 0.1 mg of α-chymotrypsin was added in two additions

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[1] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; $NaDodSO_4$, sodium dodecyl sulfate; DEAE, diethylaminoethyl.

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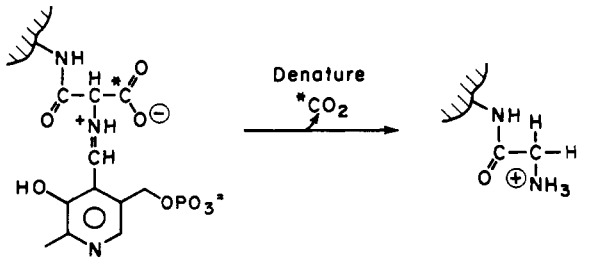


FIGURE 1: Adduct formed upon inactivation of γ-cystathionase by β,β,β-trifluoroalanine.

and incubated for 24 h at room temperature.

Alkaline Phosphatase Treatment of Chymotryptic Peptides. The major peak of the [^{3}H]phosphopyridoxyllysine-containing material from DEAE chromatography of the chymotryptic digest, CT-I, was desalted and lyophilized. The residue was resuspended in 1.5 mL of 0.015 M $NaHCO_3$, and 5.4 units of alkaline phosphatase (26 units/mg) was added. Following a 4-h incubation at room temperature, the alkaline phosphatase was removed by gel filtration on Sephadex G-75 (0.6 × 30 cm) in 0.05 M NH_4OH and lyophilized.

Identity of [^{3}H]Pyridoxyllysine in NaB^{3}H$_4$-Reduced γ-Cystathionase. A tryptic digest of NaB^{3}H$_4$-reduced γ-cystathionase was hydrolyzed in 6 N HCl at 105 °C for 24 h. A sample of the hydrolysate which contained chemically synthesized pyridoxyllysine (Heyl et al., 1948) was spotted on Whatman 3MM paper and electrophoresed for 30 min at 3 kV at pH 3.5 (pyridine/acetic acid/H_2O, 0.4:4:76), 6.5 (pyridine/acetic acid/H_2O, 8:0.24:72), and 8.9 [1% $(NH_4)_2CO_3$]. The pyridoxyllysine was visualized by fluorescence under a UV lamp, and the radioactive material was detected with a Tracerlab 4π scanner.

Cyanogen Bromide Cleavage. The lyophilized carboxymethylated protein or tryptic digest was dissolved in 70% formic acid to a final concentration of 5–10 mg/mL. An amount of cyanogen bromide equal to 50 times the weight of the protein or peptide was added, and the reaction was maintained at room temperature for 24 h. The reaction mix was diluted with 8 volumes of H_2O and lyophilized.

Inactivation of γ-Cystathionase by β,β,β-Trifluoroalanine in ^3H_2O and Incorporation of Tritium into the Adduct. γ-Cystathionase (25 mg, 310 units/mg) in 0.2 M potassium phosphate, pH 7.5, was lyophilized and resuspended in 1.2 mL of ^3H_2O (10^{11} cpm/mL). Trifluoroalanine (2.75 μmol) was added to the enzyme solution and allowed to react for 90 min at room temperature. The yellow enzyme solution turned orange. Guanidine hydrochloride was added to a final concentration of 6 M, and after 2 h at room temperature, the ^3H_2O was removed by bulb-to-bulb distillation. The residue was dissolved in H_2O and dialyzed vs. five changes of 2 L of H_2O for a total of 48 h and then vs. 6 M guanidine hydrochloride. The tritiated protein was carboxymethylated as before, except that guanidine hydrochloride (6 M) was used as the denaturant. The carboxymethylated protein contained 10^5 cpm. A portion of this material (40 000 cpm) was hydrolyzed in 6 N HCl at 105 °C for 24 h. The HCl was removed by rotary evaporation. The residue was taken up in H_2O and the precipitate removed by centrifugation. The hydrolysate (30 000 cpm), to which 5 mg of glycine was added, was chromatographed on Dowex-50 (0.6 × 72 cm, H^+ form). The column was eluted with HCl according to the procedure of Hirs et al. (1954). The fractions were assayed by ninhydrin and for radioactivity.

Purification of Peptides. Peptides were purified by gel filtration in 0.05 M NH_4OH and by DEAE-cellulose chromatography. Chromatography was performed in the dark. Peptide pools were concentrated by lyophilization and salts removed by Sephadex G-10 chromatography in 0.05 M NH_4OH. Purity of a peptide preparation was assessed by NH_2-terminal analysis (Gray, 1972) or by paper electrophoresis at pH 8.9. Peptides were detected on paper by fluorescamine (Lai, 1977).

Amino Acid Analysis. Peptides were hydrolyzed under vacuum in 6 N HCl for 20 or 72 h at 110 °C. Amino acid analysis was performed on a Beckman 121 MB analyzer.

Peptide Sequencing. Automated Edman degradations were performed with a Beckman Model 890C sequencer equipped with a cold trap. The modified single cleavage program of Hunkapillar & Hood (1978) was used. The major change was the substitution of cyclohexane for benzene. Polybrene (4 mg) was added to the spinning cup and, after being dried, was subjected to one complete degradation cycle. The peptide was added to the cup in dilute triethylamine solution (0.45 mL), dried, and then run through the appropriate number of degradation cycles. The anilinothiazolinone derivatives were converted to the phenylthiohydantoin (PTH) amino acids at 80 °C in 1 M aqueous hydrochloric acid. The PTH-amino acids were detected and quantitated on a Hewlett-Packard high-pressure liquid chromatograph (Model 1084 B) equipped with an Hitachi variable wavelength detector and an 18 cm × 4.0 mm i.d. Zorbax–CN column.

Results

Isolation and Sequence of a Pyridoxal Peptide of γ-Cystathionase Reduced with Sodium Borohydride. As in other pyridoxal 5′-phosphate dependent enzymes, the cofactor is bound as a Schiff base to a lysine residue of γ-cystathionase (Fischer & Krebs, 1959). The holoenzyme was reduced with NaB^{3}H$_4$ and digested with trypsin to obtain a peptide which includes the active-site lysine. The tryptic digest was acid hydrolyzed to amino acids in the presence of carrier pyridoxyllysine. The hydrolysate was examined by paper electrophoresis at pH 3.5, 6.5, and 8.0. In all cases, the radioactive material cochromatographed with pyridoxyllysine.

Tryptic peptides of the NaB^{3}H$_4$-reduced enzyme were prepared from 27 mg of γ-cystathionase (347 units/mg) and fractionated on Sephadex G-50. Fractions containing the [^{3}H]phosphopyridoxyllysine peptide were pooled and further digested with α-chymotrypsin. The chymotryptic digest was chromatographed on DEAE-cellulose, and the elution profile is shown in Figure 2A. The major peak of radioactive material, CT-I, eluted at 12.1 $m\Omega^{-1}$ (23 °C).[2] CT-I was then treated with alkaline phosphatase to remove the phosphate group from the pyridoxyl ring, and rechromatographed on DEAE-cellulose (Figure 2B). The radioactively labeled peptide now eluted at 9.4 $m\Omega^{-1}$ (23 °C). Amino-terminal analysis of this peptide revealed a single NH_2-terminal alanine. Yields at each purification step are given in Table I. The sequence of this chymotryptic peptide is Ala-Leu-Gly-Ala-Asp-Ile-Cys-Met-Cys-Ser-Ala-Thr-pyridoxyl-Lys-Tyr.

Isolation and Sequence of a Pyridoxal Peptide by Cyanogen Bromide Cleavage of γ-Cystathionase Reduced with Sodium Borohydride. γ-Cystathionase (60 mg, 310 units/mg) was reduced with NaB^{3}H$_4$, digested with trypsin, and chromatographed on Sephadex G-50 (1.2 × 85 cm) as before. The

[2] Due to technical problems encountered in removing salts, we were unable to purify the minor peak, CT-II. Because the parent tryptic [^{3}H]pyridoxyllysine peptide appeared homogeneous by gel filtration and by chromatography on DEAE-cellulose, Dowex-50, and HPLC, it is likely that CT-II was generated from a nonspecific cleavage by chymotrypsin and that it represents a fragment of or overlaps CT-I.

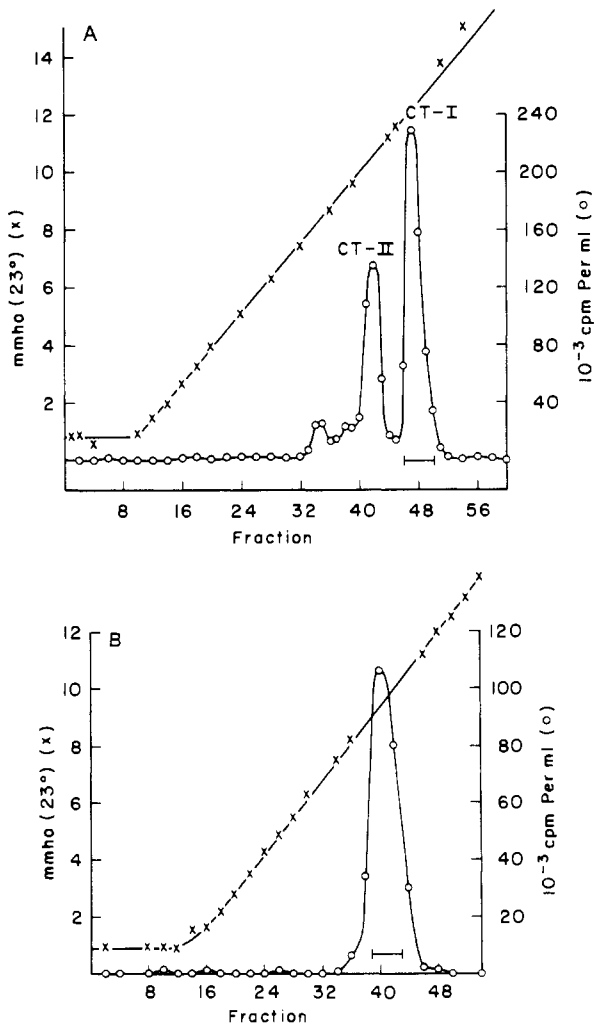


FIGURE 2: Purification of a pyridoxyl peptide from trypsin and chymotrypsin digestion of NaB^3H_4-reduced γ-cystathionase. (A) Elution profile of chymotryptic peptides chromatographed on DEAE-cellulose (0.6 × 20 cm) equilibrated in 0.015 M $NaHCO_3$ (pH 8.3). The peptides were eluted with a linear NaCl gradient consisting of 75 mL of 0.015 M $NaHCO_3$ and 75 mL of buffer and 0.4 M NaCl. (B) DEAE-cellulose chromatography of CT-I following treatment with alkaline phosphatase. Experimental conditions were the same as in (A).

elution profile resembled that of Figure 2A, and a single radioactive peak, eluting at 55 mL, was obtained. Fractions of this peak were treated with CNBr, and a fragment containing the [^{3}H]pyridoxyllysine residue was purified by chromatography on Sephadex G-50 (Figure 3A) and DEAE-cellulose (Figure 3B). Paper electrophoresis at pH 8.9 of this material, CB-I, revealed a single peptide which cochromatographed with the tritiated label. The sequence of this peptide was Cys-Ser-Ala-Thr-pyridoxyl-Lys-Tyr. The cleavage after tyrosine was probably due to a contaminant in the trypsin. In another experiment, sequencing of this CNBr peptide revealed that the tyrosine is followed by methionine. Recoveries of the [^{3}H]pyridoxyllysine-containing material during purification of CB-I are noted in Table I.

Isolation and Sequence of a Cyanogen Bromide Fragment Containing the Residue Labeled by β,β,β-Trifluoroalanine. According to the mechanism for inactivation of γ-cystathionase by trifluoroalanine, the α hydrogen of the inactivator moiety of the adduct is derived from solvent. Furthermore, upon denaturation of the inactivated enzyme, decarboxylation occurs, and an additional solvent proton is incorporated into the resulting glycyl adduct (see Figure 1). In previous experi-

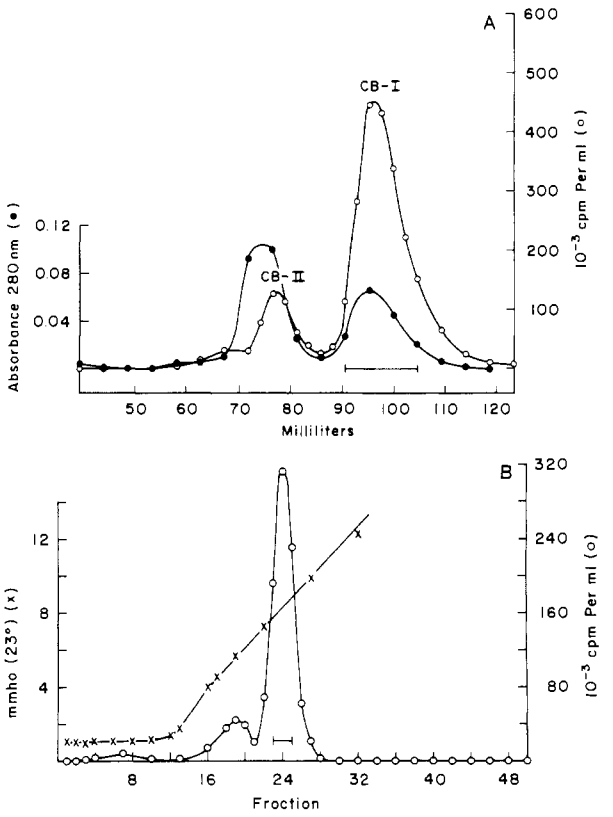
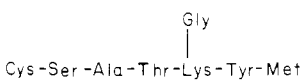


FIGURE 3: Purification of a pyridoxyllysine peptide from CNBr cleavage of NaB^3H_4-reduced γ-cystathionase. (A) Gel filtration of CNBr cleavage fragments of a partially purified tryptic peptide on Sephadex G-50 (1.2 × 85 cm) in 0.05 M NH_4OH. (B) DEAE-cellulose chromatography of CB-I. The column (0.6 × 12 cm) was equilibrated in 0.025 M $NaHCO_3$, and peptides were eluted with a linear 150-mL NaCl gradient, as in Figure 2A.

ments, [^{3}H]glycine was recovered from the amino acid hydrolysate of inactivated enzyme which had been denatured in ^3H_2O (Silverman & Abeles, 1977). Therefore, for introduction of a radioactive label into the adduct, both the inactivation of γ-cystathionase (25 mg) by trifluoroalanine and subsequent denaturation of the protein were performed in ^3H_2O. By this procedure, 10^5 cpm were incorporated into the protein. A portion of this material was acid hydrolyzed to amino acids to confirm that the tritium label was in the glycyl adduct. The hydrolysate, to which carrier glycine was added, was chromatographed on Dowex-50. The elution profile showed a single peak of radioactive material, which cochromatographed with glycine. The pooled glycine peak contained 84% of the total radioactive material applied. The remaining tritiated protein was cleaved with CNBr, and the peptides were chromatographed on Sephadex G-50 (Figure 4). The major radioactive peak, designated TF-I,[3] eluted in a position similar to the pyridoxyl peptide, CB-I (Figure 3A). TF-I was treated with trypsin and purified on Sephadex G-25 (0.7 × 90 cm) in 0.05 M NH_4OH. Yields obtained during purification of this peptide are given in Table I. The following sequence was assigned to TF-I:



In accordance with this assignment and the structure proposed for the adduct (see Figure 1), [^{3}H]PTH-glycine and PTH-(carboxymethyl)cysteine were released on the first round of

[3] Because of the small amount of radioactive material, no attempt was made to purify TF-II, which had similar elution characteristics to CB-II.

Table I: Purification of Active-Site Peptides

peptide	step	total radioactivity[a] (cpm × 10^{-4})	yield[b] (%)
tryptic digest of 27 mg of NaB^3H_4-reduced γ-cystathionase		335	100
	G-50	298	89
chymotryptic digest			
CT-I	DEAE	190	57
CT-II		66	20
CT-I	DEAE	90	27
tryptic digest of 60 mg of NaB^3H_4reduced γ-cystathionase		1500	100
	G-50	1100	73
CNBr cleavage	G-50 starting material	640	43
CB-I	G-50	300[c]	20
CB-II		48[d]	3
CB-I	DEAE	160	11
CNBr peptides of trifluoroalanine-inactivated γ-cystathionase		4.0	100
	G-50 starting material	2.8	70
TF-I	G-50	2.13	53
TF-II		0.56[d]	14
TF-I	G-25	1.68	42

[a]Includes lyophilization and desalting on Sephadex G-10. [b]Based on radioactivity. [c]30% loss upon lyophilization. [d]Represents total amount of radioactive material in the peak, excluding lyophilization and desalting.

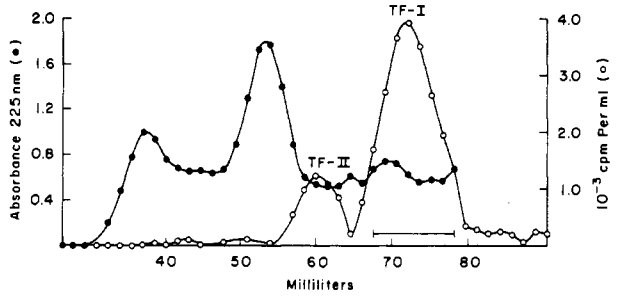


FIGURE 4: Gel filtration of CNBr fragments of γ-cystathionase inactivated by trifluoroalanine. The lyophilized CNBr peptides were resuspended in 3 mL of 0.05 M NH_4OH, and the soluble fragments representing 70% of the total radioactive material were applied to a Sephadex G-50 column (1.1 × 90 cm) equilibrated in 0.05 M NH_4OH.

sequencing. Previous studies had established that the glycine moiety is bonded by amide linkage to an amino group of the enzyme (Silverman & Abeles, 1977). Since this peptide does not include the NH_2 terminal of the protein, [^{3}H]glycine must be bound to the ε-NH_2 group of the lysine residue. Furthermore, the failure of trypsin to cleave between lysine and tyrosine provides support that the lysine residue was modified. The amino acid sequence of TF-I is the same as that determined for the pyridoxal peptides. Therefore the residue of γ-cystathionase which is labeled upon suicide inactivation by trifluoroalanine is the lysine residue which binds to pyridoxal phosphate.

Discussion

The results reported here establish that the ε-NH_2 group of the active-site lysine residue which forms a Schiff base with pyridoxal phosphate is capable of interacting with the β carbon of the suicide inactivator, β,β,β-trifluoroalanine. Therefore, it is likely that this group can interact with the β carbon, and possibly the α and γ positions, of normal substrates. In the reaction catalyzed by γ-cystathionase, protonation and deprotonation occurs at C_α, C_β, and C_γ of the substrates (Davis & Metzler, 1972). In addition, protonation of a leaving group is probably required. Because the suicide inactivators, trifluoroalanine and propargylglycine, modify two distinct nucleophilic amino acids at the active site of the enzyme (Washtien & Abeles, 1977; Silverman & Abeles, 1977), we favor a mechanism by which at least two bases mediate these functions. We suggest that the lysine residue may function as one of these bases. Since propargylglycine interacts with the enzyme at the γ carbon, it is more likely that the ε-NH_2 group of the lysine residue would donate and abstract protons at the α and/or β positions.

The participation of a lysine residue as a general acid or base in catalysis has been proposed for other pyridoxal enzymes. In aspartate transaminase, the lysine residue which forms a Schiff base with pyridoxal phosphate is covalently labeled by the suicide inactivators β-chloroalanine (Morino & Okamoto, 1973; Morino & Tanase, 1978), serine *O*-(methyl sulfate) (John et al., 1973), and vinylglycine (Gehring et al., 1977). The ability of the ε-NH_2 group to interact with inactivators led to the suggestion that this lysine residue may function as a proton-transfer agent in the normal transamination reaction (Morino & Okamoto, 1973; Morino & Tanase, 1978). While others have evidence favoring that a histidine rather than this lysine residue abstracts the α proton from substrate (Peterson & Martinez-Carrion, 1970; Martinez-Carrion et al., 1979), additional studies (Yamasaki et al., 1975; Ford et al., 1980) including models of the active site of the transaminase, based on the X-ray crystallographic structure, make it unlikely that a histidine residue acts as the proton acceptor. With pyridoxamine–pyruvate transaminase (Dunathan, 1971) and cystathionine γ-synthase (Posner & Flavin, 1972), a large isotope effect was observed for proton transfer catalyzed by these enzymes. This isotope effect can be explained by the participation of a polyprotic base, and it was suggested that a lysine residue was acting as the base for the proton transfer for these enzymes (Dunathan, 1971; Posner & Flavin, 1972).

It should be noted that γ-cystathionase contains the sequence -Ser-X-X-Lys(pyridoxal-P)-, a feature that is common to pyridoxal enzymes (Tanase et al., 1979). In mitochondrial aspartate transaminase, X-ray diffraction studies indicated that the serine residue is associated with the phosphate group of the cofactor (Ford et al., 1980).

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Nitrogen-15 Nuclear Magnetic Resonance Investigation of Nitrite Reductase–Substrate Interaction†

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ABSTRACT: Nitrogen-15 nuclear magnetic resonance (^{15}N NMR) spectroscopy at 30.4 MHz was employed to determine the interaction of the substrate nitrite (97.2% enriched) with bacterial nitrite reductase, denoted cytochrome cd_1, from *Pseudomonas aeruginosa*. The addition of ferric enzyme to nitrite did not alter the chemical shift of the bulk nitrite resonance, nor was it possible to observe a new resonance from a hypothetical bound form. However, the spin–lattice relaxation time (T_1) was lowered from 13.2 to 2.7 s, and the spin–spin relaxation time (T_2) was halved. Values of T_1 were measured by progressive saturation and values of T_2 by line widths. Control experiments involving ferric cytochrome *c* and metmyoglobin demonstrated that the perturbations did not arise from the bulk paramagnetic properties of the protein solutions. Variable enzyme/substrate ratios were measured to assess the strength of interaction. The most reasonable model consistent with the data proposes a weak association between nitrite and ferric reductase with a value of 1.3 M^{-1} for the association constant.

The dissimilatory nitrite reductase in many denitrifying bacteria is a cytochrome called cytochrome cd_1. The enzyme in native form is a dimer composed of two subunits of ca. 60 000 daltons with each subunit containing one heme *c* and one heme d_1 as prosthetic groups (Kuronen et al., 1975). In the catalytic cycle the enzyme alternates between oxidized and reduced states as it accepts electrons from a donor, usually a bacterial cytochrome *c*, and transfers them to nitrite, reducing it predominantly to nitric oxide (Wharton & Wintraub, 1980). In such a system, a pertinent question to ask is whether the substrate NO_2^- first binds to oxidized enzyme, which then is reduced by a donor, or whether the enzyme is first reduced and then interacts with substrate. Visible spectroscopy has been extensively used to demonstrate that reduced enzyme is fully capable of an interaction with nitrite that includes the transfer of reducing equivalents (Yamanaka & Okunuki, 1963; Silvestrini et al., 1979). However, the binding constant to the reduced form has not been measured, and any attempts would be ambiguous because of enzyme turnover. The available data do not rule out significant binding to the oxidized form where such an event may not be evident with light spectroscopy. Some electron paramagnetic resonance (EPR) data have been suggestive of an interaction with the oxidized form (Muhoberac & Wharton, 1980).

Nuclear magnetic resonance (NMR) spectroscopy of the ^{15}N nucleus has not been widely employed in biological studies because of low natural abundance (0.38%) and a relative

† From the Department of Chemistry, Illinois Institute of Technology, Chicago, Illinois 60616. *Received February 5, 1982.* Supported by Grants GM-23869 and GM-26071-0251 from the National Institutes of Health.

0006-2960/82/0421-3794$01.25/0