Functional Tyrosyl Residues of Carboxypeptidase A. The Effect of Protein Structure on the Reactivity of Tyrosine-198†

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ABSTRACT: Coupling of bovine carboxypeptidase A with diazotized 5-amino-1*H*-tetrazole increases esterase activity, decreases peptidase activity slightly, and modifies one tyrosyl residue. Subsequent nitration of the azoenzyme has no further effect on esterase activity, decreases peptidase activity markedly, and modifies a second tyrosyl residue. Analysis of the azopeptides isolated from a chymotrypsin digest of the doubly modified enzyme by affinity, ion exchange, and high pressure liquid chromatography indicates that the principal residue modified by diazo-1*H*-tetrazole is Tyr-248. Analysis of the nitropeptides isolated by similar procedures indicates that nitration occurs mainly at Tyr-198. This residue becomes susceptible to modification only as a consequence of a conformational change that accompanies azo coupling of Tyr-248. These results describe a unique example of the influence of protein structure on the reactivity of functional amino acid residues and illustrate an important aspect of chemical modification of enzymes.

Carboxypeptidase A from bovine pancreas was the first enzyme found to have functional tyrosyl residues at its active center. The selective acetylation of two of its 19 tyrosyl residues with acetic anhydride or *N*-acetylimidazole (Riordan & Vallee, 1963; Simpson et al., 1963) abolished its peptidase activity and increased its esterase activity. At that time it could not be determined if one or both of these residues is critical to catalytic function but subsequently the activities of carboxypeptidase were shown to be altered when only one tyrosyl residue is modified with either diazonium-1*H*-tetrazole (Sokolovsky & Vallee, 1967) or tetranitromethane (Riordan et al., 1967). Prior acetylation completely prevents the functional effects of either azo coupling or nitration; hence, the tyrosyl residues modified by diazonium-1*H*-tetrazole and tetranitromethane were thought to be among those that are acetylated.

The changes in activities on nitration of carboxypeptidase differ from those that occur on coupling with diazonium-1*H*-tetrazole. Both modifications increase esterase activity to about 180% of the native enzyme. But while nitration decreases peptidase activity substantially (to <15%), diazonium coupling alters it only slightly. These findings suggested that the two reagents reacted with different tyrosyl residues, a deduction supported by the results of consecutive modifications (Riordan et al., 1967). Thus, nitration of the azotetrazole-coupled enzyme had no further effect on esterase activity but decreased peptidase activity to that characteristic of nitrocarboxypeptidase. Moreover, spectral analysis of the product revealed the presence of one azotyrosyl and one nitrotyrosyl residue per protein molecule. Since nitration of the native enzyme has been shown to occur at Tyr-248 (Muszynska & Riordan, 1976), azo coupling was conjectured to affect the second, active site tyrosyl residue.

We have now identified the tyrosyl residue in carboxypeptidase that is modified by diazo-1*H*-tetrazole. Tetrazoylazotyrosyl peptides have been isolated by immunoaffinity chromatography and analyzed. Alignment with the known sequence of the enzyme (Bradshaw et al., 1969a) indicates that the residue predominantly modified is, in fact, Tyr-248, not a second active site tyrosine as originally suspected. The nitrotyrosyl peptides from sequentially modified nitroazocarboxypeptidase were also isolated and analyzed and the predominant site of nitration is Tyr-198. Thus it is likely the second active site residue. Since nitration of *native* carboxypeptidase has no effect on Tyr-198, this residue must become susceptible to electrophilic substitution only subsequent to the coupling of diazonium-1*H*-tetrazole with Tyr-248.

It seems reasonable to propose that azo coupling induces a series of conformational changes that create a microenvironment conducive to the nitration of Tyr-198. This phenomenon is a novel demonstration of the critical influence of protein structure on the chemical reactivity of amino acid side chains and is pertinent to the mode of action of this enzyme.

Materials and Methods

Bovine carboxypeptidase A (EC 3.4.12.2), prepared according to the procedure of Anson (1937) was obtained from Worthington Biochemical Co. (Code: COA) and purified by adsorption on a *p*-aminobenzylsuccinate-Sepharose 4B conjugate affinity column (Peterson et al., 1976). Carboxypeptidase A prepared according to Cox et al. (1964) was obtained from Sigma Chemical Co. Tetranitromethane and 5-amino-1*H*-tetrazole were supplied by Aldrich. All other chemicals were of reagent grade or the highest purity available. Adventitious metal ions were removed from all buffers and substrates by extraction with 0.01% dithizone in CCl_4 (Thiers, 1957). Protein concentrations were determined from absorbance at 278 nm with a Zeiss M4-QIII spectrophotometer. Molar absorptivities of 6.42×10^4 and 6.50×10^4 M^{-1} cm^{-1} were used for native (Simpson et al., 1963) and azocarboxypeptidase, (Sokolovsky & Vallee, 1967), respectively. The concentration of modified carboxypeptidase was also determined by amino acid analysis.

Peptidase activities were measured by the ninhydrin method of Auld & Vallee (1970) with Z-Gly-L-Phe and Bz-Gly-Gly-L-Phe in 1 M NaCl–0.05 M Tris-HCl (pH 7.5) at 25 °C. Esterase activities were determined by titration of the protons released on hydrolysis of 5 mM Bz-Gly-L-OPhe in 0.2 M NaCl

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with 0.1 N NaOH using a Radiometer autotitrator (Auld & Holmquist, 1974).

Tetrazolylazotyrosine and nitrotyrosine were measured using respective molar absorptivities of 8.70×10^3 M^{-1} cm^{-1} (483 nm) and 4.45×10^3 M^{-1} cm^{-1} (428 nm) at pH $\geq$11. Nitrotyrosine was also determined by amino acid analysis (Giese & Riordan, 1975).

Absorption spectra were recorded with a Cary 14 spectrophotometer and circular dichroic spectra with a Cary 61 dichrograph.

5-Amino-1*H*-tetrazole was converted to the diazonium salt according to published procedures (Sokolovsky & Vallee, 1966; Riordan & Vallee, 1972).

N-Acetyl-3-tetrazolylazo-L-tyrosine was synthesized as described by Sokolovsky & Vallee (1966), mp 141 °C (dec). Anal. Calcd for $C_{12}H_{13}N_7O_4 \cdot H_2O$: C, 42.7; H, 4.5; N, 29.1. Found: C, 42.97; H, 4.61; N, 29.01.

Affinity Adsorbents. Bovine serum albumin and rabbit γ-globulin (Miles) conjugated with 3-nitro-L-tyrosine via 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (Helman & Givol, 1971) contained 21 and 50 mol of ligand per mol, respectively. Antisera were generated by injecting white New Zealand rabbits with 1 mg of the albumin conjugate, emulsified with complete Freund's adjuvant (Difco) in multiple intracutaneous sites. Additional injections of 0.5 mg were made after 2 and 5 weeks. The sera were tested for antibodies by double immunodiffusion assay against the rabbit γ-globulin conjugate. Antitetrazolylazotyrosyl antibodies were isolated from whole, pooled sera by adsorption on a column of tetrazolylazotyrosyl-rabbit γ-globulin immobilized on Sepharose 4B. Proteins bound nonspecifically were washed off the column with 0.14 M NaCl–0.01 M phosphate (pH 7.4). Antitetrazolylazotyrosyl antibodies were eluted rapidly with 1 M NH_4OH and the eluate was immediately adjusted to pH 7. The immunoglobulins were then dialyzed against 0.2 M sodium citrate, pH 6.5, concentrated by ultrafiltration to 10 mg/mL, and immobilized on CNBr-activated Sepharose 4B (Wilchek et al., 1971). Anti-3-nitro-L-tyrosyl antibodies were produced and isolated in the same way using nitrotyrosyl bovine serum albumin and nitrotyrosyl rabbit γ-globulin as antigens, respectively.

Azo Coupling of Carboxypeptidase. Carboxypeptidase, 6×10^{-5} M in 1 M NaCl–0.67 M $KHCO_3$ (pH 8.8), was coupled with a sevenfold molar excess of diazotized 5-amino-1*H*-tetrazole at 0–4 °C (Sokolovsky & Vallee, 1967). The reaction was quenched after 30 min by addition of excess Tris-HCl (pH 8.0) and the product tetrazolylazotyrosylcarboxypeptidase (hereafter referred to as azocarboxypeptidase) was dialyzed against 0.2 M NaCl–0.05 M Tris-HCl (pH 8.0) at 4 °C.

Nitration of DHT-Carboxypeptidase. The azocarboxypeptidase solution was concentrated to 10 mg/mL by ultrafiltration (Amicon PM-10 membrane) and nitrated with a fourfold excess of tetranitromethane at pH 8, 20 °C. After 45 min the reaction was terminated by gel filtration through Bio-Gel P-4 (Riordan et al., 1967).

Peptide Isolation and Identification. Modified carboxypeptidase was cleaved with CNBr in 70% formic acid and the fragments were separated by gel filtration on Sephadex G-75 in 0.1 M propionic acid (Nomoto et al., 1969). Chymotrypsin digestion was carried out on the F_1 fragment (residues 104–301) after succinylation (Johansen et al., 1972). The protein (5–10 mg/mL in 0.1 M NH_4HCO_3) was incubated with 2% (w/w) α-chymotrypsin (3× crystallized, Worthington) at 37 °C in the presence of 1 mM $CaCl_2$. After 6 h another 1% chymotrypsin was added. After 24 h the digest was briefly heated to 100% to destroy residual chymotrypsin activity and then lyophilized.

Tetrazolylazotyrosyl or nitrotyrosyl peptides were isolated by affinity chromatography on their respective immobilized antibodies. The digest was dissolved in 0.05 M NH_4HCO_3 and applied to a 1.5 × 10 cm column of the appropriate antibody–Sepharose 4B conjugate. Unretarded peptides were washed through with 0.05 M NH_4HCO_3. The adsorbed peptides were then removed with 1 M NH_4OH and lyophilized.

Anion-exchange chromatography was performed on DEAE-cellulose with a linear ionic strength gradient and on Dowex AG1-X2 with a linear gradient of decreasing pH and increasing pyridine concentration (Bradshaw et al., 1969b).

High pressure liquid chromatography was carried out with a Waters ALC-CPG-204 chromatograph. Peptides were separated on micro-Bondapak C-18 (2 × 30/0.3 cm) with a 0.01 M sodium acetate (pH 4.0)–acetonitrile gradient. The effluent absorbance at 340 nm was monitored with a Beckman Model 25 spectrophotometer. Fractions were collected manually for rechromatography and further analysis.

Paper electrophoresis was performed at 50 V/cm for suitable periods on a Gilson Electrophorator, Model D, with acetic acid–formic acid–water (100:30:870, v/v) as buffer and Varsol as the heat exchanger. Whatman 3 MM paper was used for both preparative and analytical separations. A 1:5 (v/v) aqueous dilution of buffer was used for wetting the paper. Separated peptides were located with fluorescamine (Mendez & Lai, 1975).

Silica gel and microcrystalline cellulose coated glass plates (Merck) were used for thin layer chromatography. Solvent systems were 1-butanol–pyridine–acetic acid–water (15:10:3:12, v/v), and chloroform–methanol–30% ammonia (2:2:1, v/v). Peptides were visualized with ninhydrin or fluorescamine.

Peptide Analysis. Purity was checked by N-terminal analysis using dansylation (Gray, 1967) and two-dimensional thin-layer chromatography on polyamide sheets (Woods & Wang, 1967). Acid hydrolysis was carried out with glass-distilled HCl or 3 N *p*-toluenesulfonic acid (Liu & Chang, 1971) in sealed, evacuated tubes at 110 °C for 24 h. Amino acid analyses were performed on a Durrum D-500 automatic analyzer.

Results

Reaction of carboxypeptidase with a sevenfold molar excess of diazonium-1*H*-tetrazole generates one tetrazolylazotyrosyl residue per protein molecule, as determined by spectral analysis. Amino acid analysis confirms the modification of one tyrosyl residue (Table I) as well as 4–5 lysyl residues. The peptidase activity of the modified enzyme is 85% and the esterase activity is 185% relative to the native enzyme when assayed under standard conditions. After dialysis against metal-free buffer (0.05 M Tris–0.2 M NaCl, pH 8.0) the zinc content of the modified protein is 1 g-atom per mol.

Nitration of azocarboxypeptidase with a fourfold excess of tetranitromethane also modifies one tyrosyl residue as shown by the difference spectrum of the azo and nitroazoenzyme (Figure 1) and by amino acid analysis (Table I). Concomitantly, peptidase activity is reduced to about 9% relative to the native enzyme, but esterase activity is virtually unchanged.

Azo coupling in the presence of the inhibitor, β-phenylpropionate, 0.1 M, prevents modification of tyrosine and the changes in enzymatic activity (Table I). Similarly, nitration of azocarboxypeptidase in the presence of the same concentration of inhibitor essentially precludes modification of tyrosine with minimal effects on activity (Table I).

TABLE I: Tetrazolylazotyrosine and Nitrotyrosine Contents and Catalytic Activities of Native, Azo-, and Nitroazocarboxypeptidase.

Enzyme	AzoTyr/mol AAA[a]	AzoTyr/mol Spectrum	NO_2Tyr/mol AAA[b]	NO_2Tyr/mol Spectrum	$V/V_c \times 100$ Peptidase[c]	$V/V_c \times 100$ Esterase[d]
Native	0	0	0	0	100	100
Azo	1.0	1.1	0	0	85	185
Azo[e]	0	0.1	0	0	100	100
Nitroazo	1.1	1.1	0.9	1.0	9	180
Nitroazo[f]	1.1	1.0	0.1	0	82	180

[a] Determined as loss of tyrosine by amino acid analysis. [b] Amino acid analysis. [c] Assayed with 0.02 M Z-Gly-L-Phe or Bz-Gly-Gly-L-Phe according to Auld & Vallee (1970). [d] Assayed with 0.005 M Bz-Gly-L-OPhe according to Auld & Holmquist (1974). [e] Modified in the presence of 0.1 M β-phenylpropionate. For conditions, see Materials and Methods. [f] Azocarboxypeptidase nitrated in the presence of 0.1 M β-phenylpropionate.

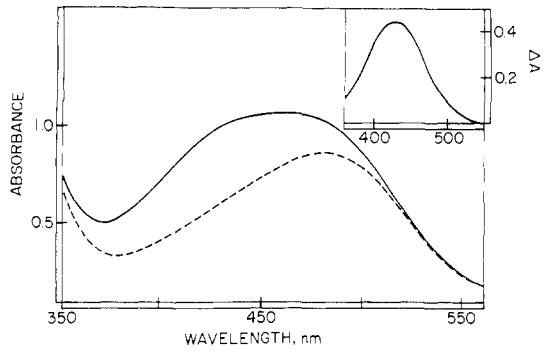

FIGURE 1: Absorption spectra of azocarboxypeptidase (- - -) and nitroazocarboxypeptidase (—) in 0.1 N NaOH both at 1×10^{-4} M. The inset represents the difference spectrum of nitroazocarboxypeptidase minus azocarboxypeptidase.

Identification of Tyrosyl Residues Modified. Gel filtration of the CNBr fragments obtained from nitroazocarboxypeptidase reveals that 90% or more of both the nitro- and the azotyrosyl residues are located in the F_1 fragment (Nomoto et al., 1969), as judged by absorbance at 340 nm (Figure 2). The nitro- and azotyrosine-containing peptides were isolated from a chymotrypsin digest of the F_1 fragment by immunoaffinity chromatography on their respective antibody-Sepharose 4B conjugates with yields of better than 90% in each instance (Figure 3). Thus, the breakthrough fraction centered at 13 mL (Figure 3A) that is obtained by immunoaffinity chromatography of the entire digestion mixture on an antitetrazolylazotyrosyl antibody-Sepharose column (1.5 × 10 cm) is composed largely of unlabeled peptides and also nitrotyrosyl peptides as indicated by the absorbance at 480 nm and by spectral and amino acid analysis. By the same criteria, the adsorbed fraction eluting with 1 M ammonia and emerging at about 34 mL (Figure 3A) contains only tetrazolylazotyrosyl peptides and is free of nitrotyrosine. Nitrotyrosyl peptides are separated from unlabeled peptides by rechromatography of the breakthrough fraction from Figure 3A on a 1.5 × 10 cm column of antinitrotyrosyl antibodies immobilized on Sepharose 4B (Figure 3B). Now the breakthrough fraction contains only unlabeled peptides while the nitrotyrosyl peptides elute with 1 M ammonia. As expected the adsorption spectrum of this second fraction is consistent with the presence of only nitrotyrosine. There is no evidence for any peptide containing a doubly modified tyrosyl residue.

Ion-Exchange Chromatography of Azotyrosyl Peptides.

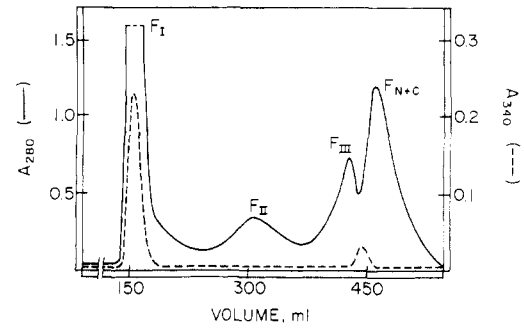

FIGURE 2: Separation of cyanogen bromide fragments of nitroazocarboxypeptidase on Sephadex G-75. A 3-μmol sample of nitroazocarboxypeptidase treated with CNBr according to Nomoto et al. (1969) was applied to a 2.5 × 95 cm column of Sephadex G-75 equilibrated and developed with 0.1 M propionic acid. Fractions of 5 mL were collected and absorbance was measured at 280 nm and 340 nm.

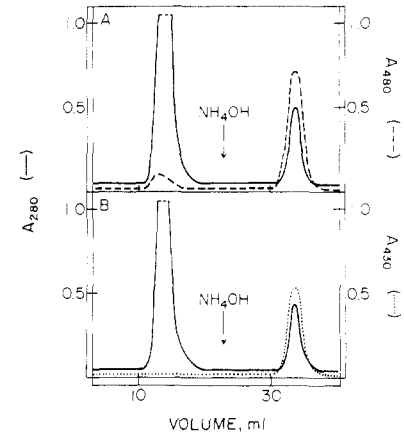

FIGURE 3: Isolation of tetrazolyazotyrosyl and nitrotyrosyl peptides by immunoaffinity chromatography. A 3-mL sample, containing 0.4 μmol of azotyrosine and nitrotyrosine, obtained by chymotrypsin digestion of the *N*-succinyl-F_1 fragment, was applied to an anti-tetrazolylazotyrosine-antibody-Sepharose 4B column (1.5 × 10 cm) equilibrated with 0.01 M NH_4HCO_3 (A). After the main peak of absorbance at 280 nm, containing nonlabeled and nitrotyrosyl peptides, was fully eluted, the azopeptides were eluted with 1 M NH_4OH. The main peak was then applied to an anti-nitrotyrosyl-Sepharose 4B column (B). After elution of the nonlabeled peptides with 0.01 M NH_4HCO_3, the nitrotyrosyl peptides were desorbed with 1 M NH_4OH. Absorbance was measured at 280, 480, and 430 nm.

The azotyrosyl peptide fraction obtained by affinity chromatography (Figure 3A) was applied to a column of Dowex AG1-X2, and eluted with a double linear pH and ionic strength gradient (Figure 4). Under these conditions, no material absorbing at 340 nm, the λ_{max} for tetrazolylazotyrosine, emerged during the first gradient but three major (ii, III, and IV) and three minor (I, V, and VI) peaks were detected in the second gradient. The overall recovery of 340 nm absorbance was essentially quantitative (>95%).

Fraction I, corresponding to 4% of the A_{340} material, produced a single spot on thin-layer chromatography and had an amino-terminal glycyl residue, as determined by dansylation (Gray, 1967) and subsequent thin-layer chromatography (Woods & Wang, 1967). This, together with its amino acid composition (Table II), is uniquely compatible with the sequence of residues 207–211 of carboxypeptidase (Bradshaw et al., 1969a). Hence, Tyr-207 is one of the residues modified, at least in part, by diazonium-1*H*-tetrazole. Fraction II, corresponding to 11% of the A_{340} material, also yielded essentially one spot on thin-layer chromatography. Its composition and N-terminal isoleucine (Table II) identify its origin as residues 247–250 and Tyr-248 as the residue modified.

TABLE II: Amino Acid Composition of Tetrazolylazotyrosyl Peptides I–IV from a Chymotrypsin Digest of the Succinyl-F_1 CNBr Fragment.

Amino acid	I	(207–211)[a]	II	(247–250)[a]	III	(246–252)[a] + (247–253)[a]	IV	(247–257)[a] + (246–257)[a]
Asp							2.0	2
Thr	1.7	2			0.7	1	0.8	1
Ser					1.6	2	3.4	4
Glu	0.6	1	0.9	1	2.1	2	2.2	2
Gly	1.0	1			2.8	3	3.8	4
Ala			1.1	1	2.1	2	2.0	2
Ile			0.9	1	1.7	2	3.5	4
Tyr		1		1		2		2
AzoTyr[b]	1.0		1.0		2.0		2.0	
N terminus	Gly		Ile		Thr, Ile		Thr, Ile	
C terminus	ND		ND		ND		Trp	
Rel yield (%)	4		11		22		48	

[a] Composition of this sequence of carboxypeptidase as determined by Bradshaw et al. (1969a). [b] Determined by spectral analysis.

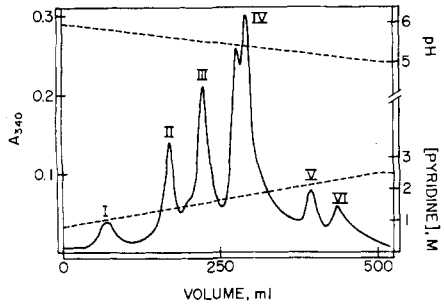

FIGURE 4: Chromatography of azotyrosyl peptides on AG1-X2. The azotyrosyl peptides (1.8 μmol), purified by immunoaffinity chromatography, were applied to a 0.9 × 55 cm column of AG1-X2 (acetate form), equilibrated with 3% pyridine at 45 °C. The column was developed with two 500-mL gradients, linear in pH and pyridine concentration: (I) 3% 1 M pyridine, pH 9–6; (II) 1–2.5 M pyridine, pH 6–5; flow rate = 25 mL/h. The pH was adjusted with acetic acid. Fractions of 2.5 mL were collected and absorbance was measured at 340 nm. Only gradient II is depicted in the figure since no material absorbing at 340 nm was eluted in gradient I.

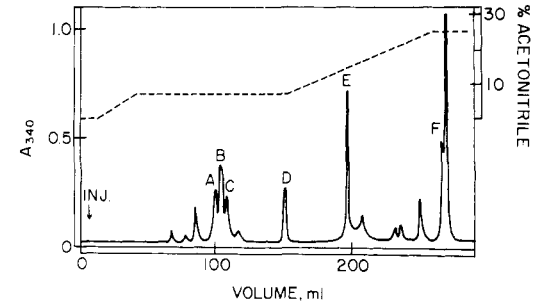

FIGURE 5: Chromatography of azotyrosyl peptides on reverse phase packing. Azotyrosyl peptides (0.6 μmol) were chromatographed on Micro-Bondapak C_{18} (Waters Associates) using 3.8 mm × 30 cm columns. Gradient elution with 0.01 M sodium acetate, pH 4.0, to 90% acetonitrile in 0.1 M sodium acetate, pH 4.0, as indicated (- - -); flow rate = 2 mL/min. Absorbance was measured at 340 nm and fractions were collected manually. Rechromatography was performed with 6% acetonitrile in 0.01 M sodium acetate, pH 4.0.

Fraction III is apparently composed of two, closely related azotyrosyl peptides. The amino acid composition is not compatible with a single, unique sequence segment but is consistent with an approximately equimolar mixture of two overlapping heptapeptides, one encompassing residues 246–252, the other residues 247–253. The presence of two N termini, Thr and Ile, also supports this view. Moreover, identical peptides were found by high-pressure liquid chromatography of the azopeptides obtained from the affinity column (Figure 5). In both cases the residue modified is Tyr-248. Based on the peak area of fraction III, these two peptides comprise about 22% of the total azotyrosyl content of the F_1 cyanogen bromide fragment.

The largest peak of A_{340} material eluting from AG1-X2 appears in fraction IV (Figure 4). It accounts for almost half of the azotyrosyl absorbance and, like fraction III, seems to contain equimolar amounts of two closely related peptides. One of these derives from that part of the sequence of the enzyme extending from Ile-247 through Trp-257, while the second begins at Thr-246 and also extends through Trp-257. Amino-terminal analysis indicates the presence of both Thr and Ile, whereas carboxyl terminal analysis, by digestion with carboxypeptidase A, demonstrates the presence of just Trp. Tryptophan was also found to be present by magnetic circular dichroic analysis of fraction IV prior to acid hydrolysis (Holmquist & Vallee, 1973) and by amino acid analysis after hydrolysis with *p*-toluenesulfonic acid. Since these peptides contain only one tyrosyl residue, the predominant site of azo modification must be at Tyr-248. Fractions V and VI, which together accounted for little more than 10% of the tyrosyl absorbance, were not homogeneous on thin-layer chromatography and their amino acid compositions could not be assigned to unique peptides. No attempt was made to assign them to any particular segment of the sequence.

High-Pressure Liquid Chromatography of Azotyrosyl Peptides. The azotyrosyl peptide fraction obtained by affinity chromatography (Figure 3A) was also fractionated by high pressure liquid chromatography on a micro-Bondapak C-18 column using an acetate buffer (pH 4.0)–acetonitrile gradient (Figure 5). Four effluent regions were subjected to further analysis. Rechromatography of the peak at 100 mL (A) gave one major component whose amino acid composition and N terminus indicated that it derives from that segment of the sequence containing residues 207 through 211. It likely corresponds to fraction I from ion-exchange chromatography (Figure 4). The fractions eluting at 105 (B) and 110 mL (C) could also be resolved further by rechromatography, and these were found to contain residues 246 through 252 and 247 through 253, respectively, and, as indicated previously, correspond to fraction III from AG1-X2 chromatography (Figure 4). The fraction emerging at 150 mL (D) was essentially pure and was found to correspond to residues 247–250, analogous to fraction II described above. It was not possible to make a

TABLE III: Amino Acid Composition of Nitrotyrosyl Peptides Obtained from Nitroazocarboxypeptidase by Paper Electrophoresis.

Amino acid	1	(162–165)[a]	2	(197–198)[a]	3	(193–198)[a]	4	(194–198)[a]	5	(196–198)[a]
Thr	0.9	1								
Ser	0.8	1	0.8	1	2.0	2	1.9	2	0.9	1
Glu	0.8	1								
Ile					0.9	1	0.9	1		
Leu					0.9	1			0.5	
His					1.0	1	0.9	1	1.0	1
Lys									0.5	
Tyr		1		1		1		1		1
NO_2Tyr	1.0		1.0		1.0		1.0		1.0	
Rel yield (%)	17		28		14		26		16	

[a] Bradshaw et al. (1969a).

TABLE IV: Amino Acid Composition of Nitrotyrosyl Peptides Obtained from Nitroazocarboxypeptidase by Ion-Exchange Chromatography.

Amino acid	ND-1	(194–198)[a]	ND-2	ND-3	(270–277)[a]	ND-4	(246–248)[a]	ND-5	(197–198)[a]	ND-6	(193–198)[a]	ND-7	ND-8	(247–248)[a]
Asp				1.2	1									
Thr				0.9	1	0.8	1							
Ser	1.8	2						0.7	1	1.7	2			
Glu				1.1	1									
Gly				1.3								0.7		
Ile	1.1	1				0.7	1			1.0	1		0.9	1
Leu				1.0	1					0.9	1			
His	0.8	1								0.9	1	1.5		
Arg				2.2	2									
Tyr		1			1		1		1		1			1
NO_2Tyr	1.0		1.0	1.0		1.0		1.0		1.0		1.0	1.0	
Rel yield (%)	23		5	12		4		25		17		5	9	

[a] Bradshaw et al. (1969a).

definitive assignment for the peak at 195 mL (E). Its amino acid composition was suggestive of the region of carboxypeptidase containing Tyr-277 but there may have been contamination with some other material as well. This fraction did not contain histidine, however, and therefore could not derive from the region around Tyr-198.

About half of the 340-nm absorbance elutes from the high-pressure liquid chromatograph at 270 mL. Amino acid analysis of this fraction reveals the presence of two peptides, one containing the 12 residues from Thr-246 through Trp-257 and the other extending from Ile-247 through Trp-257. Thus, this fraction is analogous to peak IV from AG1-X2 chromatography which also represented about 50% of the total azotyrosyl absorption eluting from that column.

Separation of Nitrotyrosyl Peptides. The nitrotyrosyl peptide fraction obtained by affinity chromatography (Figure 3B) was purified further by gel filtration on a 250-mL column of Sephadex G-25. Approximately 35% of the A_{430} material emerged as a single peak near the front and the remaining 65% as a low molecular weight fraction close to the included volume. High voltage paper electrophoresis was employed to attempt separation of the nitrotyrosyl peptides in both fractions. No separation was obtained with the first fraction due to heavy streaking but the second fraction gave five distinct bands. These were eluted and analyzed for amino acid composition (Table III). The first band accounts for 17% of the total number of micromoles of nitrotyrosine eluting from the electropherogram. Its amino acid composition and N-terminus are most consistent with that segment of the sequence of carboxypeptidase containing residues 162–165 indicating that in azocarboxypeptidase Tyr-165 is one of the tyrosyl residues that is nitrated by tetranitromethane. The second band contained the dipeptide Ser-NO_2Tyr which likely (vide infra) represents residues 197 and 198 but could arise from identical sequences at positions 41–42, 237–238, and 258–259. The yield of this band was 28%. Band three had a composition uniquely consistent with the sequence from Leu-193 to Tyr-198 and band four had one less residue and was thus Ser-194 to Tyr-198. The yields of these two peptides were 14 and 26% respectively or a total of 40% definitely attributable to the region encompassing Tyr-198. Band five could not be identified as a single specific peptide based on amino acid composition. The predominant constituents were Ser and His in addition to nitrotyrosine suggesting that it mostly represents the sequence from His-196 to Tyr-198. However, the presence of smaller quantities of Lys and Gly indicates that this band might also contain a minor amount of peptide arising from the sequence His-166 to Tyr-169. The portion of this band attributable to the Tyr-198 peptides is estimated to be 70%. Since band five in toto accounts for 16% of the nitrotyrosyl peptides separated by paper electrophoreis, the combined yield of Tyr-198 peptides would be about 78%, assuming that the dipeptide Ser-NO_2Tyr also derives from this region.

A second sample of the nitrotyrosyl peptide fraction from affinity chromatography was subjected to purification by ion-exchange chromatography on a 0.9 × 25 cm column of DEAE-cellulose (DE-32) equilibrated with 0.005 M NH_4HCO_3. Peptides were eluted with a 600-mL linear gradient from 0.01 to 0.5 M NH_4HCO_3 and four main fractions absorbing at 430 nm were collected. Each of these was pooled

and further purified by paper electrophoresis as previously described. The amino acid compositions of the resulting electrophoretic bands are listed in Table IV. The first chromatographic peak yields a single electrophoretic band, ND-1, whose amino acid composition, Ser, Ile, His, and NO_2Tyr, corresponds to the sequence from Ile-195 to Tyr-198. The second chromatographic peak gives two bands, one of which is nitrotyrosine, ND-2, and the other, ND-3, is characterized by a high Arg content and clearly corresponds to the sequence from Glu-270 to Tyr-277. Peak three from DEAE-cellulose separates into four yellow bands when subjected to paper electrophoresis. The first of these, ND-4, has a composition corresponding to the sequence Thr-Ile-NO_2Tyr and can only arise from nitration of Tyr-248. The very low yield of this peptide is consistent with the fact that, in this enzyme preparation, Tyr-248 is largely blocked with the diazo reagent. A second band ND-5 is the dipeptide Ser-NO_2Tyr which, as indicated previously, most likely derives from Tyr-198. The third electrophoretic component of this chromatographic fraction, ND-6, has a composition uniquely compatible with the sequence from Leu-193 to Tyr-198, and the last band, ND-7, contains Gly, His, and NO_2Tyr but could not be associated with any known sequence of the enzyme. Electrophoresis of peak 4 from DEAE-cellulose resulted in one major band, ND-8, containing equal amounts of Ile and NO_2-Tyr corresponding to residues 247–248. The relative yields of all of these peptides, Table IV, indicates that the residue nitrated predominantly is Tyr-198.

Discussion

These results demonstrate a novel consequence of chemically modifying an enzyme: azo coupling of one tyrosyl residue of carboxypeptidase, Tyr-248, induces another, Tyr-198, to undergo a transformation in its chemical reactivity. Tyr-198, which is virtually unreactive in the native enzyme, becomes susceptible to nitration only after Tyr-248 has been modified with 5-diazo-1*H*-tetrazole. This change in chemical properties would seem to reflect different microenvironments for Tyr-198 in the native and azoenzymes, i.e., a change in protein conformation. Thus, this unsual phenomenon illustrates an important feature of enzyme modification. Chemical approaches to the study of enzymes are employed widely to identify amino acid side chains that participate in the catalytic process (Vallee & Riordan, 1969). In general, loss of activity accompanying modification of a particular residue is taken as evidence that the residue is an essential constituent of the enzyme's active site. However, depending on the role of the residue in catalysis, modification could affect activity adversely, favorably, or not at all. Moreover, it is often assumed that the modification does nothing more than alter the physiochemical properties of the residue in question and does not disturb protein structure. In fact, these studies of carboxypeptidase indicate that a critical effect of azo coupling of Tyr-248 with 5-diazo-1*H*-tetrazole is the perturbation of the environment of Tyr-198. Thus, in this case, modification of a residue in one part of the molecule exerts an effect through a conformational change in another part. The basis for these conclusions will be considered in the following discussion.

It is clear from the data presented that coupling of carboxypeptidase with diazo-1*H*-tetrazole occurs predominantly at Tyr-248. This finding is consistent with previous studies using diazoarsanilic acid (Johansen et al., 1972; Wilchek et al., 1971) and with other evidence that Tyr-248 has unique reactivity toward chemical modification (Roholt & Pressman, 1967; Muszynska & Riordan, 1976). On the other hand, nitration of azocarboxypeptidase seems to occur largely at Tyr-198. This conclusion is based on the recovery of two peptides, Ser-194 to Tyr-198 and Leu-193 to Tyr-198, in a total yield of 40%, the recovery of another peptide, which is most probably His-196 to Tyr-198, in about 11% yield, and the recovery of the dipeptide, Ser-NO_2Tyr, in 28% yield. It seems likely that the last mentioned dipeptide represents Ser-197 and Tyr-198. Other choices are residues 41–42, 237–238, and 258–259. However, the first of these would be located in F_{III}, the cyanogen bromide cleavage fragment encompassing residues 23–103, which was shown not to contain any nitrotyrosine, and the second contains the least accessible tyrosyl residue in the entire molecule (Quiocho & Lipscomb, 1971).

Thus, only Tyr-259 remains a possible alternative though in all the studies on tyrosine modification of carboxypeptidase a peptide containing this residue has never been identified. Moreover, it, too, is relatively inaccessible and it is thus most probable that the peptide is Ser-197-Tyr-198.

The suitability of diazonium salts for incorporating chromophoric groups into proteins is well established (Pauly, 1904; Tabachnik & Sobotka, 1959, 1690). Most of these reagents are relatively nonspecific (Riordan & Vallee, 1972) but diazonium-1*H*-tetrazole, when first described, was thought to be selective for histidyl residues in proteins (Horinishi et al., 1964). Subsequently it was found to have the same broad reactivity as other diazonium compounds (Andres & Atassi, 1973) but nevertheless it has proven useful for examining the accessibility of tyrosyl and histidyl residues in proteins (Sokolovsky & Vallee, 1966) as well as for specifically modifying functional residues in enzymes (Sokolovsky & Vallee, 1967; Sogin & Plapp, 1976). It has the advantage that it cannot undergo self-condensation thus facilitating the spectral quantitation of its reaction stoichiometry. In addition, the nitrogen atom in the 1 (4) position can serve as a ligand to stabilize metal–azophenolate complexes. It was of interest, of course, that diazonium-1*H*-tetrazole modifies a single tyrosyl residue in carboxypeptidase, alters its esterase but not peptidase activity, and does not prevent its subsequent nitration with tetranitromethane (Sokolovsky & Vallee, 1967; Riordan et al., 1967). This peculiar set of circumstances seems to be related to features of the active site of carboxypeptidase and their delineation could shed light on its mechanism of action.

The reactivity of amino acid side chains toward diazonium compounds and other reagents depends markedly upon their immediate chemical environment in the protein (Vallee & Riordan, 1969). In particular, active site residues are frequently far more susceptible to modification than the same kind of residue elsewhere in the protein or in a model compound. Tyr-248 in carboxypeptidase is such a residue. Under a range of conditions it behaves differently from all other tyrosyl residues in the enzyme. It reacts preferentially both with diazotized arsanilic acid (Johansen et al., 1972) and with tetranitromethane (Muszynska & Riordan, 1976) and probably with iodine and acylating agents as well (Vallee & Riordan, 1968). Since these reagents are chemically quite dissimilar it is likely that the unusual reactivity is characteristic of the residue itself. In the crystalline enzyme, Tyr-248 is at the surface of the protein with its hydroxyl group in contact with the ambient environment. But at least 15 of the remaining 18 tyrosyl hydroxyls are also accessible to solvent (Quiocho & Lipscomb, 1971). Only the hydroxyl group of Tyr-238 and, to a much lesser extent, those of Tyr-12 and -259 are not on the exterior of the molecule. Ortho substitution might be sterically hindered for perhaps 4 or 5 of the exterior tyrosyl residues and yet under the conditions employed for azo coupling only one is substituted to any appreciable extent. The only polar group close to the phenolic hydroxyl of Tyr-248 in the model of

Lipscomb et al. (1968) is the peptide NH of Gly-155. It seems unlikely that an interaction with this group would serve to activate Tyr-248. A more plausible explanation would be the proximity of a positively charged group, either the active site zinc ion or a lysyl or arginyl residue, perhaps different in the crystal and solution states, that could promote dissociation to the phenolate species. The diazonium ion is not a strong electrophile and requires considerable assistance in order to attack an aromatic ring carbon atom. The phenolate group could provide such assistance. This view has been proposed to account for the selective nitration of Tyr-248 (Muszynska & Riordan, 1976) and for the unusual ionization behavior of nitrotyrosyl-248 (Riordan & Muszynska, 1974).

Another explanation for the selective modification of Tyr-248 with diazonium-1*H*-tetrazole is based on the fact that at neutral pH the tetrazole ring carries a negative charge (White & Legg, 1976). This would not only enhance the reactivity of the diazonium salt (Luisada-Opper & Sobotka, 1963) but also permit electrostatic interaction with the positive charge of, e.g., Arg-145 at the active site and thereby direct the reagent to Tyr-248. A similar situation has been postulated for diazotized arsanilic acid (Johnsen et al., 1972). In either case the predominant factor determining selective modification is the environment of the reactive residue and, thus, it should not be surprising that diazonium-1*H*-tetrazole modifies Tyr-248.

Although a role for Tyr-248 in catalysis has not been established, its location and reactivity have attracted attention and generated considerable speculation. It has been cited as a proton donor essential for peptide hydrolysis (Lipscomb et al., 1968) but it can be coupled with two different diazonium salts with only minor effects on enzymatic activity. Moreover, nitration lowers its pK to approximately 6.3 (Riordan & Muszynska, 1974) considerably less than that of an unmodified tyrosyl residue, and, yet, depending on the substrate, the nitroenzyme can exhibit from 15 to 90% of the peptidase activity of the native enzyme (D. S. Auld, unpublished observations). Clearly, the integrity of Tyr-248 is not essential for catalysis. Nevertheless, derivatives of the enzyme, in which this residue is known to be modified, generally exhibit some change in either esterase or peptidase activity, or both. The changes have not been related to the pK of the modified residue and they differ for different substrates, particularly in the case of peptides versus esters.

The most drastic changes in carboxypeptidase activity occur on acetylation or iodination. In both instances peptidase activity is virtually abolished but since neither reaction is selective, these changes cannot be attributed to modification of Tyr-248 alone. In fact, the present findings clearly demonstrate that while the activity of carboxypeptidase can be altered by modification of Tyr-248 it undergoes additional alteration by the subsequent modification of Tyr-198. It has not been established which tyrosyl residues are modified by acetylation but competition experiments strongly imply that Tyr-248 is one of them. Prior acetylation prevents nitration, iodination, and coupling with either diazotized arsanilic acid or diazo-1*H*-tetrazole, reactions that are all known to occur with Tyr-248. Moreover, acetylation of tetrazolylazocarboxypeptidase (Sokolovsky & Vallee, 1967) increases esterase and decreases peptidase activities to values characteristic of acetylcarboxypeptidase. Since Tyr-198 is quite susceptible to modification with tetranitromethane (providing Tyr-248 has been coupled with azotetrazole), it seems likely that it would also be susceptible to acetylation under these circumstances. Hence, the changes in activity observed by Sokolovsky & Vallee (1967) on acetylation of tetrazolylazocarboxypeptidase could be due to acetylation of Tyr-198. This would also imply that acetylcarboxypeptidase is modified at Tyr-198 because of its similar enzymatic activities. Moreover, it is known that the inhibitor, β-phenylpropionate, protects two tyrosines from acetylation and the only two tyrosyl residues in the vicinity of the active site are Tyr-248 and -198. In fact, the present studies show (Table I) that β-phenylpropionate protects against nitration of Tyr-198. It seems likely that modification of either tyrosyl residue would alter activity in some way, but thus far it has not been possible to modify Tyr-198 without first modifying Tyr-248.

What accounts for this unusual change in reactivity of Tyr-198? It does not appear to be readily susceptible to modification in the native enzyme. In their studies with carboxypeptidase crystals, Johansen et al. (1972) found that approximately 95% of the incorporation of diazotized arsanilic acid occurred at Tyr-248 with the rest probably at Tyr-19. Under somewhat different conditions and using the enzyme in solution, Wilcheck et al. (1971) found that Tyr-277 was modified as well. Muszynska & Riordan (1976) demonstrated that Tyr-248, -169, -240, and -277 could be nitrated with tetranitromethane, and in the present study coupling with diazotized 5-amino-1*H*-tetrazole occurs mostly at Tyr-248 but to some extent at Tyr-207 and probably -277. Iodination of carboxypeptidase crystals at least partially modifies Tyr-19, -42, -234, -238, -248, and -277 (unpublished results of F. A. Quiocho, 1967, cited in Quiocho & Lipscomb, 1971). In none of these instances, however, has evidence been found for any significant modification of Tyr-198 (or Tyr-259, vide supra). The only direct evidence for modification of Tyr-198 is that described here on nitration of tetrazolylazocarboxypeptidase. Presumably the presence of the azo group on Tyr-248 potentiates the reactivity of Tyr-198.

Inspection of the three-dimensional model of carboxypeptidase constructed according to the coordinates tabulated by Quiocho & Lipscomb (1971) leads to a plausible rationale. The enhanced reactivity of Tyr-248 in the crystalline state is likely due to its proximity to a positive charge. In fact, the phenolic hydroxyl group is able to come very close to the guanidino group of Arg-145 by rotation about its C_α–C_β bond. In solution, where the conformation of the enzyme is somewhat different (Johansen & Vallee, 1971, 1973), the hydroxyl group may be closer to the zinc ion. In either state the positive charge can lead to enhanced reactivity. On the other hand, Tyr-198 is located in a hydrophobic pocket in the native enzyme. It is shielded from the closest positive charge, Arg-71, by the aromatic ring of Phe-279. Although relatively accessible to reagents such as tetranitromethane and diazo-1*H*-tetrazole, it does not react because it is probably un-ionized.

Coupling of Tyr-248 with diazo-1*H*-tetrazole introduces a bulky substituent that could bring about a specific conformational change. Unlike arsanilazotyrosine-248, the tetrazolyl derivative does not appear to form an azophenol metal complex with the active site zinc ion. The enzyme solution is yellow rather than red at pH 8.0 and there is no circular dichroic extremum centered at 510 nm (Cueni, 1974). Instead, it appears that steric crowding by the tetrazolyl group could cause Arg-71 to move slightly toward Phe-279 (Figure 6). The latter would then move out of this now unfavorable environment by rotating about its C_α–C_β bond allowing Tyr-198 to move closer to Arg-71. Proximity to the positive charge of the guanidino group would promote ionization of Tyr-198 and enable it to be nitrated.

It should be noted that similar attempts at consecutive chemical modification have been carried out with arsanilazocarboxypeptidase (Kagan & Vallee, 1969). Nitration of the azoenzyme had essentially no further effect on either esterase

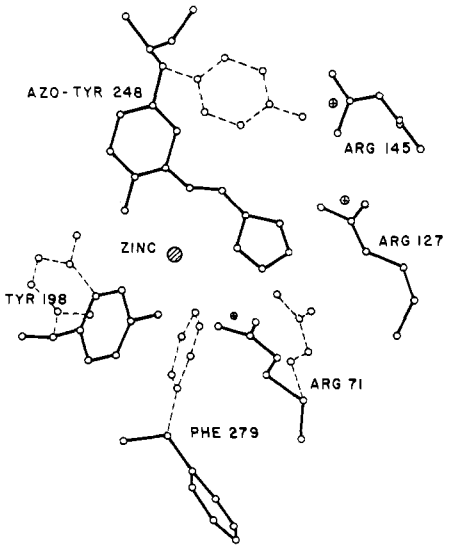

FIGURE 6: Schematic representation of residues at the active center of azocarboxypeptidase. The dashed symbols refer to the relative positions, prior to azo coupling, of those residue side chains thought to undergo a change in conformation. This scheme is from an Ortep plot of selected residues of carboxypeptidase constructed with atomic coordinates of Lipscomb supplied by the Brookhaven National Laboratory Protein Data Bank.

or peptidase activity and only a small fraction of a nitrotyrosyl residue could be detected in the product. The fact that the arsanilazotyrsoyl residue forms an intramolecular coordination complex with the active site zinc ion, whereas the tetrazolylazotyrosyl one does not, strongly suggests that the particular location of the azo group is critical for potentiating the reactivity of Tyr-198.

Model building studies have implicated the region around Tyr-198 and Phe-279 as important for binding of longer substrates in both a productive mode and in modes responsible for kinetic anomalies (Hartsuck & Lipscomb, 1971). Hence, modification of this residue would be expected to result in alterations of enzymatic activity. In particular, peptidase activity might decrease owing to impaired substrate binding while esterase activity could appear to increase by relief of substrate inhibition. Kinetic analyses of the doubly modified enzyme are consistent with these views (D. S. Auld, unpublished results).

In summary, we have described a unique example of the influence of protein structure on the reactivity of functional amino acid residues. A conformational change induced as a consequence of the chemical modification of one residue generates a microenvironment conducive to the modification of a second. This could be a rather fortuitous circumstance peculiar to carboxypeptidase and might not have been recognized except for the effect on enzymatic activity. However, because of these activity changes, the possibility must be considered that substrate binding, particularly substrates longer than Gly-Tyr, can also perturb Phe-279 in a similar way and that the subsequent effect on Tyr-198 may, in fact, be important for the catalytic process.

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Superoxide Dismutase, a Study of the Electronic Properties of the Copper and Zinc by X-Ray Absorption Spectroscopy†

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ABSTRACT: The x-ray absorption for copper and zinc in oxidized and reduced superoxide dismutase, as well as in various model compounds, was studied. Upon reduction of the protein, the added electron affects the copper site almost exclusively, while the zinc remains virtually unchanged. Reduction decreases the charge on the copper atom [toward Cu(I)] and changes the configuration of the copper site so that it becomes less symmetric. An analysis of the copper absorption observed with the oxidized enzyme and a comparison with that for $Cu(II)(imid)_4$ suggests that the copper is not simply ligated to four imidazoles. The addition of H_2O_2 to superoxide dismutase reduces the copper to Cu(I), while oxygen addition to the peroxide-reduced protein restores the copper to Cu(II).

A number of proteins from both aerobic (Fridovich, 1975) as well as strictly anaerobic organisms (Hewitt and Morriss, 1975; Asada et al., 1976; Hatchikian and Henry, 1977) have been found to effect the decomposition of superoxide, a partially reduced form of dioxygen. This catalytic process consists of the oxidation of the oxygen atoms of one molecule of superoxide to the oxidation level of dioxygen and the reduction of the oxygen atoms of another molecule to the oxidation level of hydrogen peroxide. All these enzymes contain one or more metal atoms at the active site (Keele et al., 1970), either manganese, iron (Yost and Fridovich, 1973) or copper and zinc (McCord and Fridovich, 1969).

Higher organisms utilize the copper-containing enzyme. This protein also contains zinc in equal amounts to the copper (Carrico and Deutsch, 1970). X-ray crystallographic analysis (Richardson et al., 1975a,b) has shown that bovine superoxide dismutase takes the form of a dimeric polypeptide with two active sites, each site containing a copper atom surrounded by four imidazole ligands, one of which is also ligated to a zinc atom.

At a resolution of 2.8 Å, the electron-density map suggests that each copper atom is coordinated to histidine-44, -46, -61, and -118, as deduced from the protein's sequence (Steinman et al., 1974). The imidazole from histidine-61 binds both to the copper atom and to the zinc atom as well. This close proximity of metals is consistent with spectral and magnetic studies (Fee, 1973; Rotilio et al., 1974; Moss and Fee, 1976). The zinc atom is further coordinated to histidine-69 and -78 and aspartate-81. Individual copper–zinc pairs are separated from one another by 34 Å.

In the isolated protein, the copper atom is divalent and exhibits an optical absorption near 680 nm which is characteristic of the cupric chromophore (Mann and Keilen, 1939; Carrico and Deutsch, 1969). The copper atom also exhibits an EPR[1] spectrum which is indicative of a small departure from axial symmetry (Rotilio et al., 1971; Fee and Gaber, 1972) having a rhombicity of 21% as defined by Blumberg and Peisach (1974).

During enzymatic turnover, the intensity of the 680-nm absorption changes at rates which are consistent with overall catalytic efficiency, thus implicating the metal sites in an oxidation–reduction cycle (Fielden et al., 1973). It is not known whether both metals change their valence during catalysis, although because of the inherent stability of divalent zinc (Cotton and Wilkinson, 1962) it is attractive to hypothesize that only the copper changes oxidation state. Even here, however, it is not known with certainty whether the copper shuttles between di- and trivalent oxidation states or between di- and monovalent states during actual catalysis, although the latter case is quite generally assumed (for example, Fee and Ward, 1976). It should be noted, however, that Cu(III) can be stabilized by peptide ligands (Margerum et al., 1975), and, furthermore, trivalent copper has been proposed to form during the catalytic action of galactose oxidase (Hamilton et al., 1973), another copper-containing enzyme.

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[1] Abbreviations used: EPR, electron paramagnetic resonance; LEFE, linear electric field effect; EDTA, (ethylenedinitrilo)tetraacetic acid.

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