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Environment and Conformation Dependent Sensitivity of the Arsanilazotyrosine-248 Carboxypeptidase A Chromophore†

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ABSTRACT: Reaction of carboxypeptidase A crystals with diazotized arsanilic acid uniquely modifies Tyr-248 to form a monazo derivative, which—in solution—forms an intramolecular inner-sphere coordination complex with the active site zinc atom. Arsanilazocarboxypeptidase exhibits spectral properties that are closely similar to those of the model complex, tetrazolylazo-*N*-carbobenzoxytyrosine Zn^{2+}, with a distinctive maximum at 510 nm. In addition, its circular dichroic spectrum reveals a negative extremum at this wavelength, also characteristic of this complex. Both spectra are exquisitely responsive to pH changes and serve to monitor formation and dissociation of the metal–azophenol complex. Two pK_{app} at 7.7 and 9.5 delineate the pH range over which the probe characteristics most effectively gauge conformational features of the active center of arsanilazocarboxypeptidase. Other environmental parameters, *e.g.*, substrates and inhibitors, as well as crystallization of the enzyme also critically influence the formation and dissociation of the complex; the response of the probe suggests that they induce conformational movement of the azoTyr-248 residue away from the zinc atom. The now available chemical, functional, and structural data bearing on the spatial relationships of Tyr-248 and Zn, both thought critical to catalysis, are evaluated, based on spectra of arsanilazo- and nitrocarboxypeptidase crystals and solutions as well as on detailed kinetic analyses of the native enzyme in both physical states and based on the X-ray structure analysis of the native enzyme and its Gly-L-Tyr complex. Collectively all of the data show that the conformation of carboxypeptidase in crystals differs from that in solution. Moreover, reexamination of the original X-ray maps reported in 1968 and thought to preclude a Tyr-248 · Zn interaction now leads to the conclusion that in up to 25% of the molecules in the crystals Tyr-248 interacts with the active site zinc atom (W. D. Lipscomb (1973), *Proc. Nat. Acad. Sci. U.S. 70,* 3797). Thus, even in the crystals the enzyme exists in at least two different conformations. In one of these Tyr-248 is near while in the other it is far from the zinc atom. The spectral effects of Gly-L-Tyr and β-phenylpropionate on solutions of arsanilazo- and of nitrocarboxypeptidase demonstrate that during the catalytic process Tyr-248 moves *away* from the zinc atom. This implies a mechanistic role for Tyr-248 different from that postulated on the basis of X-ray crystallographic analysis. Indeed, the proximity of Tyr-248 to the zinc atom, when altered by substrates and inhibitors, may reflect certain of the properties characteristic of the entatic, active site.

Chemical modification of carboxypeptidase A with diazotized arsanilic acid has successfully served a number of experimental objectives. In crystalline carboxypeptidase this reagent couples selectively with Tyr-248, a residue in the active site of the enzyme. The resultant product retains both peptidase and esterase activity (Johansen and Vallee, 1971, 1973; Auld and Holmquist, 1973). Further, absorption and circular dichroic spectra of such azochromophores have proven remarkably effective in probing the local and overall conformation of carboxypeptidase A (Vallee *et al.*, 1971), procarboxypeptidase (Behnke and Vallee, 1971), and carboxypeptidase S, the product of proteolytic cleavage with subtilisin (Riordan and Livingston, 1971). The absorption maxima between 330 and 390 nm and that at 485 nm are characteristic of the protonated and ionized azoTyr-248 species, respectively, and the corresponding circular dichroic bands reflect local asymmetry. But most importantly, the yellow arsanilazotyrosine-248 moiety of carboxypeptidase forms a characteristic red chromophore with an absorption maximum at 510 nm by complexing with the zinc atom present at the active site of the enzyme. In solutions of zinc arsanilazotyrosine-248 carboxypeptidase[1] the azophen-

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[1] In order to simplify presentation, zinc carboxypeptidase, zinc arsanilazocarboxypeptidase, the zinc azoenzyme and apocarboxypeptidase,

ol ligand and zinc are suitably juxtaposed to allow formation of an intramolecular azophenol · Zn coordination complex which is optically active due to vicinal features and/or inherent asymmetry of the complex. However, removal of zinc or changing the mutual orientation of or distance between the zinc atom and the azoTyr-248 residue, *e.g.*, by crystallizing the enzyme, abolishes the spectral manifestations of the complex, which probes the vicinity of its constituents in an "all or none" manner (Johansen and Vallee, 1971, 1973). This intramolecular coordination complex also responds to environmental factors, such as hydrogen ion concentration, substrates and inhibitors, denaturing agents, and the physical state of the enzyme which affect its conformation-dependent functional properties. The chromophore, moreover, is well suited to studies of steady state and transient state catalysis and to the examination of the effects of spectral perturbants such as substrates, pseudosubstrates, and inhibitors both by kinetic and equilibrium methods (Johansen and Vallee, 1971).

The properties of this intramolecular azoTyr-248 · Zn carboxypeptidase coordination complex are closely similar to those of azophenol · metal complex ions which serve as models and define the chemical basis of the spectral changes. Azophenols have not been used widely as reagents for the study of structure–function relationships in enzymes, and the chemical basis of their attractive spectral properties has not been discussed frequently in the biochemical literature. However, 2 decades ago Klotz employed ternary azopyridine–metal–protein complexes as model systems for metal-catalyzed enzymatic reactions (Klotz and Ming, 1953; Hughes and Klotz, 1956), and his earlier conclusions regarding the properties of azophenol · metal complex ions are quite analogous to those reached based on the observations to be presented.

We here report the pH dependence of spectra of zinc azocarboxypeptidase, apoazocarboxypeptidase, and of two azophenols, arsanilazo-*N*-acetyltyrosineamide and tetrazolylazo-*N*-carbobenzoxytyrosine (TAT) and its complexes with Zn^{2+}, Cd^{2+}, Hg^{2+}, and Mn^{2+}. In addition, circular dichroic and absorption titrations of the zinc azoenzyme with glycyl-L-tyrosine and β-phenylpropionate demonstrate the probe characteristics of the azochromophore. The data suggest that the spectra of azophenol · metal complex ions and intramolecular complexes can be effective probes to examine local structure–function relationships of metalloenzymes.

Materials and Methods

Carboxypeptidase A_γ, isolated by the method of Anson (1937), was obtained as an aqueous crystal suspension (Worthington Biochemical Corporation). Carboxypeptidase A_α was isolated by DEAE-cellulose chromatography from acetone powder of bovine pancreas according to the method of Cox *et al.* (1964) or, alternately, was obtained as a crystal suspension (Sigma Chemical Company). In all instances the crystals were washed three times with metal-free distilled water and recrystallized before use.

Zinc arsanilazocarboxypeptidases were prepared and characterized as described (Johansen and Vallee, 1971, 1973; Johansen *et al.*, 1972).

Apoarsanilazocarboxypeptidase was prepared by soaking crystals in 1,10-phenanthroline (Auld and Holmquist, 1974).

Protein concentration was measured by the absorbance at 278 nm, based on a molar absorptivity at 278 nm of 6.42×10^4 M^{-1} cm^{-1} (Simpson *et al.*, 1963) for native carboxypeptidase, and 7.32×10^4 M^{-1} cm^{-1} for zinc arsanilazocarboxypeptidase (Johansen and Vallee, 1971).

Monotetrazolylazo-*N*-carbobenzoxytyrosine was prepared and purified as described by Sokolovsky and Vallee (1966). Its concentration was determined from the absorbance at 416 nm, using a molar absorptivity of 4.39×10^3 M^{-1} cm^{-1}. β-Phenylpropionate was recrystallized from water. Glycyl-L-tyrosine and guanidinium hydrochloride were purchased from Mann Research Laboratories, Inc., $ZnSO_4 \cdot 5H_2O$, $CdSO_4 \cdot 8H_2O$, $MnSO_4 \cdot 5H_2O$, and $HgCl_2$ were "specpure" reagents from Johnson Matthey Co., Ltd. Stock solutions of each salt in metal-free distilled water were made up to a concentration of 10^{-3} M. All other chemicals were reagent grade.

All buffers used were freed of trace metal contamination by extraction with 0.1% dithizone in carbon tetrachloride (Thiers, 1957). Glassware and cuvets were cleaned by soaking in 1:1 nitric and sulfuric acids, followed by rinsing in metal-free distilled water.

Absorbance measurements at single wavelengths were obtained with a Zeiss PMQ II spectrophotometer. Absorption spectra were obtained with a Cary Model 14R or Model 118C spectrophotometer. For spectrophotometric pH titrations the thermostated Auld–French titration cell was utilized (Auld and French, 1970). The zinc- and apoarsanilazoenzyme, about 0.06 mM in 2 mM Tris-HCl–0.5 M NaCl buffer (pH 6.2) were titrated with aliquots of 0.1 M NaOH to result in pH increments of 0.2–0.4. The absorption spectrum between 300 and 650 nm was recorded after each addition of base. Spectral titrations with glycyl-L-tyrosine and β-phenylpropionate were performed at pH 8.5 (0.05 M Tris-HCl–0.5 M NaCl) by adding in a cuvet, microliter volumes of 0.1 M solution of the inhibitor or the substrate in the same buffer to 1.0-ml solution of the enzyme, 0.05 mM. Results were corrected for enzyme dilutions due to inhibitor or substrate addition. Spectrophotometric pH titration of TAT and of its metal ion complexes were performed as described for the enzyme. The TAT and metal ion concentrations were identical, *i.e.*, 0.09 mM in the presence of 0.1 M NaCl.

Circular dichroism measurements were performed with a Cary Model 61 recording spectropolarimeter. All measurements between 300 and 600 nm were performed in 1-ml quartz cells of 1.0-cm light path at enzyme concentrations ranging from 0.03 to 0.05 mM. Ellipticity is expressed as molecular ellipticity, $[\theta]^{25} = (\theta/10)(M/lc)$ with units of (deg cm^2)/dmol, where θ = observed ellipticity in degrees; M = molecular weight, (34,600 for carboxypeptidase A), l = path length in cm, and c = concentration in g/ml. In accord with convention (Fairclough and Vallee, 1970), molecular ellipticities are not corrected for the refractive index of the solvent. The pH dependence of the circular dichroic spectra of zinc and apoazocarboxypeptidase was deter-

apoazocarboxypeptidase, and apoarsanilazocarboxypeptidase are used interchangeably with zinc monoarsanilazotyrosine-248 carboxypeptidase and apoarsanilazotyrosine-248 carboxypeptidase, respectively, of any enzyme form. Carboxypeptidase A is referred to as native carboxypeptidase. The enzyme employed for X-ray structure analysis with a crystal habit elongated along the *a* axis is designated as the X-ray crystals. AzoTyr-248 refers to the *azophenol* of monoarsanilazotyrosine-248 and the azophenolate to its ionized species. The absorption and circular dichroic spectra with an absorption maximum and a negative circular extremum at 510 nm, respectively, are defined as the "red" intramolecular complex. At pH 8.5 and in the absence of the absorption band at 510 nm the spectrum is defined as "yellow." TAT, tetrazolylazo-*N*-carbobenzoxytyrosine; DHT, diazonium-1*H*-tetrazole; TNM, tetranitromethane.

mined from pH 6.5 to 10.8 by stepwise addition of 0.1 N NaOH to 3-ml samples. The pH was measured before and after each spectrum was recorded. Circular dichroic titrations with glycyl-L-tyrosine and β-phenylpropionate were performed as described for the absorbance titrations. Theoretical pH-titration curves were fitted to the experimental data by use of a nonlinear least-squares program written for the Hewlett Packard 9810A calculator, and kindly provided by Dr. Thayer French.

Sedimentation was carried out at 59,780 rpm in a Spinco Model E ultracentrifuge. Sedimentation coefficients were corrected to viscosity and density of water at 20°. Native and arsanilazocarboxypeptidase, 3.5 and 4.6 mg/ml, respectively, in 0.05 M Tris–1.0 M NaCl (pH 7.5), and arsanilazocarboxypeptidase, 12.6 mg/ml in 0.1 M β-phenylpropionate–0.02 M Veronal–1.0 M NaCl (pH 7.5), were subjected to sedimentation.

Results

Reaction of carboxypeptidase A crystals with diazotized *p*-arsanilic acid uniquely modifies Tyr-248, as demonstrated by the presence of 95% of the label in CNBr fragment F I and by isolation, in 80% yield, of the duodecaptide corresponding to residues 246–257 of the primary sequence (Bradshaw *et al.*, 1969; Johansen *et al.*, 1972). In solution, at pH 8.2, this zinc azoenzyme is red. The peptidase activity at pH 7.5, 0.05 M Tris–1.0 M NaCl of this derivative is 60% and the esterase activity at pH 7.5, 1.0 M NaCl, is 100% of that of the native enzyme, when Bz-Gly-Gly-L-Phe and Bz-Gly-Gly-L-*O*-Phe are the substrates, respectively. The absorption spectrum of the zinc azoenzyme has a maximum at 510 nm (Figure 1A), characteristic of the formation of an intramolecular coordination complex between the azoTyr-248 moiety and zinc. Like other coordination complexes, the formation, stability, and dissociation of this azophenol · metal complex and its spectra are sensitive to environmental factors, such as pH, substrates and inhibitors, the physical state of the enzyme, as well as any other conditions which can affect the ionization of the azophenol, modulate the conformation of the protein, or remove zinc.

In the range of pH from 6.3 to 8.5 absorbance–pH titrations of zinc arsanilazoTyr-248 carboxypeptidase generate an absorption maximum at 510 nm (ϵ 8000)[2] with an isosbestic point at 428 nm. On increasing pH to 10.8 the maximum shifts progressively to 485 nm (ϵ 10,500), characteristic of the free azophenolate ion, and two new isosbestic points become apparent at 412 and 520 nm (Figure 1A). The shift in λ_{max} and the formation of a new set of isosbestic points demonstrate the existence of at least *three* interconvertible species; that which predominates at pH 6.1 is the protonated azoTyr-248, that at ~pH 8.5 is the intramolecular azoTyr-248 · Zn complex, while that above pH 9.5 is the azoTyr-248 phenolate ion.

Over the same pH range the spectra of the *apo*azoenzyme is quite different: the 510-nm absorption band is absent, but at pH 8.5 a maximum at 485 nm becomes progressively prominent (Figure 1B). There is a single isosbestic point at 416 nm, and the pH titration curve at 485 nm fits a theoretical curve with a pK_{app} of 9.4 (Figure 1B, insert) and is consistent with *two* interconvertible species. The behavior is characteristic of the ionization of an azophenol. The titration is identical with that of monoarsanilazo-*N*-acetyltyrosineamide (*vide infra*) and of other, similar azotyrosine derivatives (Tabachnick and Sobotka, 1959).

The insert of Figure 1A shows the pH-titration curve of the zinc azoenzyme at 560 nm, where the absorbance of the complex and that of the azophenolate ion differ maximally. In contrast to the typical sigmoid titration curve for the apoazoenzyme (Figure 1B, insert) the titration curve for the zinc azoenzyme is bell-shaped with two p*K* values of 7.7 and 9.5, respectively; it is superimposable on a theoretical curve characterizing the formation and dissociation of a metal complex.

Circular dichroic spectra can vary not only in amplitude but also in sign. In the present instance they resolve the spectral contributions of the complex from that of the azophenolate ion (Figure 1C). At pH 6.9 the circular dichroic spectrum exhibits two major Cotton effects, a negative band at 340 nm ($[\theta]^{23}$, −28,000), a positive band at 420 nm ($[\theta]^{23}$, + 14,000), and a negative band at 510 nm. An increase in pH from 6 to 8.5 markedly intensifies the negative 510-nm extremum, concomitant with the decrease of the negative band at 340 nm, while that at 420 nm remains almost unaltered. These changes in circular dichroism correlate with the formation of the intramolecular azoTyr-248 · Zn complex, as is evident from the absorption–pH titration (Figure 1A). At pH 8.5 the value of the negative extremum at 510 nm is maximal ($[\theta]^{23}$, −43,000), but on increasing pH to 10.5 this is replaced by a positive extremum at 485 nm ($[\theta]^{23}$, +10,500), characteristic of the azophenolate ion, while the 420-nm band shifts to 410 nm and becomes negative ($[\theta]^{23}$, −4000). The circular dichroism–pH titration of the zinc azoenzyme at 510 nm is bell-shaped and fits a theoretical curve with two pK_{app} values of 7.7 and 9.5 (Figure 2), identical with those calculated from the absorbance–pH titration at 560 nm (Figure 1A).

Though only 25 nm separate the maximum of the complex (λ_{max} 510 nm) from that of the azophenolate ion (λ_{max} 485 nm), circular dichroism of the zinc azoenzyme can unambiguously distinguish between their extrema owing to their difference in sign. In accord with this, the apoenzyme completely lacks the negative extremum at 510 nm over the entire pH range demonstrating that its presence is a direct function of the formation of the zinc complex (Figure 1D). Up to pH 8 the apoazoenzyme exhibits only relatively small ellipticity bands at 440 and 330 nm, respectively. As pH increases above 8.5, ionization of the azophenol generates a positive band at 485 nm ($[\theta]^{23}$, +10,500) and a negative band at 410 nm ($[\theta]^{23}$, −4000). At pH 10 the circular dichroic spectrum of the apoazoenzyme is identical with that of the zinc azoenzyme demonstrating that in both cases the uncomplexed azophenolate ion is the chromophore (Figures 1C, D).

Addition of Zn^{2+}, Cd^{2+}, Hg^{2+}, or Ni^{2+} to the apoazoenzyme at pH 7.5 forms the corresponding series of metallocarboxypeptidase with absorption maxima and circular dichroic extrema, characteristic for each metal. Titrations of each metalloenzyme reveal sets of two pK_{app} values, typical for each particular metalloenzyme, as is apparent from the details of the absorption- and circular dichroism–pH titrations (Johansen *et al.*, 1973; Legg *et al.*, to be published). On standing for prolonged periods at pH values above 10, the circular dichroic bands of the apoazoenzyme disappear, due to denaturation.

The absorption–pH titrations of monoarsanilazo-*N*-acetyltyrosineamide as well as that of TAT and of its zinc complex closely resemble those of the apo- and zinc azoenzyme, respectively. Figure 3 shows the pH dependence of the visi-

[2] The absorbance and molar ellipticity values for the azoTyr-248 · Zn complex are measured at pH 8.5 and 23 ± 0.1°, those for the azophenolate ion at pH 10.8 and 23 ± 0.1°.

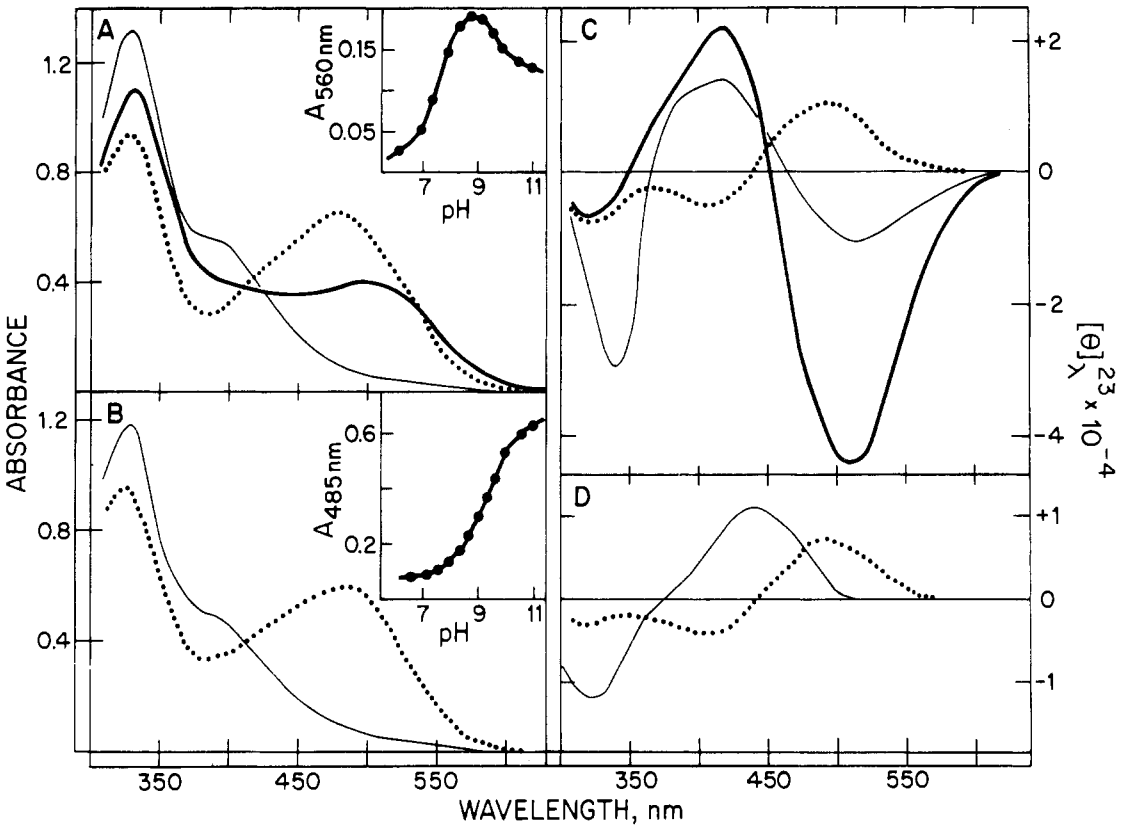

FIGURE 1: Absorption and circular dichroic spectra of zinc and apoazoTyr-248-carboxypeptidase α, γ. (A) Absorption spectra of the zinc azoenzyme at pH 6.2 (_____), pH 8.3 (▬▬), and pH 10.8 (····). The insert depicts the absorption-pH titration curve at 560 nm. (B) Absorption spectra of the apoazoenzyme at pH 7.5 (_____) and at pH 9.9 (····). The insert is the pH titration curve at 485 nm. (C) Circular dichroism spectra of the zinc azoenzyme at pH 6.9 (_____), pH 8.3 (▬▬), and pH 10.8 (····). (D) Circular dichroism spectra of the apoazoenzyme at pH 7.2 (_____) and pH 9.9 (····). Enzymes were dissolved in 2 mM Tris-HCl-0.5 M NaCl, 23°. In this and Figures 3 and 4 spectra for values of pH intermediate between pH 7 and pH 8.3 as well as between pH 8.5 and pH 10.5 have been omitted to simplify visualization of critical effects and changes.

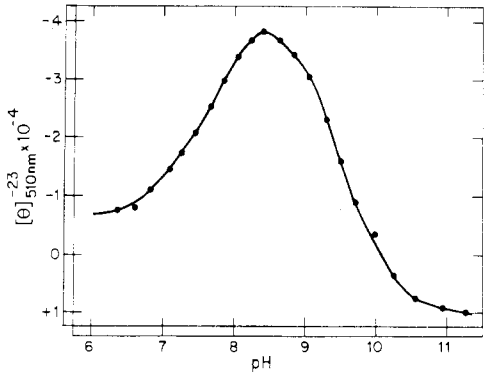

FIGURE 2: Circular dichroism-pH titration curve of the zinc azoenzyme at 510 nm in 2 mM Tris-HCl-0.5 N NaCl, 23°.

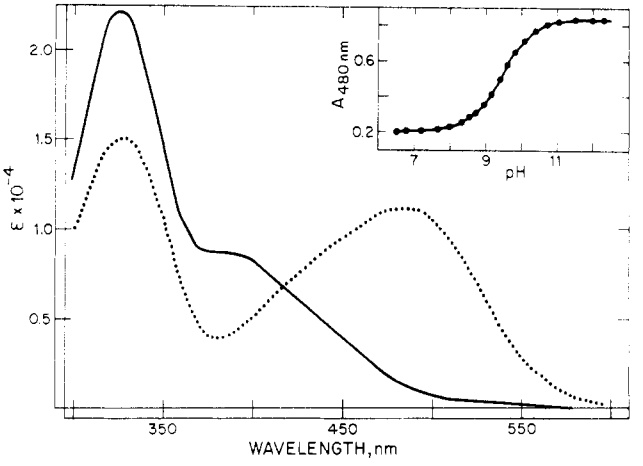

FIGURE 3: Absorption spectra of monoarsanilazotyrosine amide at pH 7.0 (_____) and pH 13 (····). The insert is the absorption pH titration curve at 480 nm.

ble absorption spectrum of monoarsanilazo-*N*-acetyltyrosineamide. At low pH the spectrum of the protonated form exhibits an absorption maximum at 325 nm (ϵ, 22,000), a shoulder at 380 nm, and a broad envelope of overlapping bands extending to 560 nm. Increasing pH generates a new absorption band at 485 nm (ϵ, 10,5000) with an isosbestic point at 415 nm. The absorbance-pH titration reflects a p*K* of 9.4 for the hydroxyl group of the azophenol (Figure 3, insert), and spectral properties characteristic both of monoazotyrosine derivatives and of apoazocarboxypeptidase (Figure 1B and insert). Study of the spectral properties of bidentate azophenol-metal complexes, *e.g.*, those of monoarsanilazo-*N*-acetyltyrosineamide · Zn^{2+}, presents problems due to their insolubility (Anderson and Nickless, 1967). However, tridentate heterocyclic and aromatic azophenols, exemplified by TAT, have proven particularly useful as metal indicators, owing to the solubility and high stability of their 1:1 metal complexes (Anderson and Nickless, 1967; Johansen and Vallee, 1971). One of the azo nitrogens, the phenoxy group of the ionized azophenol, and the N-2 nitrogen of the tetrazolyl group are thought to serve as metal ligands.

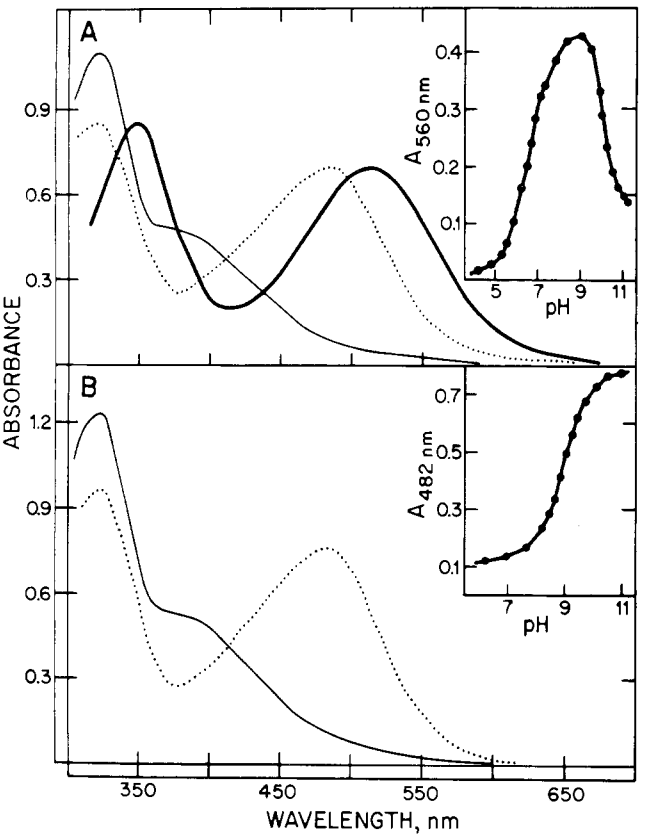

FIGURE 4: Absorption spectra of (A) the tetrazolylazo-*N*-carbobenzoxytyrosine · Zn complex (1:1) in 0.1 M NaCl at pH 4.3 (——), pH 8.5 (▬▬), and pH 11.0 (····). (B) Tetrazolylazo-*N*-carbobenzoxytyrosine in 0.1 M NaCl at pH 6.3 (——) and pH 11 (····). The insert in (A) is the absorption–pH titration curve for the tetrazolylazo-*N*-carbobenzoxytyrosine complex at 560 nm, and in (B) that for tetrazolylazo-*N*-carbobenzoxytyrosine alone at 482 nm.

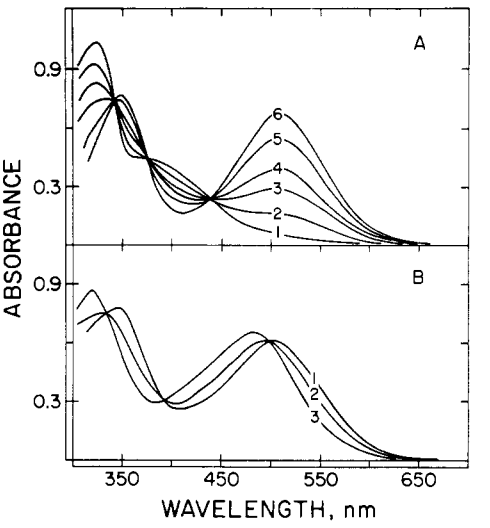

FIGURE 5: Effect of pH on the absorption spectrum of the tetrazolylazo-*N*-carbobenzoxytyrosine · Cd complex (1:1) in 0.1 M NaCl. (A) Titration in the pH range from 5.0 to 8.3 where the complex forms, and (B) titration in the pH range from 9.2 to 11.2 where the complex dissociates. Numbers in the figure indicate the pH at which the spectra were recorded: (A) (1) pH 5.0, (2) pH 5.9, (3) pH 6.3, (4) pH 6.6, (5) pH 7.1, (6) pH 8.3. (B) (1) pH 9.2, (2) pH 10.2, (3) pH 11.2.

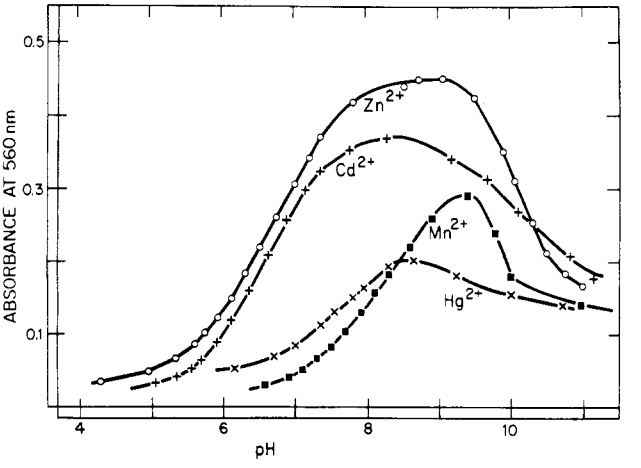

FIGURE 6: Absorption–pH titration curves of tetrazolylazo-*N*-carbobenzoxytyrosine at 560 nm in the presence of Zn^{2+} (○), Cd^{2+} (+), Mn^{2+} (■), and Hg^{2+} (x), respectively, all in ratios of 1:1 in 0.1 M NaCl.

The pH dependence of the visible absorption spectrum of TAT (Figure 4B) is very similar to that of arsanilazo-*N*-acetyltyrosineamide (Figure 3); the p*K* of this phenoxy group is 9.0 (Figure 4B, insert). In the absence and presence of equimolar concentrations of Zn^{2+} ions at pH 4.3 the spectra of TAT are identical (Figures 4A and B), since Zn^{2+} is not successful in dissociating the hydrogen ion of the azophenol at this pH. As pH increases, Zn^{2+} ions displace this proton, generating an absorption band at 510 nm which reflects the formation of the complex with isosbestic points at 341, 377, and 438 nm (Figure 4A). Further increase in pH to 11 progressively shifts the spectrum to that characteristic of the free azophenolate ion, λ_{max} 482 nm, and new isosbestic points are formed at 338, 398, and 497 nm. Substitution of Cd^{2+}, Hg^{2+}, or Mn^{2+} for Zn^{2+} generates complexes which exhibit absorption spectra typical for each metal ion and Figure 5 shows the detailed absorbance–pH titration of TAT · Cd^{2+} as an example. While the results of pH-titration are similar for all the metals investigated, the absorption maxima, and the pH range where the complexes form and dissociate are characteristic for each particular metal (Table I). Figure 6 compares absorbance–pH-titration curves for TAT in the presence of Zn^{2+}, Cd^{2+}, Hg^{2+}, and Mn^{2+}, respectively.[3] The metal complexes dissociate (or hydrolyze) in the pH range where the azophenolate ion forms. The change in absorbance as a function of pH is shown at 560 nm, the wavelength at which the absorbance of the Zn complex and that of the azophenolate ion differ maximally. The pH–titration curves of the metal

Table I: Absorption Maxima and pK_{app}'s Characterizing Formation and Dissociation of Metal Complexes of Tetrazolylazo-*N*-carbobenzoxytyrosine.

Complexing Metal	Maximum of Absorption (nm)	pK_{app}[a]	pK_{app}[b]
Zn^{2+}	510	6.7	10.1
Cd^{2+}	490	6.5	10.1
Hg^{2+}	550	7.4	9.4
Mn^{2+}	500	8.1	*c*

[a] pK_{app} characterizing formation of the complex. [b] pK_{app} characterizing dissociation of the complex. [c] Not discernible under the conditions of the experiments.

[3] Oxidation of Co^{2+} to Co^{3+} precluded analogous titrations with this ion.

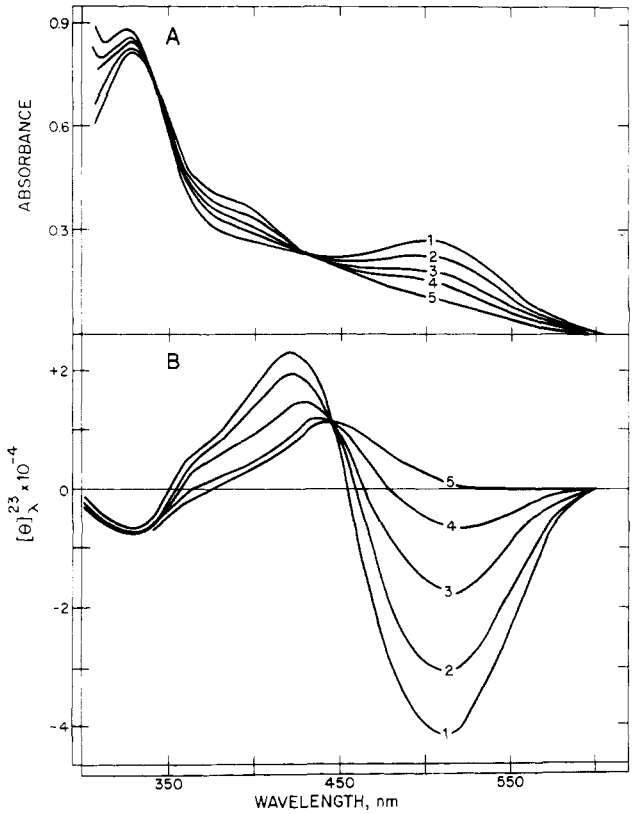

FIGURE 7: Effect of Gly-L-Tyr on the absorption spectrum (A) and circular dichroic spectrum (B) of zinc azoTyr-248 carboxypeptidase in 2 mM Tris-HCl-0.5 M NaCl (pH 8.5). Numbers indicate the Gly-L-Tyr concentrations at which the spectra were recorded: (A) (1) 0; (2) 0.45 mM; (3) 1.7 mM; (4) 3.5 mM; (5) 11.1 mM. (B) (1) 0; (2) 0.4 mM; (3) 1.4 mM; (4) 3.8 mM; (5) 6.5 mM.

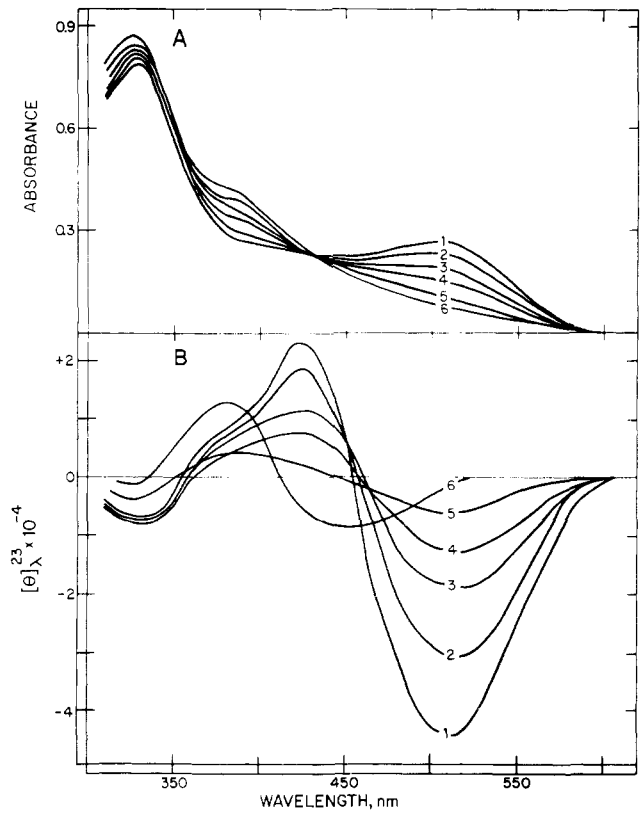

FIGURE 8: Effect of β-phenylpropionate on the absorption (A) and circular dichroic spectrum (B) of zinc azoTyr-248 carboxypeptidase in 2 mM Tris-0.5 M NaCl (pH 8.5). Numbers indicate the β-phenylpropionate concentration at which the spectra were recorded: (A) (1) 0; (2) 1.2 mM; (3) 3.8 mM; (4) 6.2 mM; (5) 12 mM; (6) 50 mM. (B) (1) 0; (2) 1.5 mM; (3) 3.8 mM; (4) 6.5 mM; (5) 13 mM; (6) 50 mM.

complexes are bell-shaped. The ascending limb reflects the formation of the complex, as the metal ion successfully competes with a proton for the ligand. With increasing pH the competition of OH^- with the azophenolate ion for the metal becomes apparent and absorption decreases. Thus, among other possible factors the steep slope of the descending limb of the Mn^{2+} titration curve at high pH is due to precipitation of Mn^{2+} hydroxide. The effect of pH on the metal complexes in the present system are quite analogous to the behavior of other, similar azodye-metal complexes (Anderson and Nickless, 1967; Klotz and Loh Ming, 1953). The data closely resemble those of the apo- and zinc azocarboxypeptidase system and provide a chemical basis for their spectra. The series of chemical events which give rise to the spectral changes observed for the azoenzyme include the displacement of a proton from the azoTyr-248 to form the azoTyr-248 · Zn complex and, at higher pH, dissociation of the complex to result in free, ionized azoTyr-248, possibly due to competition with OH^- ions (Figures 1, 4, and 6). Klotz and coworkers (Klotz and Loh Ming, 1954; Hughes and Klotz, 1956) came to similar conclusions based on their studies of a model system for metal-catalyzed enzymatic reactions, particularly of hydrolytic enzymes. Their studies of the effect of pH on the formation of ternary protein-metal-azopyridine complexes revealed that proteins such as serum albumin provide ligands to bind and thereby stabilize bidentate azopyridine-metal complexes. As a consequence, such mixed complexes form readily in the presence of proteins, just as observed in the present instance.

Exposure of the zinc azoenzyme to 5 M guanidinium chloride abolishes the maximum at 510 nm, and the absorption spectrum then becomes identical with that of the protonated azoTyr-248 moiety. Denaturation also completely abolishes all ellipticity bands of both the zinc and apoazoenzymes.

Glycyl-L-tyrosine and a number of inhibitors also abolish the 510 nm band of zinc azoTyr-248 carboxypeptidase α (Johansen and Vallee, 1971). We have now performed titrations with varying concentrations of Gly-L-Tyr and β-phenylpropionate at pH 8.5, where the intramolecular coordination complex forms maximally. Gly-L-Tyr is hydrolyzed at about 5/1000th of the rate of Cbz-Gly-L-Phe and affects the absorption spectrum of the zinc azoenzyme in a manner almost identical with that of β-phenylpropionate (Figures 7A and 8A). Gly-L-Tyr in concentrations ranging from 0.2 to 10 mM abolishes the 510-nm absorption band of the intramolecular coordination complex while concomitantly increasing absorbance at 330 and 380 nm to that characteristic of the protonated azophenol (Figure 7A). Similarly, the amplitudes both of the negative and positive ellipticity bands at 510 and 420 nm decrease, the latter shifting to 440 nm; there is a single, tight, isosbestic point at 445 nm (Figure 7B). The spectrum observed in the presence of 10 mM Gly-L-Tyr closely resembles that of the apoenzyme at pH 8.5, unaltered even on increasing the concentration of the pseudosubstrate to 20 mM. The resultant absorption and circular dichroic titrations at 510 nm result in superimposable, sigmoid titration curves with an apparent dissociation constant of 9×10^{-4} M.

Addition of β-phenylpropionate in concentrations varying from 0.01 to 50 mM progressively decreases the 510-nm absorption band of the complex while increasing those at 330

and 380 nm with a tight isosbestic point at 428 nm. At a β-phenylpropionate concentration of 50 mM, the spectrum becomes identical with that of the protonated azophenol (Figure 8A). In fact, overall, the changes in absorption as a function of both β-phenylpropionate and Gly-L-Tyr concentrations are virtually superimposable (Figures 7A and 8A) on those of the zinc azoenzyme as a function of pH (Figure 1A).

Concentrations of β-phenylpropionate up to 4 mM decrease both the amplitudes of the negative ellipticity band at 510 nm and that of the positive band at 420 nm, with an isosbestic point at 450 nm. At yet higher concentrations the amplitude of both bands decreases further, but the isosbestic point gradually shifts to higher wavelengths. Furthermore, the extremum at 420 nm gradually shifts to 380 nm, and a new negative band at 450 nm is formed. These complex titration spectra suggest the presence of at least two modes of binding of β-phenylpropionate. The amplitudes of the circular dichroic and absorbance changes at 510 nm on titration with β-phenylpropionate follow simple sigmoid titration curves with apparent dissociation constants of 3.1 and 3.2 mM, respectively.

High concentrations of β-phenylpropionate are known to induce polymerization of native carboxypeptidase (Bethune, 1965a,b). Hence, their effect on the sedimentation of azoTyr-248 carboxypeptidase was examined. At 10 mM β-phenylpropionate, where native carboxypeptidase exists as a polymer, the azoenzyme sediments as a single boundary with an $s_{20,w}$ = 3.3 S, a value identical with that for the native carboxypeptidase monomer. No evidence of dimers or higher polymers could be detected, analogous to results of comparable experiments with acetylcarboxypeptidase (Bethune, 1965b).

Discussion

Knowledge of the disposition of Tyr-248 with respect to the active site zinc atom of carboxypeptidase A is central to an understanding of the structure and function of the enzyme. A number of functional, chemical, and structural approaches have served to examine this problem. We here report the pH dependence of the spectra of the zinc and apoazoenzyme in solution, of the model, tetrazolylazo-*N*-carbobenzoxytyrosine and its complexes with Zn^{2+} and other metal ions and the spectral perturbation of the zinc azoenzyme by glycyl-L-tyrosine and β-phenylpropionate. We further relate and compare these results to all previous studies of this particular tyrosine and its spatial relationships to the zinc atom of the enzyme.

Spectral Properties of Azochromophores. Selective coupling of diazonium salts with tyrosyl side chains of proteins generates intensely colored derivatives (Tabachnik and Sobotka, 1959, 1960) which exhibit distinctive circular dichroic spectra (Fairclough and Vallee, 1970). The location of the extrema, the signs, and the magnitudes of the resultant extrinsic Cotton effects are characteristic of both the diazonium salt and the protein and depend critically upon the relative concentrations of reactants, ambient conditions (Fairclough and Vallee, 1970), and the physical state of the protein (Vallee *et al.*, 1971). These extrinsic Cotton effects reflect the stereochemistry of the azotyrosyl groups which are vicinally conditioned and, hence, are abolished by denaturation. Such optically active azochromophores provide excellent probes of local conformation and reveal its relevance to function (Fairclough and Vallee, 1970, 1971; Vallee *et al.*, 1971).

Azocarboxypeptidase. We have found diazotized arsanilic acid particularly useful in the study of structure–function relationships of carboxypeptidase A (Kagan and Vallee, 1969; Fairclough and Vallee, 1970). Modification with diazotized arsanilic acid can be followed through measurement of the number and nature of the residues by both amino acid and spectral analyses and by quantitative determination of the arsenic introduced into the protein. Under suitable conditions, treatment of carboxypeptidase A crystals with this reagent exclusively yields monoarsanilazotyrosine-248 carboxypeptidase (Johansen and Vallee, 1971, 1973; Johansen *et al.*, 1972). This is of particular significance since X-ray crystallographic analysis has assigned a critical, catalytic role to Tyr-248 (Lipscomb *et al.*, 1968).

All of the arsanilazoproteins which we have studied exhibit either two or three dichroic bands with extrema between 320 and 485 nm (Fairclough and Vallee, 1970). Thus far, however, only the arsanilazocarboxypeptidase family of proteins, *i.e.*, carboxypeptidase A (Kagan and Vallee, 1969; Fairclough and Vallee, 1970; Johansen and Vallee, 1971), its zymogen (Behnke and Vallee, 1971), and biologically related molecules such as carboxypeptidase S (Riordan and Livingston, 1971) or carboxypeptidase B (Sokolovsky and Eisenbach, 1972), exhibit a distinctive absorption and circular dichroic band, in general above 500 nm and at 510 nm for azocarboxypeptidase A in particular (Kagan and Vallee, 1969; Fairclough and Vallee, 1970). Therefore, the physical origin of this band was thought to differ from that of all other circular dichroic bands in this and other azoproteins (Fairclough and Vallee, 1970; Johansen and Vallee, 1971). Our investigations have established that the azophenol group gives rise to the circular dichroic bands at 320–340 and 420–450, while that at 485 nm is due to the azophenolate ion. The unique band at 510 nm reflects the formation of an intramolecular coordination complex between the azophenol of azoTyr-248 and the active site zinc atom (Johansen and Vallee, 1971, 1973). Thus, in carboxypeptidase A a diazotized arsanilic acid reactive tyrosyl residue and the active site zinc atom are suitably juxtaposed so that an intramolecular azophenolate–metal complex can form.

The Molecular Basis of Spectra of Azophenol · Metal Complexes. A number of observations and considerations demonstrate that an azoTyr-248 · Zn complex indeed is the molecular basis of the absorption and circular dichroic bands at 510 nm.

It is known that heterocyclic and aromatic azophenols form stable complexes with Zn^{2+} and other metals. The specific wavelength and extinction of the intense absorption maxima, generally also above 500 nm, are a function both of the metal and of the complexing agent (Anderson and Nickless, 1967; Vallee *et al.*, 1971). In particular, arsanilazo-*N*-acetyltyrosineamide and tetrazolylazo-*N*-carbobenzoxytyrosine and their ionized species (Figures 3 and 4) can be taken as excellent models, representative of the spectral characteristics of azocarboxypeptidase A (Figure 1A,B).

The formation of metal coordination complexes and their attendant spectra are characteristically pH dependent. In this regard, the consequences of absorption–pH–titrations of the TAT · Zn complex and of azoTyr-248 carboxypeptidase are remarkably similar. Tridentate azophenol–metal complexes such as TAT · Zn are water soluble and, hence, much more suitable for titrations over a broad range of pH than are the corresponding bidentate azo compounds. In the present study, titrations were carried out from pH 3 to 11 to detail the physical–chemical basis of the spectra of the

TAT · metal complexes and to define the range of effectiveness of their probe properties.

These data, in turn, pertain to the intramolecular arsanilazoTyr-248 · Zn complex of zinc azocarboxypeptidase. Hydrogen ion titrations of TAT complexes with Zn^{2+}, Cd^{2+}, Hg^{2+}, and Mn^{2+} result in spectral maxima and values of pK_{app} for their formation and dissociation characteristic of each metal (Figure 6 and Table I). In each case, there is a bell-shaped titration curve with one pK_{app} below 8.1 and another above 9.4. Analogous data have been obtained by substituting Cd^{2+}, Hg^{2+}, or Ni^{2+} for Zn^{2+} in azocarboxypeptidase (Johansen *et al.*, 1973; Legg *et al.*, to be published). Both absorption and circular dichroism–pH titrations of the azoenzyme demonstrate two pK_{app} values, 7.7 and 9.5, characterizing the formation and dissociation of an arsanilazotyrosine-248 · Zn complex (Figure 1A).

In the absence of zinc, pH <8, TAT[4] is yellow with a maximum at 325 nm and a shoulder at 380 nm. An absorbance–pH titration reveals a pK_{app} of 9.0 for the phenolic hydroxyl group (Figure 4B). Similarly, the apoazoenzyme has a maximum at 330 nm and a shoulder at 380 nm. It exhibits two circular dichroic bands at 340 and 440 nm but none at 510 nm. Titration shows a single pK_{app}, 9.4, due to ionization of the azophenol, λ_{max}, 485 nm (Figure 1B). Thus, in both the model and the enzyme the absence or removal of the metal precludes formation of the complex and, hence, its spectral manifestations.

Quiocho *et al.* (1972) have previously performed absorption–pH titrations of zinc azocarboxypeptidase solutions, but only up to pH 9. Hence, the pK_{app} of 9.5 was not detected, and it was therefore concluded that the pH–absorbance changes did not reflect an azophenol · zinc complex. Circular dichroism–pH titrations were not performed. The data in the inserts of Figure 1A and C in conjunction with the data of Figures 4 and 6 show that the titrations of Quiocho *et al.* (1972) were too restrictive and, hence, could not serve to analyze and describe the system adequately.

For the zinc azoenzyme the two pK_{app} values of 7.7 and 9.5, apparent from both the absorption and the circular dichroism–pH titrations at either 560 or 510 nm, delineate the region of pH within which the probe characteristics effectively gauge conformational features of the active center. The shift from a single isosbestic point at 428 nm to two at 412 and 520 nm (Figure 1A) as the pH is raised above 8.5 confirms the existence of at least three species: the azophenol, its zinc complex, and the free azophenolate ion. The circular dichroism–pH titrations unambiguously differentiate the 510-nm band of the complex from the 485-nm band of the azophenolate ion, since these two bands differ in their signs (Figure 1C).

The complex can form only at pH values defined by the two pK_{app} values and, further, only when azoTyr-248 and the zinc atom are suitably juxtaposed, as must be the case with all forms of the native enzyme in solution (Johansen and Vallee, 1973). However, alterations in overall three-dimensional structure, brought about, for example, by denaturation of the protein with 5 M guanidinium chloride, will disrupt the complex and abolish the long wavelength band (Johansen and Vallee, 1971). Hence, the spectral manifestations of the system specify the proximity of azoTyr-248 and Zn^{2+} to that distance which is required for the formation of an inner-sphere coordination complex.

Effects of Gly-L-Tyr and β-Phenylpropionate on Spectra of Zinc Azocarboxypeptidase α. Substances which interact specifically at the active site might also be expected to interfere with complex formation. At pH 8.5 both Gly-L-Tyr, 10 mM, and β-phenylpropionate, 50 mM, completely abolish the characteristic 510-nm absorption band of the azoTyr-248 · Zn complex (Figures 7A and 8A). The resultant absorption spectra are indistinguishable from that of the zinc azoenzyme at pH 6.6 (Figures 1A and 7). Clearly, both agents disrupt the complex once it has formed, while their removal by dialysis allows its restoration. Apparently, both Gly-L-Tyr and β-phenylpropionate alter the mutual orientation, proximity, or topographic relationships between the azophenol and the zinc atom to preclude the existence of the complex.

The circular dichroic spectra confirm and extend such deductions. Both Gly-L-Tyr and β-phenylpropionate completely abolish the negative circular dichroic band at 510 nm (Figures 7B and 8B). Interaction of Gly-L-Tyr with the zinc azoenzyme also shifts the extremum of the circular dichroic band of the azophenol at 420 to 440 nm, signalling a change in the asymmetric environment of azoTyr-248. In fact, in the presence of Gly-L-Tyr, the circular dichroic spectrum is identical with that of the apoazoenzyme at pH 8.5, suggesting that the symmetry of the environment of azoTyr-248 is the same in both instances.[5] Importantly, there is no discontinuity in the circular dichroic–pH titration.

β-Phenylpropionate also abolishes the 510-nm ellipticity band, but at shorter wavelengths it induces different spectral effects which are concentration dependent. At concentrations up to 4 mM it decreases the amplitudes both of the band at 420 and at 510 nm, and there is one tight isosbestic point at 450 nm. But on increasing concentration from 10 to 50 mM, two new circular dichroic bands become apparent centered at 380 and 450 nm, respectively, and concomitantly, the isosbestic point continually shifts to higher wavelengths. These different circular dichroic spectra likely signal different binding modes of this inhibitor, as previously recognized from kinetics of the native enzyme and spectral studies of the cobalt enzyme. These have already established at least two concentration dependent binding modes for β-phenylpropionate, manifesting kinetically as either competitive or noncompetitive inhibition (Auld *et al.*, 1972) and, spectrally, by the successive induction in the cobalt spectrum of ellipticity bands at 543 nm and then at 580 nm (Latt and Vallee, 1971). The effect of higher β-phenylpropionate concentrations may also be compared with nuclear magnetic resonance (nmr) studies which indicate that the agent displaces a water molecule from manganese carboxypeptidase (Shulman *et al.*, 1966). Multiple enzyme–inhibitor interactions are further consistent with the previous observations that β-phenylpropionate can protect two tyrosyl residues of carboxypeptidase from acetylation (Simpson *et al.*, 1963), and X-ray crystallographic data similarly indicate two metal-dependent binding sites for the *p*-iodo derivative of β-phenylpropionate in the crystalline enzyme. Based on such effects of β-phenylpropionate (Simpson *et al.*, 1963; Vallee, 1964), we suggested that two tyrosyl residues are involved in the catalytic mechanism. The functional consequences of successive modification of the enzyme

[4] Other, similar azodyes have analogous properties.

[5] This result is of particular interest since the X-ray structure analysis has shown that upon addition of glycyl-L-tyrosine to the *apoenzyme*, Tyr-248 moves inward toward the ligands of the metal, similar to its 12 Å movement toward the zinc atom of the metalloenzyme (Steitz *et al.*, 1967).

with diazonium-1*H*-tetrazole (DHT) and tetranitromethane (TNM) (Riordan *et al.*, 1967) supported this conclusion which has now been confirmed by sequence analysis (Cueni, 1974). The data demonstrate that first DHT modifies Tyr-248 and then TNM modifies another tyrosine residue, Tyr-198, with concomitant loss of peptidase activity (Cueni and Riordan, in preparation). This novel finding clearly suggests a reevaluation of those current mechanistic proposals on the role of tyrosine in carboxypeptidase catalysis which are based solely on Tyr-248.

X-Ray Structure of Native Crystals and Spectra of Azocarboxypeptidase in Crystals and Solution. In addition to pH, denaturants, substrates, and inhibitors, the physical state of carboxypeptidase could also be expected to affect the formation of the azoTyr-248 · Zn complex. Chemical and kinetic studies have demonstrated that both tyrosine and zinc participate in the catalytic mechanism of carboxypeptidase A (Vallee, 1964; Vallee and Riordan, 1968; Vallee *et al.*, 1970). Crystallographic structure analysis, while postulating that Tyr-248, in particular, is essential to catalysis, emphasized that in the X-ray crystals of carboxypeptidase α a Tyr-248 · Zn interaction is precluded (Lipscomb *et al.*, 1968; Quiocho *et al.*, 1972). The phenolic oxygen atom of Tyr-248 and the zinc atom have been reported to be separated by 17 Å (Lipscomb *et al.*, 1968; Quiocho *et al.*, 1972), thus preventing complexation of the metal by the arsanilazo derivative.

Spectral titrations of the crystals of all known forms of the zinc azoenzyme support this hypothesis. Three different procedures have been reported for the preparation of crystalline carboxypeptidase A (Anson, 1937; Allan *et al.*, 1964; Cox *et al.*, 1964), and these represent the only forms of the bovine enzyme generally available. All of them exhibit the same specificity and specific activities. Each of these preparations contains variable amounts of carboxypeptidases A_α, A_β and A_γ (Pétra, 1970). In addition, there are two allelomorphic species of the enzyme which differ in amino acid sequence at positions 179, 228, and 305 (Pétra, 1970).

Absorption spectra of crystals can be examined only up to pH 9, since above this pH the crystals dissolve; however, between pH 6 and 9 the crystals of the α, β, γ and of the allelomorphic forms of the enzyme do not exhibit any absorption band at 510 nm (Johansen and Vallee, 1973). Thus, in all of these crystals the preponderant conformation would seem to be that in which azoTyr-248 is not liganded to the Zn atom (Johansen and Vallee, 1971), entirely consistent with the reported X-ray structure (Lipscomb *et al.*, 1968; Quiocho *et al.*, 1972). Accordingly, the conformations of azocarboxypeptidase in the crystal and solution states must be different, at least with respect to the relative locations of the zinc atom and tyrosine-248. Additional differences involving adjacent segments of the polypeptide backbone must exist as well, but the nature and extent of such differences can only be surmised at the present time.

Spectra of Nitrocarboxypeptidase. It should be appreciated that evidence for conformational differences between crystals and solutions of carboxypeptidase derives not only from the spectra of the azoenzyme but also from those of the nitroenzyme. The results of studies employing this derivative to probe the conformations of the enzyme in these two physical states fully support the above conclusions. Nitration of carboxypeptidase crystals modifies Tyr-248 to form nitroTyr-248 carboxypeptidase. Its esterase activity increases to 180% and its peptidase activity decreases to 20% of the native enzyme (Riordan and Muszynska, 1974). At pH 7 nitrocarboxypeptidase crystals absorb maximally at 360 nm, but as the pH is increased to 8.5 this absorption progressively shifts to 428 nm with an isosbestic point at 380 nm, typical of ionization of a nitrophenol. The pK_{app} of the hydrogen ion titration of the crystals is 8.2. In solution, however, the pK_{app} is 6.3, a shift of nearly 2 pH units, another clear indication of markedly different environments of nitroTyr-248 in the crystal and solution states.

Kinetics of Native Carboxypeptidase Crystals. Detailed kinetic analyses of the native enzyme have now provided evidence that even without these chemical modifications the conformation of carboxypeptidase crystals and solutions differ. The physical state of the enzyme critically affects the catalytic rate constant, k_{cat}, both of α and γ carboxypeptidase (Spilburg *et al.*, 1974). Significantly, there are virtually no changes in the Michaelis constant, K_M. For the six substrates examined, k_{cat} decreases from 20- to 1000-fold on going from solutions to crystals. Moreover, for any given substrate, crystallization of the enzyme also drastically alters other features of the kinetic profile compared to that found in solution. Substrate inhibition, prominent for many substrates in solution, has not been observed with the crystals. In fact, while the kinetics of both Cbz-Gly-Gly-L-Phe and Bz-Gly-L-*O*-Phe are characterized by substrate inhibition in solution, they are marked by substrate activation for the crystals. Thus, over and above the diminution of k_{cat}, crystallization of the enzyme alters productive and nonproductive peptide and ester binding to carboxypeptidases α and γ.

Therefore, the substrate concentration–velocity profiles and the corresponding kinetic constants turn out to be important functional indices of the conformational differences of the native enzyme in the two physical states, consistent with the chemical and spectral evidence from chemically modified derivatives, *i.e.*, azo- and nitrocarboxypeptidase. As a consequence, the carboxypeptidase crystal structure does not provide a suitable basis for the mechanistic interpretations of kinetic data obtained in solution (Spilburg *et al.*, 1974).

Structure and Enzymatic Activity of Carboxypeptidase α Crystals, Elongated Along the "a" Axis, the "X-Ray Crystals." The question has been raised whether or not the conformational difference of crystals and solution applies to all forms of carboxypeptidase α, β, and γ and the allelomorphic variants (Quiocho *et al.*, 1972). As described above, all of these various forms of the enzyme have been modified with diazotized arsanilic acid, and in each case the resultant products exhibit spectral differences characteristic of the physical state. Thus far, only one crystal habit of one form of crystalline carboxypeptidase has been reported not to exhibit the type of behavior seen with all the others. This crystal habit of this form is the one employed for the X-ray crystallographic studies of carboxypeptidase, *i.e.*, the X-ray crystals. Compared with all those examined, these crystals are exceptional in that they are elongated along the *a* axis while all others are elongated along the *b* axis (Quiocho *et al.*, 1972). Moreover, relative to the native enzyme in solution, the X-ray crystals are reported to exhibit 30% of the activity of the native enzyme when assayed in the crystalline state, using Cbz-Gly-L-Phe as substrate. In contrast, from the data presented (Steitz *et al.*, 1967) it can be estimated that the activity of this crystal habit when assayed with Gly-L-Tyr was like that of all other known carboxypeptidase forms. However, inasmuch as details of the Cbz-

Gly-L-Phe assay were not reported and as the complex kinetic behavior of this substrate precludes meaningful comparisons of single point activity determinations, the reported difference in crystal activity may be but apparent. Since we now know that crystals and solutions differ in their modes of interaction with substrates (Spilburg *et al.*, 1974), activation or inhibition could result in either high or low activity ratios while concealing the true functional consequence of a change in physical state.

The most likely explanation for the dynamic, spectral response of the azoTyr-248 probe to the physical state of the enzyme would be that crystallization induces an *outward* movement of the azoTyr-248 ligand, away from the zinc atom, a conformational motion in a direction opposite to that inferred from the X-ray crystallographic analysis, based on the (enzyme · Gly-L-Tyr minus enzyme) electron density difference map. This difference map was obtained with calculated structure phases which were corrected for the low Gly-L-Tyr occupancy of the crystals (Lipscomb *et al.*, 1970). Since only 30% of the total number of molecules in the crystals formed a Gly-L-Tyr complex, the data were recalculated with functions designed to remove contributions of the native enzyme to the difference map and to enhance substrate features (Lipscomb *et al.*, 1970). Since in the crystal habit examined 25% of the total number of Tyr-248 residues present interact with zinc even in the absence of substrate (Lipscomb, 1973), in view of the 30% occupancy, it is essential to know to which fraction of molecules Gly-L-Tyr binds, *i.e.*, either to those 25% of the molecules in the crystals in which Tyr-248 interacts with the active site zinc atom or to the other 75% which do not. Such knowledge is crucial in deciding whether or not the differences in the conformation in Tyr-248 observed in the native enzyme and in the enzyme · Gly-L-Tyr complex reflect an increased movement of that residue on binding as proposed by Lipscomb *et al.* (1968). If Gly-L-Tyr binds only to those molecules in which Tyr-248 is already complexed to zinc the data would allow inferences completely different from those then drawn.

Spectra of the Azocarboxypeptidase "X-Ray Crystals" The one major difference between the atypical X-ray crystals and all others is the fact that subsequent to modification with diazotized arsanilic acid at pH 8.2 these crystals are red while all others are yellow at this pH (Quiocho *et al.*, 1972). This is particularly surprising in view of the fact that it was the X-ray structure analysis of these very crystals—prior to modification, of course—that assigned 17 Å as the distance between the phenolic oxygen and the zinc atom.

A point has been made of the fact that the X-ray structure analyses were carried out at pH 7.4 (Quiocho *et al.*, 1972; Lipscomb, 1973), though, evidently, some structure analyses were performed at pH 7.5 and even 8.0 (Lipscomb *et al.*, 1968). However, no X-ray structure analyses were performed on the arsanilazotyrosyl enzyme, either at pH 7.4 or pH 8.2. Since the modified X-ray crystals have been described as almost yellow at pH 7.4 (Quiocho *et al.*, 1972), it now remains to explain the molecular basis of their red color at the higher pH.

One possibility is that a residue other than Tyr-248 was modified. However, this is not satisfactory, since one is still left with a red spectrum of unexplained origin but which responds to pH changes as does an azophenol · metal complex.

Another explanation could be that a different derivative might have formed. Indeed, the curious band at 540 nm present in the absorption spectrum of the azo derivative of the X-ray enzyme (Quiocho *et al.*, 1972) is reminiscent of that of bisazotyrosine at pH 8.2 (Tabachnick and Sobotka, 1960). However, thus far we have been unable to find conditions which would allow formation of bisazotyrosine while also restricting modification to a single tyrosyl residue of carboxypeptidase.

In an attempt to resolve this important, pressing question it has been speculated further that differences in intermolecular interactions, primarily steric in nature, in the packing of molecules of these various crystals could account for the differences observed (Lipscomb, 1973). Since there are no known intermolecular interaction differences among crystal habits of this enzyme, this postulate must be considered less than satisfactory, since it does not seem susceptible to experimental examination or verification at this time.

In this regard, past interpretations of the electron density maps have consistently excluded the possibility that Tyr-248 might be able to interact with the zinc atom of the enzyme, as indicated above. This was thought to require such major repositioning of the extended polypeptide chain in the region of Tyr-248 as to render this possibility highly unlikely (Lipscomb *et al.*, 1968; Quiocho *et al.*, 1972).

However, Lipscomb (1973) has reexamined his maps originally reported in 1968 (Lipscomb *et al.*, 1968) and now reports that a region of electron density immediately adjacent to the zinc atom represents an interaction of Tyr-248 of up to 25% of the molecules with the active site zinc atom. Simultaneously model building now also detects that the conformation of the polypeptide backbone near Tyr-248 is much more flexible than had been believed possible, enabling the phenolate group of Tyr-248 to bind to the zinc atom. The results of these revisions are entirely consistent with the deduction that carboxypeptidase can exist in at least two different conformations in one of which Tyr-248 is near, while in the other it is distant from the zinc atom. The crystals and solutions represent extremes and different proportions of at least two major conformations in the two states (Johansen and Vallee, 1971, 1973).

It is also possible, of course, that the intramolecular azoTyr-248 · Zn complex differentiates not only between different conformations in crystals and in solution but even between different conformations of different crystal habits.

Tyr-248 · Zinc Interaction and Its Relation to Function. Crystallographic verification of an enzyme conformation in which Tyr-248 does interact with the active site zinc atom has now led to the postulate that it is this conformation which cannot be enzymatically active (Lipscomb, 1973). Based on this reappraisal of the X-ray data, these crystals then exhibit two conformations, one in which Tyr-248 is 17 Å away from the zinc atom, and another, constituting up to 25% of the total, in which this residue interacts with zinc (Lipscomb, 1973). Further, this latter conformation is said to be enzymatically inactive (Lipscomb, 1973), a deduction which is not easily reconciled with the report that these crystals are one-third as active as the native enzyme in solution (Quiocho *et al.*, 1972; Lipscomb, 1973). Beyond this, however, this particular or any other functional conclusion can hardly be reached based solely on the analysis of the structure of the native enzyme. That structure, moreover, the only one now available, is deduced from the one crystal habit of the α form which is found infrequently and for all practical purposes is unavailable for study. By definition, activity can only manifest itself in the presence of substrate.

of course. The reasoning employed to deduce that the enzyme is inactive when the Tyr-248 · Zn bond is formed (Lipscomb, 1973) can only lead to the conclusion that *all* conformations which differ from that which pertains when substrate is present must be inactive. Hence, the predominant conformation of the native enzyme crystals, *i.e.*, that in which Tyr-248 is 17 Å away from the zinc atom, must also be inactive, since in that instance this residue is too far from the zinc atom to assist in catalysis. The difficulty seems to be primarily one of semantics, since structure analysis of the native enzyme alone can at best lead to speculations on the *potential* for catalysis.

The dynamic probe characteristics of the azoenzyme are particularly helpful here, since they allow direct and simultaneous inspection of the pertinent structural and functional features of the enzyme. In solution, zinc arsanilazocarboxypeptidase retains the potential for catalysis (Johansen and Vallee, 1971, 1973) and at pH 8.2 it actively catalyzes substrate hydrolysis. Moreover, the rate of spectral changes as a function of pH is entirely compatible with catalytic rate constants of substrate hydrolysis (Harrison, Auld and Vallee, in press), contrary to earlier reports (Quiocho *et al.*, 1972).

What then is the role of Tyr-248 in the mechanism of action of carboxypeptidase? In the crystalline state it would appear that this residue undergoes a conformational change involving rotation about its C_α–C_β bond which moves the phenolic hydroxyl group some 12 Å to place it near the scissile bond of the substrate (Lipscomb *et al.*, 1968). As a consequence of these observations, Tyr-248 has been thought to be a proton donor facilitating the hydrolysis of peptides (Lipscomb *et al.*, 1968). Our present studies like earlier ones (Riordan *et al.*, 1967) also indicate that Tyr-248 undergoes a conformational change during the catalytic process. However, the spectral effects of Gly-L-Tyr and β-phenylpropionate on solutions of azocarboxypeptidase show that in their presence tyrosine *moves away* from the zinc atom. Presumably, binding of substrate triggers a series of rearrangements of the backbone which moves Tyr-248 out of the substrate pocket.

Movement of Tyr-248 on binding of β-phenylpropionate has already been deduced from studies with nitrocarboxypeptidase (Riordan *et al.*, 1967). The inhibitor was shown to shift the pK_{app} of the nitrotyrosyl residue from 6.3 to about 7.0. In view of the pH–absorbance titrations of crystalline nitrocarboxypeptidase (*vide supra*) these studies with this derivative also make it likely that this shift in pK_{app} indicates a movement of the nitrotyrosyl residue *away* from the zinc atom.

Whatever the sequence of events, it is apparent that a 12 Å movement of Tyr-248 toward the zinc atom is not an obligatory feature of the mechanism of action of the enzyme. In fact, it would seem more probable that on binding substrates in solutions of the native enzyme tyrosine-248 must also move away from the zinc atom. In turn, this direction of movement indicates a role for Tyr-248 in the mechanism of action of carboxypeptidase different from that postulated previously (Lipscomb *et al.*, 1968, 1970).

Indeed, the proximity of Tyr-248 to the zinc atom, when altered by substrates and inhibitors, may reflect certain of the properties characteristic of the entatic, active site (Vallee and Williams, 1967; Vallee *et al.*, 1971). The pH dependence of both ester and peptide hydrolyses catalyzed by several different modified carboxypeptidases makes it most unlikely that Tyr-248 functions as a proton donor. Yet so many different tyrosyl modifications affect activity that the participation of this residue in catalysis cannot be excluded. Its interaction with the zinc atom would now seem to be a critical feature of the active site, and this interaction may well determine the course of events leading to products.

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Mitochondrial Adenosine Triphosphatase. Location of Sulfhydryl Groups and Disulfide Bonds in the Soluble Enzyme from Beef Heart†

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ABSTRACT: The soluble beef heart mitochondrial ATPase (F_1) contains eight sulfhydryl groups and two disulfide bonds. *N*-Ethylmaleimide has been used to radioactively label the sulfhydryl groups before and after cleavage of the disulfide bonds by dithiothreitol. After subjecting the labeled protein to polyacrylamide gel electrophoresis in sodium dodecyl sulfate and measuring radioactivity in each of the separated subunits, the location of all the sulfhydryl groups and the disulfide bonds may be specified. The conclusions are supported by direct examination of depolymerized, unreduced, enzyme by polyacrylamide gel electrophoresis. The results also indicate that current ideas regarding the overall subunit structure of this enzyme may be incorrect, and this is discussed in light of new data presented here.

The oligomycin-insensitive ATPase from beef heart mitochondria is a soluble enzyme (called "F_1") which is believed to carry the catalytic site at which ATP is hydrolyzed or synthesized in mitochondria (Pullman *et al.*, 1960; Penefsky *et al.*, 1960; Racker, 1970). The structure of the enzyme is complicated, and has recently been reviewed (Senior, 1973a). This large spherical enzyme complex, of molecular weight around 360,000, contains five different types of tightly bound subunits and a sixth, more loosely bound subunit called the "specific inhibitor protein." The overall subunit structure in the molecule is not known. One suggestion (Senior and Brooks, 1971) has been made, based on the relative staining intensity of the different subunits after separation by polyacrylamide gel electrophoresis and on their molecular sizes.

The native, unreduced enzyme is known to contain eight sulfhydryl groups and two disulfide bonds (Senior, 1973b). In this paper I have described experiments using radioactive *N*-ethylmaleimide, which labels the sulfhydryl groups. The locations of the sulfhydryl groups on the various subunits, and the probable positions of the disulfide bonds have been specified. Further, the data suggest that our previous proposal for the overall subunit structure may be incorrect, and other arrangements are discussed.

Materials and Methods

Preparation of ATPase. The enzyme was prepared from beef heart mitochondria as described previously (Senior and Brooks, 1970) with a slight modification at step 4 (Brooks and Senior, 1972). The eluate at step 4 was directly taken through step 6 (heat step). The specific activity of the enzyme, assayed as described (Senior and Brooks, 1970), was 112 μmol of ATP hydrolyzed per min per mg.

N-Ethylmaleimide. Nonradioactive material was purchased from Eastman Kodak, Rochester, N.Y. *N*-Ethyl[1-^{14}C]maleimide and *N*-ethyl[2-^{3}H]maleimide were purchased from New England Nuclear. Solutions (20–30 mM)

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