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Conformations of Arsanilazotyrosine-248 Carboxypeptidase $A_{\alpha,\beta,\gamma}$. Comparison of Crystals and Solution

(intramolecular coordination/circular dichroism and absorbance–pH titration)

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ABSTRACT **The spectra of the α, β, and γ forms of zinc monoarsanilazotyrosine-248 carboxypeptidase A are indistinguishable. At pH 8.2 their crystals are yellow, while their solutions are red, λ_{max} 510 nm. Absorption and circular dichroism–pH titrations of the modified zinc and apoenzymes demonstrate that the absorption band at 510 nm is due to a complex between arsanilazotyrosine-248 and the active-site zinc atom. Two pK_{app} values, 7.7 and 9.5, characterize the formation and dissociation of this arsanilazotyrosine-248·Zn complex. On titrations of the apoenzyme, the absorption band at 510 nm is completely absent at all pH values. Instead, there is a single pK_{app}, 9.4, due to the ionization of the azophenol, λ_{max} 485 nm. Substitution of other metals for zinc results in analogous intramolecular coordination complexes with absorption maxima and circular dichroism extrema characteristic of the particular metal. Similar data and conclusions have been derived from studies of heterocyclic azophenol·metal complexes.**

The present studies demonstrate that the conformation of the crystals of all generally available α, β, and γ forms of the arsanilazoenzyme differs from that of their solutions. The spectra of the modified x-ray crystals, however, differ from those of all other carboxypeptidase forms and crystal habits studied. The internal consistency of their data, their interpretation, and the conclusions of Lipscomb and coworkers [*Proc. Nat. Acad. Sci. USA* (1972) 69, 2850–2854] are examined. Dissimilar chemical modification or conformation is thought to underlie these differences.

The arsanilazotyrosine-248·zinc complex is a sensitive, dynamic probe of environmental conditions. Its response to changes in pH and physical state of the enzyme suggest different orientation of the arsanilazotyrosine-248 side chain in solution from that in the crystal. This finding calls for reexamination of the basis of the substrate-induced conformation change which has been thought to be critical to the mechanism, postulated on the basis of the x-ray structure analysis performed at pH 7.5.

The integration of functional data obtained in solution with the structure derived from crystals remains one of the important problems in discerning the mechanism of action of an enzyme. We have searched for means that in solution could simultaneously gauge activity and the structural dynamics of the active center under a wide range of environmental conditions. Several chromophoric probes have been found particularly helpful in this regard (1, 2).

In relating the specific structure of carboxypeptidase A_α EC 3.4.2.1 to the catalytic mechanism of carboxypeptidase in general, the location of Tyr-248 with respect to the active-site Zn atom has been thought critical (3, 4). When specifically coupled with diazotized arsanilic acid, solutions of carboxypeptidase A_γ exhibit an absorption spectrum with a maximum at 510 nm (red) thought to be indicative of an intramolecular coordination complex between mono-arsanilazotyrosine-248 and Zn†. This complex can be dissociated and its accompanying spectrum can be abolished by crystallization of the enzyme, removal of the metal, or interposition of substrates or by inhibitors (2).

We have now examined the absorption and circular dichroism spectra of all three forms, α, β, and γ, of zinc arsanilazotyrosine-248 carboxypeptidase. Their crystals are all yellow, and their solutions all exhibit the same characteristic red absorption maximum at 510 nm, as observed previously for the γ enzyme (2, 5). Moreover, the pH dependence of this spectrum provides detailed information regarding the molecular basis of its origin. These data demonstrate that the orientation and mutual proximity of azotyr-248 and Zn in the crystals of α, β, and γ azocarboxypeptidase differ from that of their solutions, as reported earlier (1, 2, 5).

Recently, Lipscomb and coworkers (4), using conditions and forms of the enzyme comparable to ours, i.e., crystals of A_α and A_γ elongated along the *b*-axis, successfully repeated these experiments and obtained identical spectral results; the spectra of the crystals differed from those of the solutions. However, using the unusual crystalline form of carboxypeptidase A_α, elongated along the *a*-axis which served for x-ray analysis, the same spectra were found for the crystals and solution. This has raised some important questions: Is the molecular basis of the 510-nm spectrum the same or different for the various modified carboxypeptidase forms? Alternatively, are the crystal structures of these various enzyme forms having different crystal habits the same or different? The present work raises doubts about the interpretation and conclusions of Lipscomb and coworkers (4).

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† To simplify nomenclature, zinc carboxypeptidase A (the zinc azoenzyme) and apoazocarboxypeptidase A (the apoazoenzyme) are used interchangeably with zinc monoarsanilazotyrosine-248 carboxypeptidase and apoarsanilazotyrosine-248 carboxypeptidase, respectively, of any enzyme form. Carboxypeptidase A_α used for x-ray structure analysis and with a crystal habit elongated along the *a* axis (4) was designated the x-ray crystal or enzyme. Azo-Tyr-248 refers to mono-arsanilazotyrosine-248, the azophenolate ion to its ionized species. The absorption spectrum of zinc monoarsanilazotyrosine-248 carboxypeptidase is defined as "yellow" in the absence of an absorption band at λ_{max} 510 nm, as "red" in its presence.

MATERIALS AND METHODS

Procedures to isolate, characterize, and crystallize the various forms of carboxypeptidase A and to prepare their arsanilazo derivatives have been published (2, 6–9). Apoenzymes were prepared by soaking crystals in 1,10-phenanthroline (Auld, D. S., to be published).

The α and β forms of carboxypeptidase are much more soluble than the γ enzyme (10). Hence, different conditions, critical for labeling them with a single arsanilazotyrosine-248 residue per molecule (9) had to be used for each form and crystal type of the enzyme.

Precautions to prevent contamination by adventitious metal ions (11) were taken throughout. The thermostated titration cell of Auld and French (12) was adapted to the Cary model 14R recording spectrophotometer for pH titrations from pH 6.0–11.0. Zinc– or other metal–carboxypeptidase (and the corresponding apoenzyme), about 60 μM in 0.02 M Tris·HCl–0.5 M NaCl buffer (pH 6.0) at 23° ± 0.1 was titrated with aliquots of 0.1 N 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. Circular dichroic spectra were obtained with a Cary model 61 spectropolarimeter. Measurements between 300 and 650 nm were performed in 1-cm cells at protein concentrations of 30–60 μM. The pH dependence of the circular dichroic spectra of zinc and apoazocarboxypeptidase was also determined from pH 6.5–10.8 by stepwise addition of 0.1 N NaOH to 3-ml samples. The pH was measured with a CK 2321 Radiometer (Copenhagen) electrode before and after each spectrum was recorded. Mono-tetraazolyl-*N*-carbobenzoxytyrosine was prepared as described (13) and its concentration was determined by use of $\epsilon_{416} = 4.39 \times 10^3\ M^{-1}\ cm^{-1}$. Spectrophotometric pH titrations of mono-tetraazolyl-*N*-carbobenzoxytyrosine and its zinc complex were performed as for the enzyme; 0.1 mM each of mono-tetraazolyl-*N*-carbobenzoxytyrosine and $ZnSO_4 \cdot 7\ H_2O$ was used. Theoretical 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.

RESULTS AND DISCUSSION

The α, β, and γ zinc azoenzymes, modified according to criteria described (2, 9), have identical spectra and activities. The crystals are yellow but turn red when dissolved at pH 8.2 as expected when azoTyr-248 and zinc form a complex (2, 5). Hence, the particular enzyme form used will be indicated only for comparative purposes. The red solution λ_{max} 510 nm (ϵ = 8,000)‡, changes to yellow on (*a*) crystallization, (*b*) removal of zinc, (*c*) addition of substrate, (*d*) addition of inhibitors, (*e*) denaturation of the enzyme, or (*f*) lowering of the pH below 6 (2).

Spectral titration (Fig. 1*A*) of zinc azocarboxypeptidase generates an absorption maximum at 510 nm between pH 6.3 and 8.5. When the pH is raised to 10.8, the maximum shifts progressively to 485 nm (ϵ = 10,500), typical for the free azophenolate ion. Significantly, over the lower pH range there is but one isosbestic point, at 428 nm, while over the higher range there are two, at 412 and 520 nm. These data provide direct evidence for the progressive formation of at least three

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

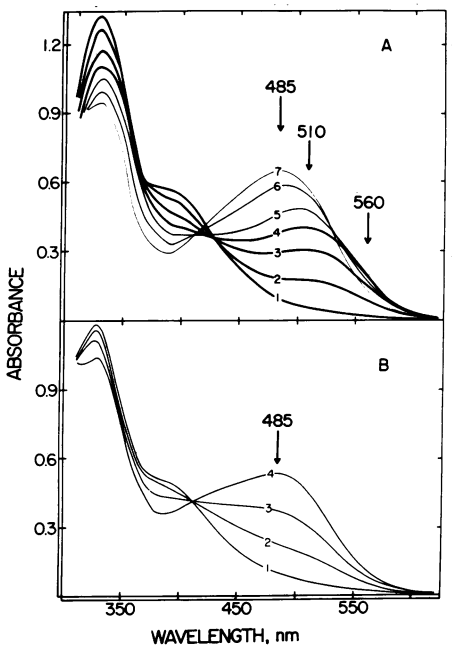

FIG. 1. Effect of pH on the absorption spectrum of (*A*) zinc arsanilazotyr-248 carboxypeptidase$_{\alpha,\beta,\gamma}$ and (*B*) apoarsanilazotyr-248 carboxypeptidase$_{\alpha}$ both in 2 mM Tris·HCl–0.5 M NaCl. The *numbers* indicate the pH at which the spectra were recorded: (*A*) (1) pH 6.2; (2) pH 7.3; (3) pH 7.9; (4) pH 8.3; (5) pH 8.8; (6) pH 9.6; (7) pH 10.8. (*B*) (1) pH 7.5; (2) pH 8.7; (3) pH 9.3; (4) pH 9.9. At higher pH values, the apoenzyme denatures. The *arrows* in this and succeeding figures identify maximal absorbances (or differences).

interconvertible species, i.e., the azophenol, the complex, and the azophenolate ion. Titration of the apoenzyme over the same pH range elicits a completely different spectral response (Fig. 1*B*). As would be expected in the absence of zinc, no 510-nm absorption band appears. Instead, just the absorption band of the azophenolate at 485 nm is generated (2, 5), and, significantly, only after pH is increased above pH 8.5. A plot of absorbance at 485 nm against pH reveals a pK of 9.4 (Fig. 2*C*), characteristic of the ionization of the arsanilazophenol and identical to that of mono-arsanilazo *N*-acetyl and *N*-carbobenzoxytyrosine amides (about 9.4; to be published) and other similar mono-arsanilazotyrosine derivatives (14).

Fig. 2*A* shows the pH titration curves of the zinc azoenzyme at 485 and 510 nm and Fig. 2*B* that at 560 nm, the wavelength where the absorbance of the complex and the azophenolate ion differ maximally (Fig. 1*A*). When examined over a wide pH range, the titration at neither 485 nm nor 510 nm fit a theoretical titration curve for a single pK. Furthermore, that at 560 nm readily reveals two pK values at 7.7 and 9.5, respectively, and fits a theoretical curve consistent with the existence of an intermediate complex.

A pH-titration curve of the x-ray enzyme at one wavelength only, 510 nm, and over a narrow pH range, was performed by Lipscomb and coworkers (4) and interpreted to indicate the existence of only a single ionization, pK_a 7.78 ± 0.04 for zinc azocarboxypeptidase$_{\alpha}$ and pK_a 7.72 ± 0.64 for the modified γ enzyme. Their pH titrations were apparently insensitive to the substantial contribution of the azophenolate species to the 510-nm absorbance (Fig. 2*A*). They were performed at very low absorbance and were not extended beyond

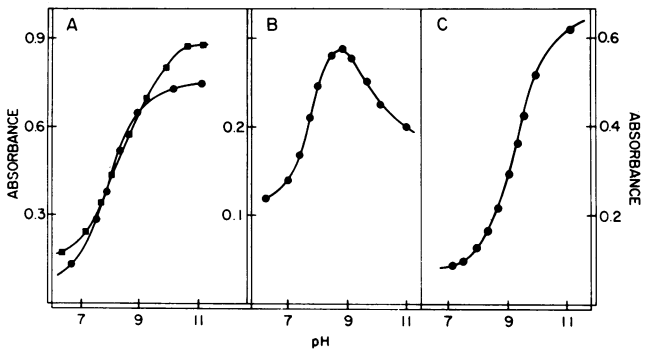

FIG. 2. Absorbance-pH titration from the spectra in Fig. 1*A* and *B* of (*A*) zinc arsanilazotyr-248 carboxypeptidase$_{\alpha,\beta,\gamma}$ at 485, 510 and (*B*) 560 nm, respectively, and of (*C*) apoarsanilazotyr-248 carboxypeptidase$_{\alpha}$ at 485 nm. The value at pH 11 is calculated.

pH 9, where the spectral characteristics of the azophenolate ion would have become obvious (Fig. 2*A*). These titrations are incomplete and do not permit the mechanistic deductions made (4).

Circular dichroic measurements can vary not only in amplitude but also in sign. In the present instance they definitively resolve the spectral contribution of the complex from that of the azophenolate ion (Fig. 3*A*). The negative ellipticity band at 510 nm, specific for the azotyr-248·Zn complex, is generated as pH increases from 6 to 8.5, where the value of the negative extremum at 510 nm is maximal, $[\theta]^{23^\circ}$ −43,000. At higher pH values a positive band for the azophenolate ion is found at 485 nm, $[\theta]^{23^\circ}$ +10,500. Though the absorption maxima of these two species are separated by only 25 nm, owing to their difference in sign, they can be distinguished unambiguously in the zinc azoenzyme by means of circular dichroism (Fig. 3*A*). In accord with this, the apoazoenzyme only exhibits the positive rotation at 485 nm associated with the azophenolate species. The apoazoenzyme completely lacks the large negative ellipticity band at 510 nm over the entire pH range (Fig. 3*B*), demonstrating the dependence of this negative ellipticity band on the formation of the zinc complex.

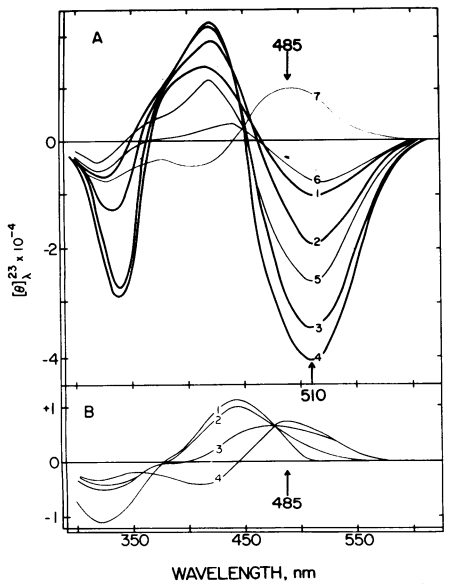

FIG. 3. Effect of pH on the circular dichroism spectrum of (*A*) zinc arsanilazotyrosine-248 carboxypeptidase$_{\alpha,\beta,\gamma}$ and (*B*) apoarsanilazotyrosine-248 carboxypeptidase$_{\alpha}$ in 0.2 mM Tris·HCl–0.5 M NaCl. The *numbers* indicate the pH at which the spectra were recorded: (*A*) (1) pH 6.9; (2) pH 7.3; (3) pH 7.9; (4) pH 8.3; (5) pH 9.4; (6) pH 9.8; (7) pH 10.8. (*B*) (1) pH 7.2; (2) pH 8.4; (3) pH 9.0; (4) pH 9.9.

The bell-shaped circular dichroism-titration curve at 510 nm (Fig. 4) for the zinc azoenzyme fits a theoretical curve for the ionization of two groups having pK_{app} values, 7.7 and 9.5, identical to those obtained from the absorbance–pH titration at 560 nm (Fig. 2*B*). Moreover, addition of cobalt, nickel, cadmium, or manganese to the apoenzyme results in a series of different metallocarboxypeptidases with absorption and circular dichroic extrema characteristic for each metal. Their titrations reveal sets of two pK values typical of each particular metal (5).

The results of spectrophotometric pH titrations of monotetraazolyl-*N*-carbobenzoxytyrosine and its Zn complex (Fig. 5*A* and *B*) resemble those of the apo and zinc azoenzymes, respectively, to a remarkable degree (Fig. 1*A* and *B*). With increasing pH the maximum of the complex at 512 nm (ϵ = 8.700) shifts to 482 nm (ϵ = 8.700) until the spectrum becomes identical to that of the azophenolate ion. A plot of absorbance at 560 nm against pH is bell-shaped, reflecting the formation and dissociation of the complex (Fig. 5*A*, *insert*). The titration curve of mono-tetraazolyl-*N*-carbobenzoxytyrosine alone indicates the ionization of a single proton, pK 9.0 (Fig. 5*B*, *insert*).

The series of chemical events that give rise to these spectral data include the displacement of the azophenolic hydroxyl group proton to form the zinc complex and, at higher pH, dissociation of the complex to give the azophenolate ion. We conclude, therefore, that the formation of a coordination complex between arsanilazotyr-248 and zinc accounts for the characteristic 510 nm absorption and circular dichroic bands of arsanilazocarboxypeptidase.

Such data call for a reevaluation of the circumstances that have led to current views regarding those structural features of carboxypeptidase believed critical for its function. Even though the native α-carboxypeptidase crystals, used for x-ray diffraction, are found infrequently and their crystal habit is unusual (4), their analysis has provided the only structure of carboxypeptidase now available. The structure determination places Tyr-248 away from the active site with its OH group some 17 Å from the zinc, but within 4–5 Å when the substrate glycyl-L-tyrosine intervenes between them (3). Their mutual

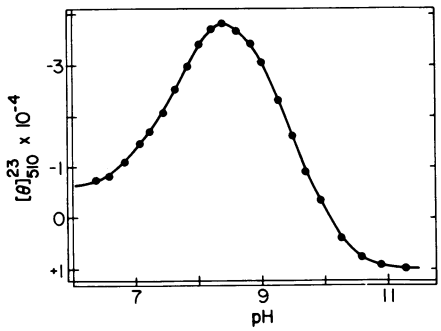

FIG. 4. Circular dichroism–pH titration from the spectra in Fig. 3 of zinc arsanilazotyrosine-248 carboxypeptidase at 510 nm in 2 mM Tris·HCl–0.5 M NaCl.

relationship in the x-ray crystals would be expected to pertain to all forms and crystal habits of the enzyme, and the underlying changes have served as the basis for general mechanistic considerations. However, differences between the spectral properties of the arsanilazo derivatives of these and the crystals of all other carboxypeptidase forms have been reported (4).

It is important to keep in mind that virtually all mechanistic studies in solution have used the α, β, and γ carboxypeptidases as crystals elongated along the *b*-axis, available in quantity from multiple sources. Their specificities and specific activities are identical. The spectral properties of their azoderivatives are also identical, as are the activities of their crystals and solutions, respectively. Table I summarizes these spectral properties of all carboxypeptidases examined thus far (2, 4, 5). The spectra in solutions and, hence, the proximity and mutual orientation of Tyr-248 and Zn, differ from those in the crystals. There is one exception, however, the modified x-ray crystals, although yellow at pH 6.7, nearly yellow at pH 7.5, are red at pH 8.2, as is their solution (4). Based in part on the failure to observe a second pK_{app}, Lipscomb and coworkers (4) have suggested that an "ionization-induced conformational change of the modified Tyr-248 from the yellow arsanilazotyrosine to the red form of arsanilazotyrosine" accounts for all phenomena owing to the ionization of a single proton. However, the present results (Figs. 1–4) show that the 510-nm absorbance and ellipticity bands of zinc azocarboxypeptidase are characterized by two pK_{app} values at 7.7 and 9.5, which define the formation and dissociation of an azoTyr-248·Zn complex, not a single proton ionization. Absorbance and circular dichroism–pH titrations over the pH range from 6–11 of the zinc and apoazocarboxypeptidase used for x-ray structure studies would readily establish the molecular basis of the crystal spectra of this material.

TABLE 1. *Color characteristics of arsanilazotyr-248 carboxypeptidase$_{\alpha,\beta,\gamma}$*

Carboxypeptidase form	pH	Color		Mode of enzyme preparation (Ref.)	Modified by§
		Crystal	Solution		
γ	7.5	Yellow	Red	(6)	A
γ	8.2	Yellow	Red	(6)	A
γ	8.2	Yellow	Red	(6)‡	A
γ	8.2	Yellow	Red	(6)	A
γ	8.2	Yellow	Red	(6)	B
α	8.2	Yellow	Red	(4)	B
α*	7.5	Yellow†	Yellow†	(4)	B
α*	8.2	Red	Red	(4)	B
α	8.2	Yellow	Red	(7)	A
β	8.2	Yellow	Red	(8)	A
apo γ	8.2	Yellow	Yellow	(6)	A
apo β	8.2	Yellow	Yellow	(8)	A
apo α	8.2	Yellow	Yellow	(7)	A

Red denotes the presence of a 510-nm absorption band and yellow its absence.

* X-ray crystals (see text footnote †).

† Described in ref. 4 as "nearly yellow."

‡ Courtesy of Drs. Michael Hass and Hans Neurath, University of Washington, Seattle, Wash.

§ Modifications and spectral studies of the enzymes were performed either by Johansen and Vallee (*A*) or Lipscomb and coworkers (*B*).

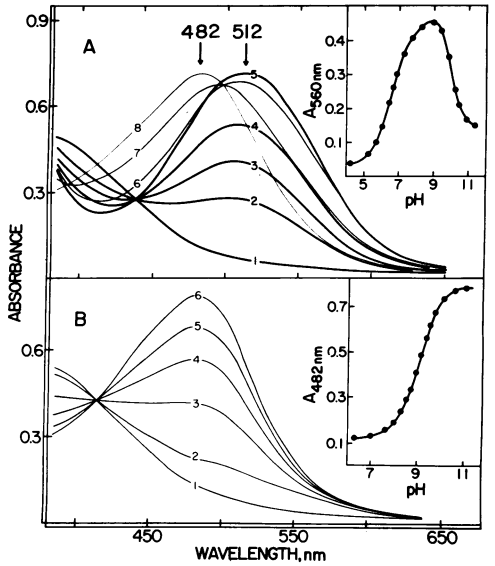

FIG. 5. Effect of pH on the absorption spectrum of (*A*) tetrazolylazo-*N*-carbobenzoxytyrosine $+Zn^{2+}$ and (*B*) Tetrazolylazo-*N*-carbobenzoxytyrosine in 0.1 M NaCl. The *insert* in (*A*) depicts the pH titration curves at 560 nm for the mono-tetraazolyl-*N*-carbobenzoxytyrosine·Zn complex and in (*B*) at 482 nm for mono-tetraazolyl-*N*-carbobenzoxytyrosine alone. (*A*) (1) pH 4.3; (2) pH 6.1; (3) pH 6.5; (4) pH 7.0; (5) pH 8.5; (6) pH 9.5; (7) pH 10.3; (8) pH 11.0. (*B*) (1) pH 6.3; (2) pH 8.2; (3) pH 8.9; (4) pH 9.3; (5) pH 9.7; (6) pH 11.

Interpretations seem closely interwoven with experimental details and conditions used for the experiments with the various enzyme forms. Collectively, existent observations lead to two predominant, alternate hypotheses which could account for different results. They are based either on the molecular basis underlying the spectra or on the conformations of different enzyme forms and their crystal habits.

The first hypothesis would be that the molecular basis of the (crystals and solution) spectra of the modified x-ray crystals elongated along the *a*-axis differ from those of all other readily available zinc azocarboxypeptidase forms. Indeed, the spectra that are superficially similar do differ significantly. The color of the x-ray azocarboxypeptidase crystals at pH 7.4 is described as "nearly yellow" but the spectrum contains a prominent absorption band at 540 nm (4), purple in the sense of Tabachnick and Sobotka (13). Neither the crystals nor the solutions of any of the readily available α, β, and γ zinc azocarboxypeptidases exhibit such spectral ambiguities. pH titration of their crystals, moreover, does not generate bands at 510 nm or higher wavelengths, up to pH 8.8, where the crystals dissolve. Additional data would be required to discern the origin of the 540-nm band. We have emphasized earlier the importance of spectral, amino-acid, and arsenic analyses, as well as sequence determination, in order to uniquely characterize the product of azocoupling as the mono-arsanilazotyrosine-248 derivative (2, 5). This detailed information, necessary to differentiate, e.g., contributions to the spectrum, of bis- and mono-azotyrosine, respectively, is not available for the modified x-ray crystals, the arsanilazo content of which was only determined by absorption spectra (15); the modified product needs to be characterized further. Hence, comparison

with the spectra of other azocarboxypeptidases, as here described, is not possible now, though it seems critical to interpretation regarding a possible contribution of bis-azotyrosine to the spectrum.

A second hypothesis would suggest that the active-site structure of the x-ray crystals differs from that of other crystal habits of carboxypeptidase A_α. This possibility cannot be dismissed, of course. However, there are inconsistencies when the x-ray structure and the spectral data of the modified x-ray crystals are compared. The pK values for the color change of the modified x-ray crystals and their solution are similar, 7.78 (4). If the unlabeled tyrosine of the native enzyme behaves in similar fashion, a substantial fraction of Tyr-248 would be expected to be complexed to zinc at pH 7.5. However, the structure determination places it 17 Å away and postulates that substrate is required to induce a change in its conformation. The information that these crystals are 30% as active as the native enzyme in solution (4) is particularly surprising in this regard, since the enzyme–substrate complex with glycyl-L-tyrosine and the failure of 90% of it to be hydrolyzed after 20 days has served to recognize the substrate-induced conformation change (16). If suitably characterized monoarsanilazo Tyr-248 x-ray crystals exhibit 510-nm absorbance at pH 8.2, the assumption that an azoTyr-248·Zn· complex has formed could satisfactorily account for the observation.

It is conceivable that the x-ray structure determination at pH 7.5 and 4° may not pertain at pH 8.2 or for other forms of the enzyme or their crystal habits. Such considerations might also indicate that a structure, static with respect to environmental conditions, need not pertain when such parameters change. The dynamic response of the spectral azoprobe would seem to reflect the structure-dependent function of the enzyme over a wider range, rendering comparisons somewhat incongruous. Verification of either or both hypotheses would call for much more extensive structural documentation than is currently available.

While such information is being acquired, it is now clear that the conformation of the crystals of the widely available α, β, and γ azocarboxypeptidases *does* differ from that of their solutions. The intramolecular azoTyr-248·Zn coordination complex may even differentiate between different conformations of different crystal habits.

The data presented indicate the need to reexamine earlier conclusions based on the structure analysis of the native x-ray crystals, i.e., that substrate is required to induce a conformational change involving an inward movement of tyrosine 248 toward the zinc atom as an obligatory aspect of the mechanism of carboxypeptidase-catalyzed hydrolysis (17). The present results reinforce our earlier conclusion (1, 2) that documentation of conformational changes as well as the judgment of their significance awaits the development of approaches that can define structural details of enzymes in solution with a precision comparable to that feasible for their crystals.

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