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Differences between the Conformation of Arsanilazotyrosine 248 of Carboxypeptidase A in the Crystalline State and in Solution

(circular dichroism/azoprobe/visible spectrum)

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ABSTRACT **Coupling of carboxypeptidase A crystals with diazotized arsanilic acid specifically labels tyrosine 248, an active-site residue of the enzyme. Many azophenols are yellow and their zinc complexes are red; the "yellow" absorption spectrum of zinc arsanilazocarboxypeptidase crystals is characteristic of the arsanilazotyrosyl group, not of the zinc complex. This is consistent with the interpretation of x-ray data on native crystals of carboxypeptidase A, indicating that tyrosine 248 and the zinc atom are too far apart to form a complex. However, the enzyme in solution is red, denoting the formation of a complex between zinc and arsanilazotyrosine 248. The most likely interpretation of the data is that the orientation of arsanilazotyrosine 248 in solution and in the crystal is different. If the unlabeled tyrosine 248 of native carboxypeptidase undergoes similar changes, these data may bear upon the low activity of the enzyme in the crystalline state and on the catalytic mechanism of the enzyme based on the crystal structure. The opportunities for analogous spectrochemical studies of other, similar systems are pointed out.**

The increasing importance of x-ray crystallography in studies of structure–function relationships of enzymes has generated considerable interest in the effect of crystallization on conformation. Knowledge of the relative conformations of enzymes in the solid state and in solution is now beginning to accumulate (1). For example, the properties of carboxypeptidase have been studied in particular detail from this point of view (2–4). The specific activity of carboxypeptidase in the crystalline state is only about 0.3% of that in solution. Further, the two physical states differ in the ease of zinc removal and in the enzymatic consequences of inorganic and organic modifications, though the basis of these phenomena has remained obscure. Hence, search for additional effects of the two states on conformation seemed indicated.

We have emphasized the value of spectrochemical probes in the assessment of changes in local protein conformation. The coupling of tyrosyl residues with diazonium salts has proven particularly useful in this regard (5, 6). We have now used diazotized arsanilic acid for the specific modification of tyrosine 248, an active-site residue of carboxypeptidase A (7). The resultant azophenol is capable of forming complexes with metal atoms (8), a circumstance that we have used to delineate conformational details of Tyr 248 in crystals and in solution. This azoprobe signals changes in conformational properties coincident with alterations of the enzyme's physical state. The data may bear upon the very low activity of the native crystals.

METHODS

A 50-fold molar excess of freshly prepared diazotized *p*-arsanilic acid was added to a suspension of carboxypeptidase crystals in 0.01 M $KHCO_3$, pH 8.8, 0°C and, after 3 hr, the reaction was stopped by centrifugation. The modified crystals were washed five times with metal-free water, suspended in 0.05 M Tris·HCl (pH 8.2), and fractionated as described by Richards *et al.* (2). Both the quantitation of amino-acid residues modified and the preparation of apoenzyme have been described (9). Tetrazolylazo-*N*-carbobenzoxytyrosine was prepared as described (10). Circular dichroic spectra were obtained with a Cary model 60 spectropolarimeter and absorption spectra with a Cary model 14R recording spectrophotometer. The absorption spectra for the modified carboxypeptidase crystals were corrected for light scattering (11), with a suspension of fractionated unmodified crystals as reference.

RESULTS

Zinc arsanilazocarboxypeptidase, the product of coupling native carboxypeptidase crystals with diazotized arsanilic acid, contains one arsanilazotyrosine, but no other amino-acid residues are modified (Table 1). Furthermore, the reaction is virtually specific for Tyr 248, as demonstrated by quantitative sequence analysis. About 95% of the label is present in fragment F_1, obtained by cleavage with cyanogen bromide (14). The peptide Thr-Ile-DAATyr-Gln-Ala-Ser-Gly-Gly-Ser-Ile-Asp-Trp, corresponding to residues 246–257 of native carboxypeptidase (15), was isolated from a chymotryptic

TABLE 1. *Number of residues modified and arsenic incorporation upon coupling carboxypeptidase crystals with diazotized arsanilic acid*

Amino-acid residue	Spectral analysis*	Amino-acid analysis†
Tyr	1.0 ± 0.1	0.9
His	0.05	0
Lys	—‡	0

$$\text{As}\ \frac{\text{g atom}}{\text{mol}} = 1.04$$

* Tabachnick, M., and H. Sobotka (12).
† Spackman, D. H., W. H. Stein, and S. Moore (13).
‡ Does not apply.

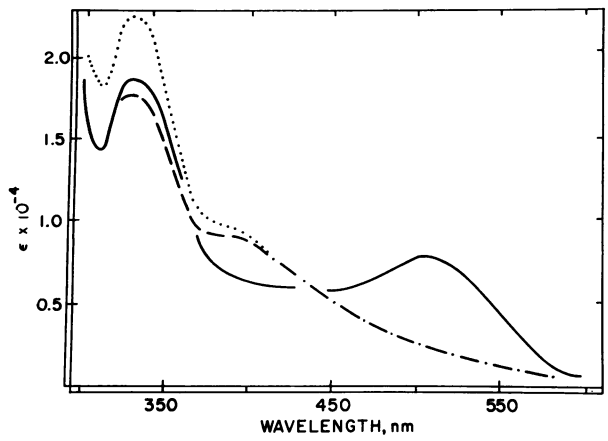

FIG. 1. Absorption spectra of zinc arsanilazocarboxypeptidase (——) and apoarsanilazocarboxypeptidase (···) dissolved in 0.05 M Tris·HCl–1 M NaCl (pH 8.2), and of zinc arsanilazocarboxypeptidase crystals suspended in 0.05 M Tris·HCl (pH 8.2) (– – –). The differences at high absorbance between the zinc arsanilazoenzyme crystals and the corresponding apoenzyme in solution are due to uncompensated light scattering and absorption flattening of the crystal suspension (11).

digest of fragment F_1 by affinity chromatography on an arsanilazotyrosyl-antibody–Sepharose column (16) (Johansen, J. T., D. M. Livingston, and B. L. Vallee, to be published). The yield of the peptide was 80%.

In the crystalline state, zinc arsanilazocarboxypeptidase* is yellow, but it turns red when it is dissolved; recrystallization restores the yellow color. These changes are clearly reflected in the corresponding absorption spectra (Fig. 1). In solution, an additional absorption band appears, centered at 510 nm, that accounts for the red color.

Removal of the active site zinc atom from the enzyme with 1,10-phenanthroline abolishes the absorption band at 510 nm and turns the solution yellow (Fig. 1). The resultant absorption spectrum of this enzyme in solution is very similar to that of the zinc enzyme crystals.

The circular dichroic spectra of the zinc enzyme in solution provide further information regarding the origin of the 510-nm band. The zinc enzyme exhibits one positive, and two negative ellipticity bands at 420, 335, and 510 nm, respectively (Fig. 2). Removal of the zinc atom, yielding the apoenzyme, eliminates virtually the entire circular dichroic spectrum, which is restored completely, however, by the addition of 1 g atom of zinc. The azotyrosyl moiety of carboxypeptidase apparently forms a complex with the zinc atom, characterized by an optically active absorption band with a maximum at 510 nm (Fig. 1) that generates the circular dichroic spectrum (Fig. 2).

It is well known that azophenols form colored metal-complexes, which are useful as metal indicators (8). Fig. 3 shows the absorption spectrum of one such azophenol, tetrazolylazo-*N*-carbobenzoxytyrosine, and of its Zn^{2+} complex. The spectrum of this free azophenol is remarkably similar to that of *crystalline* zinc arsanilazocarboxypeptidase and of the

* To simplify nomenclature, zinc carboxypeptidase (the zinc enzyme) and apocarboxypeptidase (the apoenzyme) are used interchangeably with zinc arsanilazocarboxypeptidase and apoarsanilazocarboxypeptidase, respectively. Unmodified carboxypeptidase A is referred to as "native".

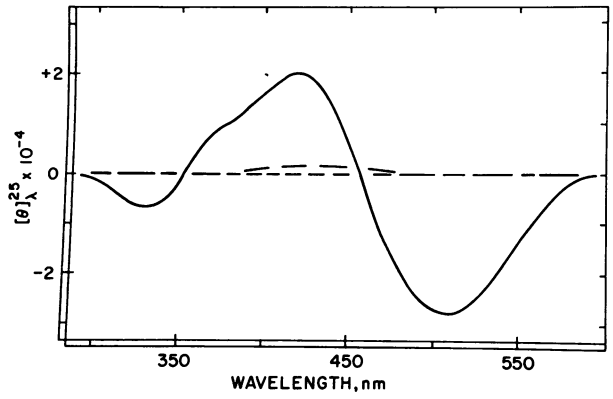

FIG. 2. Circular dichroism of zinc arsanilazocarboxypeptidase (——) and of the corresponding apoenzyme (— —) dissolved in 0.05 M Tris·HCl–1 M NaCl (pH 8.2). Over most of the spectral range examined, the baseline (– – – –) is indistinguishable from the spectrum of the apoenzyme.

apoarsanilazoenzyme in solution, while its zinc complex closely resembles that of the zinc enzyme *in solution*.

Comparisons of the difference spectra of the enzyme in solution in the presence and absence of zinc, of zinc carboxypeptidase in solution and in the crystalline state, and of the model compound, tetrazolylazo-*N*-carbobenzoxytyrosine with and without zinc (Fig. 4) indicate that a complex between the arsanilazotyrosyl moiety of zinc arsanilazocarboxypeptidase and the active site zinc atom is, indeed, formed in solution, but not in the crystalline state.

In solution, addition of substrates (e.g., glycyl-L-tyrosine or glycyl-L-phenylalanine) or inhibitors (e.g., L-lysyl-L-tyrosine, β-phenylpropionate, and L-phenylalanine), addition of 5 M guanidine, or lowering of the pH below 6 changes the red color to yellow. The absorption band becomes virtually identical to that of apocarboxypeptidase (Fig. 1). Removal of substrates or inhibitors, raising the pH to neutrality, or reconstitution of the zinc carboxypeptidase by adding Zn^{2+} to the apoenzyme reestablishes the original spectrum.

In the crystals, however, it has not, thus far, proven possible to induce the 510-nm absorption band. Variation of the ambient pH from 5 to 9, addition of LiCl or NaCl up to 2 M, addition of solvents with different dielectric constants, such as

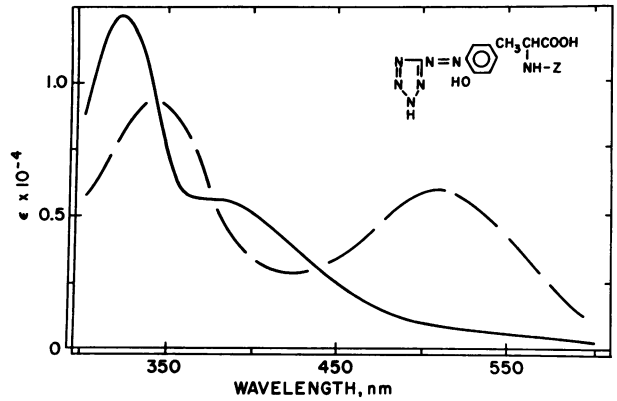

FIG. 3. Absorption spectra of tetrazolylazo-*N*-carbobenzoxytyrosine (structure given) alone (——) and in the presence of a 10-fold molar excess of Zn^{2+} ions (— —), both at pH 7.5.

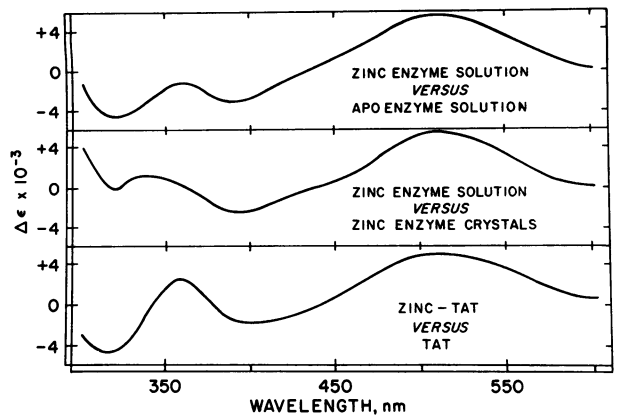

FIG. 4. Difference spectra obtained from Figs. 1 and 3 (as identified). TAT: tetrazolylazo-*N*-carbobenzoytyrosine.

ethanol, dioxane, ethylene glycol, chloroethanol, or ethylene dichloride, or addition of denaturing agents such as urea and guanidine, or addition of the substrates and inhibitors mentioned above, all fail to change the yellow color of the crystals to red.

DISCUSSION

The functional properties of enzymes in solution are frequently interpreted in terms of their three-dimensional crystal structures, based on the hypothesis that the conformations are the same in both states. This assumption is critical to the interpretation of the functional significance assigned to structural details of active sites of enzymes. It is difficult to define the precise role of amino-acid side chains in catalytic mechanisms if their positions change as a function of the physical state of the protein. Proteins have long been thought to assume multiple and readily interconvertible, but closely related, conformations (17). So far x-ray studies of enzymes have indicated a virtually unique conformation for each enzyme in the crystalline state, although certain side chains and segments of peptide chains (18–20) may be motile. There is evidence to suggest that motility of some proteins in solution may be greater than in the crystal (1, 2, 4). The documentation of such conformational changes, as well as the judgment of their significance, have presented difficulties, since methods for analyzing protein structure often require conditions that are not directly comparable with those exploring function. The question of whether or not the conformation that prevails in enzyme crystals also predominates in solution has not as yet been answered satisfactorily.

Carboxypeptidase A is particularly attractive for the examination of this problem because of the extensive information available on its composition, structure, and function (7, 21, 22). Chemical studies have shown that its zinc atom and tyrosyl residues are essential to peptide hydrolysis (21). Moreover, a critical catalytic role has been assigned to Tyr 248, thought to donate a proton to the scissile peptide bond of the substrate (7). The x-ray crystallographic studies place Tyr 248 at the surface of the molecule, with its side chain pointing away from the active site.

In solution, the distance between Tyr 248 and the zinc atom is unknown, and direct methods for such measurements have not as yet been devised. However, the introduction of a probe dependent on their interaction might signal their proximity in an "all-or-none" fashion. For this purpose, we have placed the arsanilazogroup on a single residue, Tyr 248, since such azophenols are well known to form zinc and other metal complexes, with a concomitant change in color from yellow or orange to red or purple (8). Indeed, the spectral characteristics of the apoarsanilazoenzyme and the zinc enzyme respectively, are closely similar to those of tetrazolylazo-*N*-carbobenzoxytyrosine and its zinc complex (Figs. 1 and 3)†. The product is enzymatically active and the "red" absorption spectrum denotes formation of the complex, which becomes possible due to suitable proximity and topography of its constituents. The "yellow" absorption spectrum indicates the absence of these conditions, owing to loss of zinc, or changes in the mutual orientation of, or distance between, the zinc atom and the arsanilazotyrosyl residue. Thus, denaturation with guanidine or addition of substrates or inhibitors, the latter known to interact with the zinc atom (21, 24), would be expected to alter spatial or topographic relationships, critical to the formation of the chelate. Either chelating agents or low pH can displace zinc and, indeed, on addition of the metal or on increasing the pH, the "red" absorption band is restored.

Binding of the arsanilazotyrosyl residue to the intrinsic zinc atom generates optical activity, resulting in characteristic extrinsic Cotton effects, either due to vicinal or inherent asymmetry of the complex, that reflect local conformation (Fig. 2). As expected, substrates and inhibitors perturb this circular dichroic spectrum. Removal of zinc virutally elminates it, since it is the asymmetric chelate that generates the ellipticity bands in this instance.

The absorption spectrum of these zinc enzyme crystals is characteristic of the arsanilazotyrosyl group, not of the zinc complex (Fig. 1). Conditions considered potentially capable of influencing overall or local protein conformation have failed to favor formation of the complex in the crystals. Neither changes of the polarity of the ambient solvent, nor changes of the pH or the temperature, or an increase in ionic strength produce the "red" spectrum. In the crystalline state, the electrostatic potential can be considerable, but it becomes negligible at ionic strengths of about 1 (25). Our experiments would seem to exclude electrostatic effects as a basis for the differences in spectra of the zinc arsanilazoenzyme crystals and solutions, respectively,‡ in accord with the fact that the pH activity profile of native carboxypeptidase crystals is similar to that in solution, suggesting the existence of the same electrostatic potential (4). On the basis of present knowledge, the spectral differences observed in the two states is accounted for most readily if the distance between the zinc atom and arsanilazotyrosine 248 were to be less in solution than in the crystals§.

These spectral data on zinc arsanilazocarboxypeptidase crystals are consistent with the interpretation of the x-ray data

† Other azophenols and their azophenol-zinc complexes have similar properties (8).

‡ The formation of the free arsanilazophenolate ion (pK about 9.2) under these conditions has been considered and has been eliminated experimentally (Johansen, J. T., and B. L. Vallee, to be published).

§ This could occur by movement either of the azotyrosyl residue or of the zinc atom, though the latter possibility alone is much less probable. Further, the formation of possible *inter*molecular complexes is not likely to account for the red color of the enzyme in solution, since ultracentrifugation has given no evidence for the existence of polymers.

on crystals of native carboxypeptidase and of its glycyl-L-tyrosine complex (26), making it unlikely that the introduction of the probe itself accounts for the spectral differences between the zinc arsanilazoenzyme crystals and in solution. In the native crystals, the side chain of Tyr 248 is at the surface and points away from the zinc atom, and its phenolic oxygen cannot complex with the zinc atom since they are 1.7 nm (17 Å) apart (7, 27). In fact, the selective reactivity of this residue in the crystals towards diazotized arsanilic acid may be accounted for, in part, by its location. Even on the addition of glycyl-L-tyrosine, the movement of the phenolic oxygen, by 1.2 nm (12 Å) (refs. 26, 27) would still leave a difference too great to allow formation of the complex. The carbonyl oxygen of the peptide bond of the substrate, moreover, interacts with zinc (7), which would be expected to block access to the metal binding site of arsanilazotyrosine 248.

In solution, however, the spectrum indicates that zinc and arsanilazotyrosine 248 are close enough and suitably aligned for the formation of a complex. This would suggest a different orientation of the arsanilazotyrosine 248 side chain in solution from that in the crystal, with respect to the zinc atom and this may bear upon their disposition in the native enzyme, where the activity of the crystals is about 0.3% of that in solution (2). On crystallization of the enzyme, arsanilazotyrosine 248 apparently undergoes a conformational change. If the unlabeled tyrosyl residue of the native enzyme undergoes similar changes in conformation when the crystals are dissolved, speculations on the catalytic mechanism based on crystal structure studies alone may require some reexamination.

The present data raise the question whether analogous conformational differences between solutions and crystals of enzymes in general may escape detection for want of suitable methods. Spectrochemical probes, specific for a specific site of a particular enzyme, present distinct opportunities in this regard. Clearly, the 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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