DIFFERENCES BETWEEN THE CONFORMATIONS OF NITROTYROSYL-248 CARBOXYPEPTIDASE A IN THE CRYSTALLINE STATE AND IN SOLUTION*

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In solution, nitrocarboxypeptidase A, modified at tyrosyl-248, exhibits a nitrotyrosyl pK apparent of 6.3. In the crystalline state, the pK apparent is about 8.2. This change in ionization is consistent with the hypothesis that crystallization of the enzyme causes a displacement of tyrosine-248 away from the active site zinc ion.

In order to provide a basis for the integration of functional data obtained in solution with structural information from X-ray analysis of crystals, Johansen and Vallee studied the spectral properties of arsanilazotyrosyl-248 carboxypeptidase A (1,2). They found that the modified tyrosyl residue forms an intramolecular coordination complex with the active site zinc ion when the enzyme is in solution but not when it is in the crystalline state. This suggested that in solution tyrosine-248 is close enough to the zinc atom to allow complex formation, much closer than indicated by crystallographic studies (3). The possibility that yet other chemical probes could serve to reveal such conformational differences conditioned by the physical state of the enzyme has now been investigated using nitrotyrosyl-248 carboxypeptidase. The present results indicate that the nitroenzyme also exhibits different conformations in the crystal

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and solution states, confirming the previous findings, and, further, making it unlikely that these structural differences are the consequence of the presence of either probe.

EXPERIMENTAL: Modification of carboxypeptidase A (Worthington) was carried out with the enzyme crystals (5 mg/ml) suspended in 0.05 Tris, pH 8.0, in the dark. A 3-fold molar excess of tetranitromethane was added to the stirred suspension and the reaction was stopped after 45 minutes at room temperature by centrifugation and washing the crystals with 0.05 M Tris, pH 8.0, buffer. Esterase and peptidase activities and degree of nitration were measured as reported previously (4). Spectra were recorded on a Cary 14 spectrophotometer. Spectral titrations were carried out by addition of µl quantities of 0.2 N NaOH to either solutions or crystal suspensions of nitrocarboxypeptidase in 0.002 M Tris-acetate, pH 5.5. Solutions of enzyme also contained 0.5 M NaCl. The effect of this concentration of NaCl would be to lower the apparent pK by about 0.2 pH unit. In the case of crystal titrations neutral density filters were placed in the reference beam to bring the sample absorption on scale. The amount of enzyme which had dissolved during the course of the crystal titrations was determined by centrifugation and spectral examination of the resultant supernate, generally less than 10% of total amount present in the initial suspension.

RESULTS AND DISCUSSION: Nitration of carboxypeptidase A with tetranitromethane increases its esterase activity to 180% and decreases its peptidase activity to about 20% relative to the native enzyme when assayed under standard conditions (4). These functional changes occur concomitantly with the formation of one nitrotyrosyl residue per molecule of enzyme.

At least 80% of the modification occurs at tyrosine-248 (5). A chymotrypsin digest of heat-denatured nitrocarboxypeptidase gave a mixture of soluble peptides which could be separated by immuno affinity chromatography on an antinitrotyrosyl antibody-Sepharose column into those containing

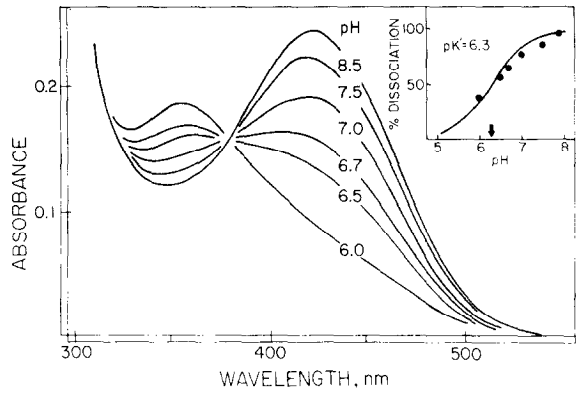

Fig. 1. Absorption spectra of a solution of nitrocarboxypeptidase A in 0.002 M Tris-acetate-0.5 M NaCl at the pH values indicated. Inset: Degree of dissociation as a function of pH. The solid line describes a normal ionization curve with a mid-point at pH 6.3 (Arrow).

nitrotyrosine and those which did not. Further purification by DEAE-sephadex chromatography resulted in three pure peptides, two of which could be identified as originating in that segment of the protein containing tyrosine-248; together they accounted for about 80% of the original nityrosyl absorption. The third peptide contained tyrosine-169.

In solution, at pH 6, nitrocarboxypeptidase exhibits a visible absorption spectrum with a maximum at 360 nm. As the pH is increased to 8.5 this band decreases and is replaced by another at 428 nm (Figure 1). There is an isosbestic point at 381 nm. This titration behavior is typical of nitrophenol ionization. A plot of absorbance at 428 nm vs pH (Figure 1, inset) follows a titration curve with an apparent pK of 6.3. This value is lower than that observed for 3-nitrotyrosine, N-acetyl-3-nitrotyrosine or o-nitrophenol, 6.8, 7.0 and 7.2, respectively (Table I).

Charge effects in the local environment of the nitrotyrosyl residue of carboxypeptidase could account for the decrease in apparent pK. Spectrophotometric titrations of a series of nitrotyrosyl containing copolymers reveal a progressive shift in the nitrotyrosyl pK as the net charge of the copolymer becomes more positive (Table I). Thus, it would appear that the low apparent pK of the nitrotyrosyl residue in nitrocarboxypeptidase

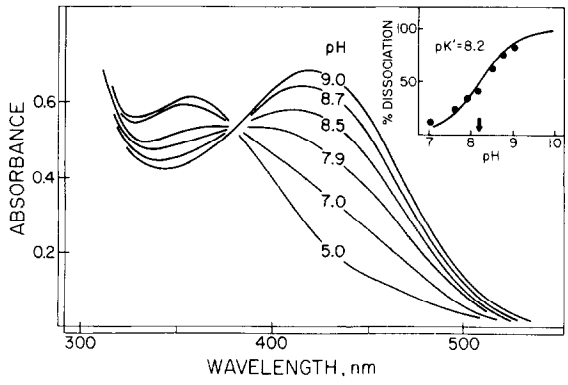

Fig. 2. Absorption spectra of a crystal suspension of nitrocarboxypeptidase A in 0.002 M Tris-acetate at the pH values indicated. Inset: Degree of dissociation as a function of pH. The solid line describes normal ionization curve with a mid-point at pH 8.2 (Arrow).

may indeed be due to its proximity to a positive charge on the enzyme.

The red color of solutions of arsanilazotyrosyl-248 carboxypeptidase has been shown to be due to the formation of an intramolecular zinc-azophenolate coordination complex (1,2). Crystals of arsanilazotyrosyl-248-carboxypeptidase are yellow because the phenolic hydroxyl group of tyrosine-248 can no longer interact with the metal ion to form the red complex. This conclusion is consistent with X-ray structural analysis which places the phenolic oxygen atom some 17 Å away from the zinc (3). The present data with nitrocarboxypeptidase indicate that in solution nitrotyrosyl-248 is near a positive charge. By analogy to the work of Johansen and Vallee this positive charge could well be the active site zinc ion. In that case, a change in physical state should affect the ionization behavior of nitrocarboxypeptidase, as is, indeed, the case. In the crystalline state titration of nitrocarboxypeptidase from pH 6.5 to 9.5 again increases absorbance at 428 nm while decreasing that at 360 nm. The isosbestic point is again at 381 nm (Figure 2). However, in contrast to the enzyme in solution, these data now follow a titration curve (Figure 2, inset) with an apparent pK of 8.2, rather than 6.3, a shift of almost 2 pH units. This dramatic shift in titration behavior is brought about solely by a change in the physical state of the enzyme.

Table I

pK' Values for Phenolic Ionization in Various Nitrophenols and Nitrotyrosyl Residues in Copolymers*

Substance	pK'
o-Nitrophenol	7.2
3-Nitrotyrosine	6.8
N-Acetyl-3-nitrotyrosine	7.0
Glu-Tyr (9:1)	7.1
Glu-Lys-Tyr (54:40:6)	6.7
Lys-Tyr (10:1)	6.2

*All pK's were estimated from spectrophotometric-pH titration data obtained with solutions of the various substances in 0.2 M Tris-acetate-0.5 M NaCl.

It would appear that in nitrocarboxypeptidase, as in arsanilazocarboxypeptidase, the conformations of tyrosine-248 in solution and in the crystalline state are different.

These data indicate that a reevaluation of current views on the structural features of carboxypeptidase catalysis is necessary. Moreover, they raise questions as to whether or not analogous conformational differences between solutions and crystals of other enzymes may escape detection for want of suitable methods. Spectrochemical probes clearly offer distinct opportunities in this regard.

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