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Similarities Between the Conformation of Arsanilazotyrosine 248 of Carboxypeptidase A_α in the Crystalline State and in Solution

(enzyme activity/stopped-flow pH-jump)

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ABSTRACT Modification of carboxypeptidase A_γ crystals (Anson) with diazotized arsanilic acid specifically labels tyrosine 248; at pH 8.2 the modified enzyme gives yellow crystals, but a red solution. It has been suggested that arsanilazotyrosine 248 forms a complex with the Zn cofactor accounting for the red color in solution, but that a complex is not formed in the crystal. However, the crystal structure of carboxypeptidase A_γ is unknown. We show here that crystals of carboxypeptidase A_α, whose crystal structure has been determined, are red both in solution and in the crystalline state (at pH 8.2) after modification with diazotized arsanilic acid. These new data are of importance in relating the structure in the crystalline state to the catalytic mechanisms, as based on the x-ray diffraction evidence.

The activity of carboxypeptidase A in the crystal and in solution has a ratio of only 1/3 for the α form, in contrast to the ratio of 1/300 for the γ form, with carbobenzoxyglycyl-L-phenylalanine as a substrate.

A pH-jump experiment monitored by stopped-flow kinetics in a split-beam apparatus has revealed a single exponential rate when a solution of arsanilazotyrosine 248 carboxypeptidase A_α at pH 6.7 (yellow) is increased to pH 8.5 (red). The rate constants obtained in this experiment are 6.1 sec^{-1} at 3.0 mg/ml and 7.2 sec^{-1} at 1.6 mg/ml concentration of enzyme.

The relationship of the three-dimensional structure to biological function of carboxypeptidase A (CPA; EC 3.4.2.1), a proteolytic enzyme that cleaves most C-terminal amino acids from peptide substrates, has been correlated (1). In particular, the three-dimensional structure of CPA_α* indicates that there are 307 amino acids (2) in the single chain, that the cofactor Zn^{++} is near the active site, and that there are only two side chains (Glu 270 and Tyr 248) of the enzyme that can approach within Van der Waals contact with atoms of the substrate at the bond to be cleaved (3). The structure of native CPA_α at pH 7.5 and 4° shows that the OH group of Tyr 248 is some 1.2 nm (12 Å) away from the position to which it moves when a substrate such as glycyl-L-tyrosine is bound. Comparison of the activity of CPA_γ, which has seven fewer amino acids at the N-terminus than does CPA_α, in solution and crystalline states, has shown that at pH 7.5 and low ionic strength the maximum specific activity of small crystals is less than that in solution by a factor of 300 (4).

Recently, Vallee and coworkers (5–8) have modified Tyr 248 of carboxypeptidase A_γ with diazotized arsanilic acid, and have found a color change from yellow in the crystalline state to red in solution at pH 8.2. Similar color changes in model azophenols indicate a color change from yellow (or orange) to red (or purple) upon complexing with zinc or other metal ions (9). Vallee and coworkers, therefore, suggest that, in solution, the azophenol complexes with the Zn of CPA_γ, but not in the crystals. They state further that these deductions are consistent with the three-dimensional x-ray structure, but raise questions concerning the interpretation of mechanisms based upon these x-ray results.

Inasmuch as several different crystal forms of bovine CPA, depending upon the source of material and the method of preparation, were available to us, we undertook similar chemical modification studies on these various forms. We were particularly interested in the behavior of the CPA_α used in x-ray diffraction studies. Further, we reexamine below the optical properties of crystal and solution phases of the arsanilazotyrosine 248 derivatives of CPA_α and CPA_γ. We also have determined the specific activity of crystals of CPA_α, and a preliminary result on the pH-jump kinetics of arsanilazotyrosine-248 CPA_α in solution is presented.

MATERIAL AND METHODS

The enzyme itself and its crystals, identical to those used in the x-ray analysis, were prepared from bovine pancreatic juice (10). The crystal habit shows elongation along the *a* axis. Similar preparations from other samples of bovine pancreatic juice nearly always yielded crystal habit showing elongation along the *b* axis. Similarly, crystals of the commercially available Anson (11) preparation (Worthington) are likewise elongated along the *b* axis. All of these crystals are monoclinic, but not all have the same unit-cell parameters. For examples, crystals of CPA_α have unit-cell parameters a = 51.41 Å, b = 59.89 Å, c = 47.19 Å and β = 97°35′, while crystals of CPA_γ have parameters a = 50.9 Å, b = 57.9 Å, c = 45.0 Å and β = 94°40′. However, the work described below pertains mainly to the x-ray preparation (CPA_α) and to the commercial preparation (CPA_γ). Although there are some physical differences between CPA_α and CPA_γ, such as solubility and reconstitution of Zn-free enzyme, nevertheless both forms have comparable sedimentation coefficients and specific activities (12).

In the modification experiments, uniform microcrystals were reacted with diazotized arsanilic acid (6), and then were washed with 0.01 M $KHCO_3$ (pH 8.5) because we found that the recommended procedure of washing with water (6) considerably disorders the crystals.

Absorption spectra were taken at 8° by a Beckman DB-GT recording spectrophotometer. The spectra of the modified CPA crystals were corrected for light scattering (13), with

Abbreviation: CPA, carboxypeptidase A.

* CPA_α has 307 amino acids, while CPA_γ has seven amino-acid residues missing from the N-terminus.

the use of a suspension of unmodified crystals as a reference. Enzymatic activity of unmodified crystals of CPA$_\alpha$ and CPA$_\gamma$ was assayed by a standard procedure (4). Analyses were made of the arsanilazo content (14).

The pH-jump, stopped-flow experiments were performed in a split-beam apparatus, a modification of that designed by Yates, McMurray, and Gutfreund (15), shown in Fig. 1. Arsanilazocarboxypeptidase A$_\alpha$ crystals were dissolved in 0.01 M Tris·maleate–1.0 M NaCl (pH 6.7), and this yellow solution was then mixed in the stopped-flow apparatus with a buffer solution of 0.2 M Tris·HCl–1.0 M NaCl (pH 8.5). The final pH was 8.4. The color change to red was followed at 510 nm.

The pK$_a$ of the change at 510 nm and the binding constant of Gly-L-Tyr at pH 8.4 were determined in the apparatus shown schematically in Fig. 2. For the pK$_a$ study, a 5.0 N solution of NaOH in a syringe was titrated incrementally to a 2-ml solution containing arsanilazo CPA$_\alpha$, 1.0 M NaCl, 0.01 M Tris·maleate (pH 6.4). For the binding constant of Gly-L-Tyr, a total volume of 112 μl of a solution of 0.03 M Gly-L-Tyr–1.0 M NaCl–0.2 M Tris·HCl (pH 8.2) was titrated continuously from a syringe to a 2.0-ml solution containing 0.66 mg arsanilazo CPA$_\alpha$,1.0 M NaCl, 0.2 Tris·HCl (pH 8.2). These data were analyzed by a nonlinear least-squares program in Fortran IV for an IBM 1130 computer. Parameters for the pK$_a$ determination were (*a*) the pK$_a$, (*b*) the absorbancy at 510 nm for the species AH, and (*c*) the absorbance at 510 nm for the species A$^-$, if we assume a single-proton ionization. Parameters for the binding of Gly-L-Tyr to arsanilazo CPA$_\alpha$ at pH 8.2 were (*a*) the dissociation constant K$_{DISS}$, (*b*) the absorbance at 510 nm at saturation of the ligand (Gly-L-Tyr), and (*c*) a correction to absorbance for dilution upon addition of ligand. A single binding site was assumed, in agreement with the x-ray results.

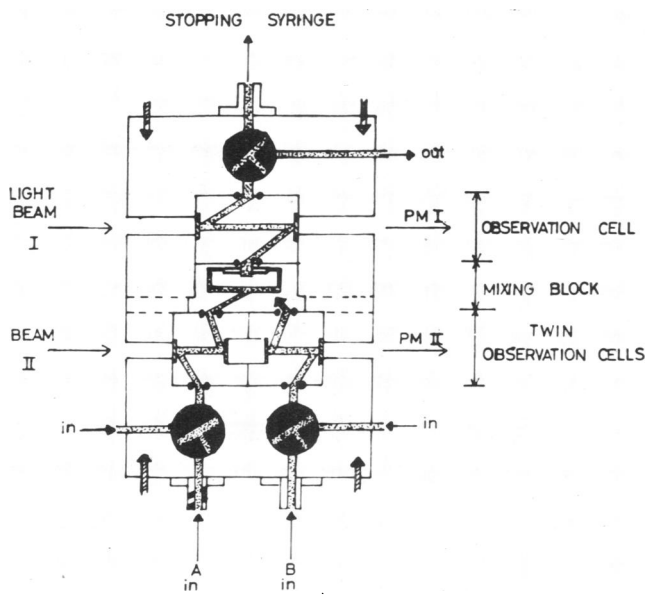


FIG. 1. Split beam stopped-flow apparatus, showing entrance of the two solutions at points labeled *A* and *B*, their absorption measurement by beam II, which passes through two independent observation cells, followed by a mixing cell within the mixing block, and by absorption measurement of the mixed solution by beam I. Differential output between photomultipliers PM I and PM II are displayed on an oscilloscope, or converted directly through a log amplifier to give a direct measure of optical absorbance.

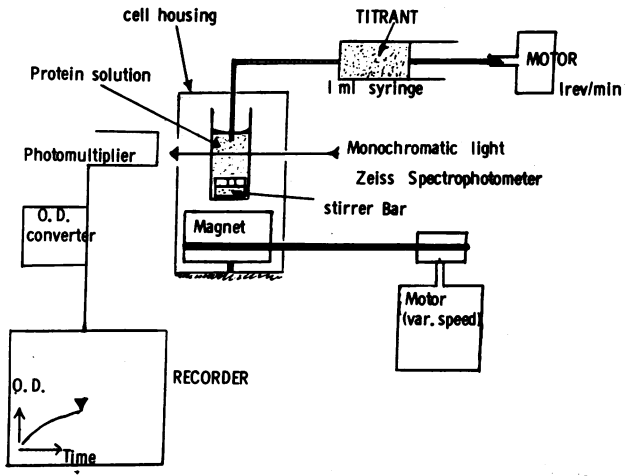


FIG. 2. Schematic diagram of apparatus for spectrophotometric measurement of ionization constants and binding constants. The diagram shown applies to the binding experiment discussed in the text. In the experiment for determination of the pK$_a$, a pH electrode (GK 23210) from a model 28 pH meter (Radiometer, Copenhagen) was inserted into the stirred cell of the spectrophotometer directly above the optical path. Aliquots of 5 N base were added incrementally from the syringe.

RESULTS AND DISCUSSION

We have repeated the experiments of Vallee *et al.*, and are in agreement with their experimental results: when crystals obtained from a commercial preparation by the method of Anson are reacted with diazotized arsanilic acid, these crystals are yellow at pH 8.2, but turn red when dissolved at pH 8.2. In the solution phase an additional band appears centered at 510 nm (Fig. 3). The same result was obtained with crystalline preparations that had the same crystal form (elongated along the *b* axis) as the commercial form, but were obtained by the method used for the crystals obtained in the x-ray analysis. The number of arsanilazo groups per enzyme molecule was close to unity (0.85–0.95) in these and subsequent experiments described below for CPA$_\alpha$. All forms of CPA modified in the solid state gave full enzymatic activity as assayed in solution at pH 7.5 by a standard procedure (16). Our results on this activity, and the number of tyrosines

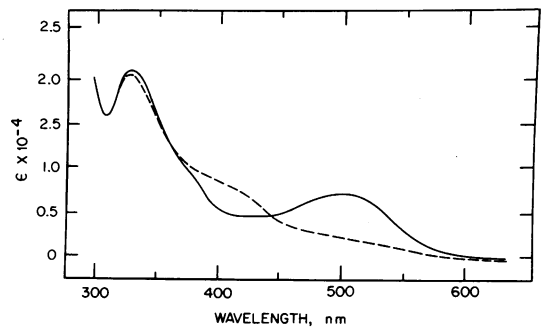


FIG. 3. Absorption spectra of zinc arsanilazocarboxypeptidase A$_\gamma$ crystals suspended in 0.02 M Tris·HCl (pH 8.2) (– – –) and of the same arsanilo-CPA dissolved in 0.02 M Tris·HCl (pH 8.2)–1.0 M NaCl (——). Native crystals were obtained from commercial preparations by the method of Anson (11).

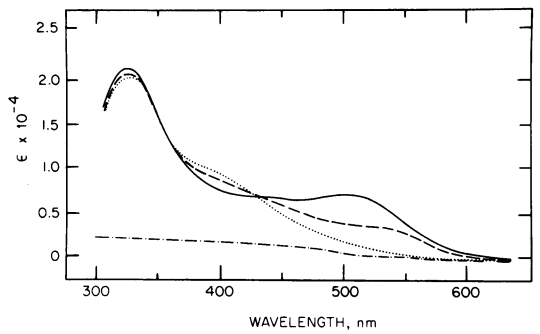


FIG. 4. Absorption spectra of a single batch of zinc arsanilazocarboxypeptidase A_α crystals suspended in 0.02 M Tris·HCl (——, pH 8.2), (– – –, pH 7.4), and in deionized water (· · · ·). Spectra were taken in the order listed, but an experiment performed in the reverse order gave similar spectra. The bottom line (–·–·) is the spectrum of the supernatant obtained after sedimentation of the crystal upon completion of the spectrum at pH 8.2.

modified, are in agreement with results of Vallee *et al.* (6–8), who studied CPA_γ.

We find that treatment of CPA_α with diazotized arsanilic acid yields red crystals at pH 8.2 and that the color remains red when the crystals are dissolved at pH 8.2. The enzyme preparation is exactly that used in the x-ray analysis, and we feel that this is the appropriate result to be interpreted in terms of the published x-ray structure. In both crystalline and solution states an absorption band is present near 510 nm

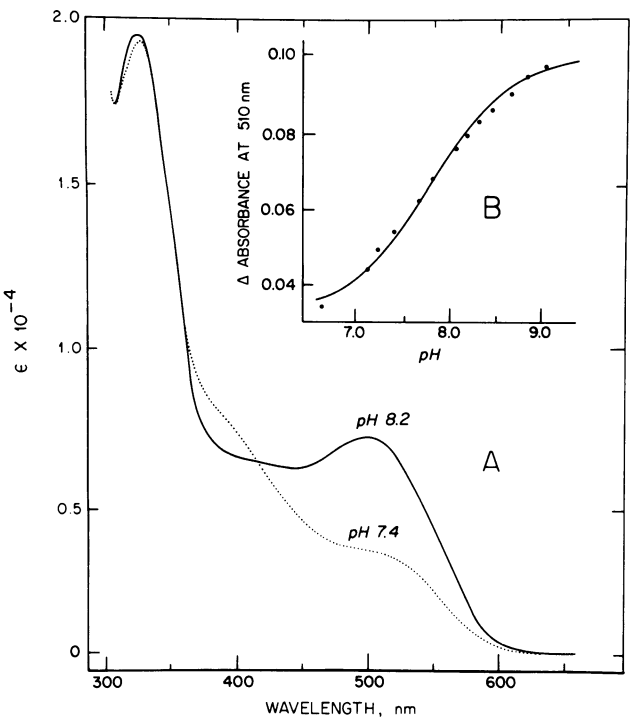


FIG. 5. (*A*) Absorption spectra of zinc arsanilazocarboxypeptidase A_α identical to that used in Fig. 4 but dissolved in 0.02 M Tris·HCl (pH 8.2)–1.0 M NaCl (——). The spectrum at pH 7.4 (· · ·) was obtained from the same solution used at pH 8.2 after dialysis to pH 7.4 with 0.02 M Tris·HCl (pH 7.4)–1.0 M NaCl. (*B*) Difference absorption spectrum at 510 nm of arsanilazocarboxypeptidase A_α as a function of pH. The computer fit is the *solid line* through the experimental points.

at pH 8.2 (Figs. 4 and 5*A*). At pH 7.5 (4°), at which the crystal structure was determined, Tyr 248 is away from the active site; at this same pH the crystals and solution of arsanilazo Tyr 248 CPA_α are nearly yellow (Figs. 4 and 5*A*). We therefore caution against a general interpretation of the results of Vallee *et al.*, and we also suggest that their results for CPA_γ are not readily interpretable in terms of the known crystal structure of CPA_α.

The red color at pH 8.2 of either crystals or solution of CPA_α can be changed to yellow by (*a*) lowering of the pH to 7.4 (Figs. 4 and 5*A*), (*b*) addition of a chelating agent, 1,10-phenanthroline, (*c*) addition of a substrate glycyl-L-tyrosine, or (*d*) addition of the inhibitor L-phenylalanine.

Measurements of the pK_a of the pH change at 510 nm in solution yielded 7.78 ± 0.04 for modified CPA_α (Fig. 5*B*) and 7.72 ± 0.04 for modified CPA_γ. For modified CPA_α crystals, roughly the same value was found. Results of the pH-jump, stopped-flow experiment (pH 6.7–8.4) described above yielded, for the single exponential rate observed, rate constants of 6.1 sec^{-1} (Fig. 6) and 7.2 sec^{-1} at enzyme (CPA_α) concentrations of 3.0 mg/ml and 1.6 mg/ml, respectively. It is probable that this rate reflects an ionization-induced conformational change of the modified tyrosine 248, from the yellow arsanilazotyrosine to the red form of arsanilazotyrosine. Since one would expect the simple ionization of the arsanilazo tyrosine 248 to be fast, and incapable of being detected by the technique used in these experiments, the rates we are observing must reflect some slow rate-limiting process. Similarly, the observed pK_a will not be the direct simple ionization of the arsanilazotyrosine but will be the pK_a of this rate-limiting process. The assignment of a unique step to this process cannot yet be made: some of the possibilities are (*a*) movement of Tyrosine 248 to the active site (1), (*b*) ligation of the arsanilazotyrosine with the zinc, (*c*) displacement of H_2O from the zinc, (*d*) other conformational changes, or some combination of these processes. The assignment of the observed rate to any step in the catalytic mechanism is also uncertain. It should be noted, however, that this rate is very similar to that observed for the rate-limiting process (k_{cat}) for peptide hydrolysis by use of substrates with reasonable turnover numbers (1). Attempts to locate this

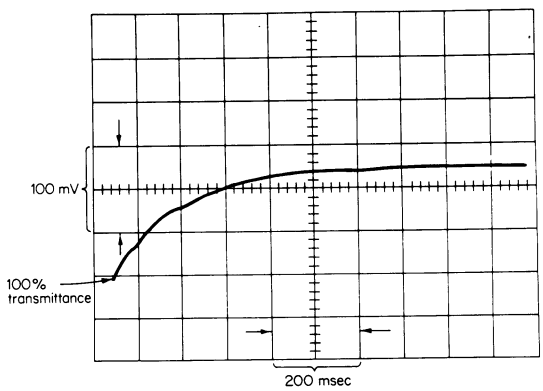
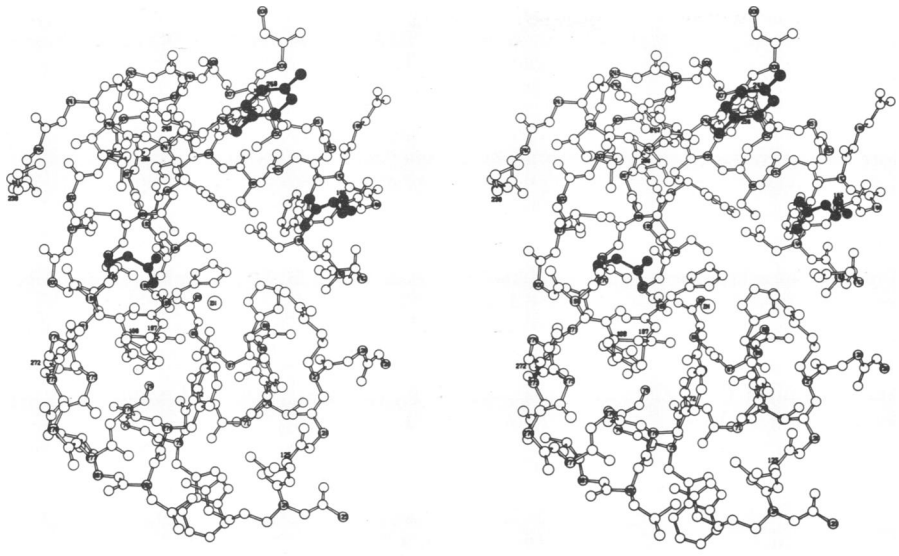
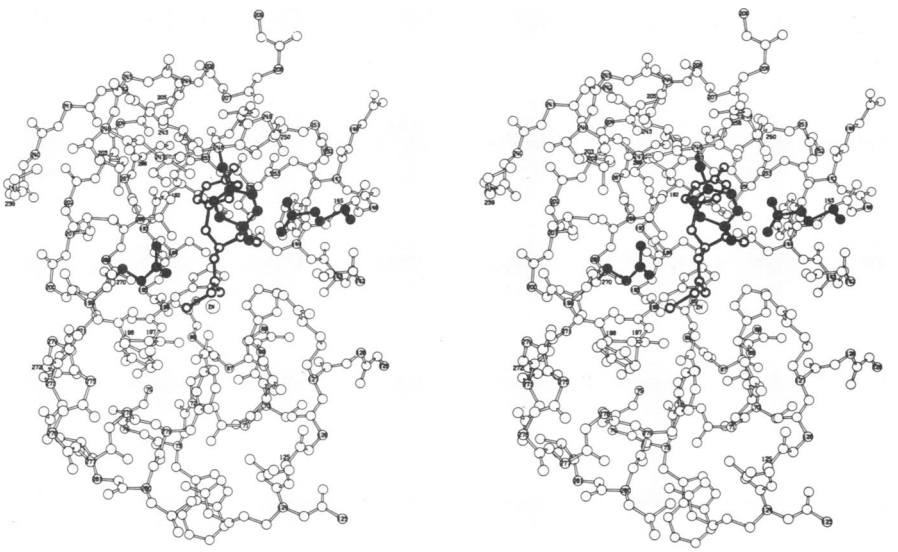


FIG. 6. pH-jump, stopped-flow experiment of the rate of change of arsanilazocarboxypeptidase A at 510 nm. 6.2 mg/ml of azo-CPA in 0.01 M Tris·maleate (pH 6.7)–1.0 M NaCl was mixed with 0.2 M Tris·HCl (pH 8.5)–1.0 M NaCl in a split-beam, stopped-flow apparatus. The final enzyme concentration and pH after mixing are 3.0 mg/ml and pH 8.4, respectively.



a

FIG. 7*a*. Stereoview of about one-quarter of the CPA molecule, showing the active site [*1–3*]. The remainder of the protein continues below, and to the left of, the β-structure on the left. The Zn^{+2} is bound to N_1 of His 69, O of Glu 72, N_1 of His 196, and to a water molecule (not shown). Side chains of Arg 145, Tyr 248, and Glu 270 are shown (as *solid discs*) before their conformational change (see Fig. 7*b*) upon addition of the peptide substrate.



b

FIG. 7*b*. Stereoview of the same region as Fig. 7*a* after Gly-Tyr is bound (*heavy circles*) and after conformational changes have occurred for residues Arg 145, Glu 270, and Tyr 248 (*solid discs*). The N-terminal NH_2 (or NH_3^+) of Gly-Tyr is hydrogen bonded to a water molecule, which is hydrogen bonded to the carboxylate group of Glu 270. The salt link from the carboxylate group of Gly-Tyr to the guanidinium group of Arg 145 consists of two hydrogen bonds. In making the large conformational change, the ring of Tyr 248 moves 8 Å and its OH group moves 12 Å by rotation of this side chain about the C_α—C_β bond by about 120°.

structural change in x-ray diffraction studies of arsanilazotyrosine-248 CPA have failed, so far, because of a tendency for crystal disorder to occur.

The dissociation constant of Gly-L-Tyr for its complex with arsanilazotyrosine-248 CPA_α was found from the experiment described above to be 7.8×10^{-4} M ($\pm 0.5 \times 10^{-4}$ M) at pH 8.2. This value is comparable with that of 7×10^{-4} M for the kinetic parameter K_M for the interaction of Gly-L-Tyr with native unmodified CPA at pH 7.5 (17). This agreement is reasonable because k_{cat} is sufficiently small that K_M is nearly an equilibrium constant for this very slowly cleaved substrate, and is comparable to K_I when Gly-L-Tyr is used as an inhibitor.

The locations of Tyr 248 relative to the active site and Zn atom of CPA is of critical importance in relating the three-dimensional structure to mechanism, in correlating the structures in the crystal and in solution, and in interpreting the spectral properties of the arsanilazotyrosine-248 derivative.

The crystallographic analysis of CPA_α (at pH 7.5 and 4°) places Tyr 248 away from the active site with its OH some 17 Å from Zn (Fig. 7*a*). When substrates or inhibitors are bound, the 120° rotation about the α-β bond of Tyr 248, plus a small movement of the peptide chain of Tyr 248, brings the OH of Tyr 248 within about 3 Å of the NH of the scissile bond of a peptide substrate (Fig. 7*b*). This movement is facilitated by the observed 2-Å conformational change of Arg 145, but seems to be otherwise unhindered both within the molecule and in the three-dimensional structure. Nevertheless, the OH of Tyr 248 is still some 4–5 Å away from Zn. We note that a further movement of Tyr 248 is required if its oxygen is to coordinate to Zn in the red complex, as suggested by Vallee and coworkers for the arsanilazo complex. This additional movement would require a large repositioning of the extended polypeptide chain around Tyr 248. In the absence of a definitive x-ray diffraction study of arsanilazotyrosine-248 CPA_α we are unsure of the structural interpretation of the

color behavior, but this behavior is the same for our modified CPA_α in both the crystal and solution.

Although no general statement can be made about comparisons of behavior of enzymes and other proteins in the crystal and in solution, the excellent review by Rupley (18) indicates that kinetic parameters are more likely to be affected than are equilibrium thermodynamic parameters. Indeed, the fact that enzymatic activity can be observed at all in the crystal is due to the large proportion of solvent in the crystal structure. Also, the active site is usually in a crevice or depression in the enzyme surface, and therefore is less likely to be in regions of intermolecular contact than other parts of the molecule. Our results here indicate a striking difference between the behavior of crystals of modified CPA_α and crystals of modified CPA_γ in a spectral change. Also, enzymatic activities of crystals of CPA_α and of CPA_γ are quite different. We therefore suggest that when activity is strongly modified in crystals of one type, it may be possible to find another type of crystal in which the modification of activity is considerably less. However, this procedure may not be helpful if very large conformational shifts occur among subunits of an enzyme complex.

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