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Intramolecular arsanilazotyrosine-248·Zn complex of carboxypeptidase A: A monitor of multiple conformational states in solution

(coordination complex/protein conformation/stopped-flow temperature jump kinetics/enzyme mechanism/spectral probe)

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ABSTRACT **The red azoTyr-248·Zn complex of arsanilazocarboxypeptidase, previously used to demonstrate differences in conformation of the enzyme in crystals and in solution, has now provided means to detect multiple conformations of the enzyme in solution by stopped-flow pH and temperature jump experiments. These studies identify two distinct processes. $E_r + H^+ \rightleftarrows E_y$ (I), is the extremely rapid, k_{fast} about 10^5 sec^{-1}, pH dependent dissociation of the metal complex. $E_y \rightleftarrows E_y'$ (II), is the much slower, k_{slow} about 5 sec^{-1}, *pH independent* interconversion of two distinct populations of protein molecules, E_y and E_y', in which the yellow azoTyr-248 is in different conformations. These two conformations can be differentiated readily by stopped-flow pH-jump experiments, since I is three to four orders of magnitude faster than II. Mathematical expressions derived from this mechanism accurately predict all observations over the pH range from 6.0 to 8.5.**

In a previous stopped-flow pH-jump experiment, Lipscomb and coworkers [Quiocho, F. A., McMurray, C. H. & Lipscomb, W. H. (1972), *Proc. Nat. Acad. Sci. USA* 69, 2850–2854] recognized only a single process with a rate constant of about 6 sec^{-1}, but not the major, very rapid rate observed here. The failure to detect this fast process led to the postulation of a number of explanations intended to account for the detection of only a single, slow rate. The present observations show that the premise for those conjectures is not valid.

The azoprobe reveals the existence of rapidly interconvertible substructures of carboxypeptidase A, and the results support the view that in solution, enzymes can adopt multiple, readily interconvertible and related conformations which could then either facilitate or impede catalysis. In crystals, rearrangement of molecular structure could be severely impaired or restricted, and crystallization might single out either active or inactive conformations. In the latter case, such crystals would have greatly reduced activities and markedly altered catalytic behavior, as is observed for carboxypeptidase A. In combination with detailed kinetic analysis of crystals, conformational analysis in solution should be a valuable guide to discern enzyme mechanisms and select crystals for x-ray structure analysis.

Aromatic azo compounds exhibit a number of characteristic spectral properties (1–3) that can be utilized as optical probes of protein structure. Previous studies from this laboratory have taken advantage of these properties to demonstrate that the conformation of arsanilazocarboxypeptidase A in solution differs from that in the crystalline state (4–6). Moreover, it was pointed out that in either of these two physical states the enzyme may exhibit multiple conformational states that might not have been detectable by the methods and approaches used in the past. In this regard, spectrochemical probes, located in specific sites of a given enzyme, were shown to present distinct opportunities for defining structural details of enzymes in solution with a high degree of precision (4, 7). The red azoTyr-248·Zn complex of azocarboxypeptidase now has proven to be a means of inspecting catalytic events and detecting multiple conformations in solution by stopped-flow, pH jump, and temperature jump experiments. Under one set of conditions, the rapid disruption of the complex due to substrate binding can be visualized directly by the disappearance of the red color (8, 9). Under another set of conditions, the probe identifies a pH independent equilibrium between two carboxypeptidase conformers, neither of which is red. These findings serve as a general model that may account for quantitative differences of conformations in solution, in crystals, and in different crystal habits.

MATERIALS AND METHODS

Carboxypeptidase A_α (Sigma Chemical Corp.) and A_γ (Worthington Biochemical Corp.) were modified with diazotized arsanilic acid according to published procedures (4, 6, 7). Both enzyme forms gave analogous results. All other chemicals were reagent grade. Precautions to prevent contamination by adventitious metal ions (10) were taken throughout. Stock solutions of azoenzyme, 5×10^{-4} M, were prepared in 1 M NaCl, pH 7, and after centrifugation, diluted into appropriate, degassed solutions prior to stopped-flow experiments.

Stopped-flow experiments were performed with a Durrum-Gibson instrument equipped with a Durrum fluorescence accessory no. 16400, a 75-W Xenon lamp and an end-on EMI 9526B photomultiplier. The instrument was calibrated with a Cary 14 recording spectrophotometer to yield analogous spectra under rapid kinetic conditions. Identical data were obtained with a Durrum stopped-flow instrument equipped to read absorbance directly, kindly furnished by Dr. Thomas C. Bruice.

RESULTS

Spectral measurements of arsanilazocarboxypeptidase† at equilibrium (4–7) and the kinetics of native carboxypeptidase (11) have demonstrated significant differences in the conformations of the enzyme in crystals and in solution. It was postulated earlier (12) that in solution, at least, enzymes

Abbreviations: Mes, 2-(*N*-morpholino)ethanesulfonic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

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† In order to simplify presentation, zinc arsanilazocarboxypeptidase, azocarboxypeptidase, and azoTyr-248-carboxypeptidase are used interchangeably with zinc monoarsanilazotyrosine-248 carboxypeptidase, of enzymes forms α or γ. AzoTyr-248 refers to the azophenol of monoarsanilazotyrosine-248, and the azophenolate to its ionized species.

Table 1. Amplitudes of fast and slow processes* (stopped-flow jumps to pH 8.5)

Initial pH	$\Delta A_{fast}:\Delta A_{slow}$	$k_{slow}(sec^{-1})$
6.0	60:40	5.0
6.6	55:45	5.0
7.1	56:44	5.0
7.6	63:37	5.0
Average	58:42	
Predicted†	56:44	

* Absorbance changes monitored spectrophotometrically at 510 nm. The initial enzyme in 5 mM Mes, 1.0 M NaCl was mixed at 25° with 0.10 M Tris, pH 8.5, 1.0 M NaCl.

† The ratio of amplitudes calculated from Eq. 2, where $pK_1 = 7.45$ and $K_2 = 1.11$.

can adopt multiple, readily interconvertible but closely related conformations. Demonstration of the validity of this hypothesis would clearly be most important in assigning functional significance to, e.g., the spatial position of the side chain of Tyr-248 in the catalytic mechanism of carboxypeptidase. The present data show that rapid kinetic studies, based on pH and temperature jump techniques (13), can detect and characterize such conformational states in solution.

Our previous stopped-flow, pH, and temperature jump studies demonstrated that the intramolecular coordination complex between azoTyr-248 and the active site zinc atom of azocarboxypeptidase A serves as a spectrokinetic probe of catalytic events (8, 9). Stopped-flow rapid mixing of azocarboxypeptidase with substrate at pH 8.5, where the intramolecular azoTyr-248-Zn coordination complex is maximally formed, abolishes 100% of the red complex within 3 msec (9). The spectral characteristics reflect the effect of the substrate, synchronized with catalytic events.

However, stopped-flow pH jump studies at 510 nm demonstrate that on mixing azoTyr-248 carboxypeptidase at pH 8.5 with pH 6.0 buffer, 97% of the red azoTyr-248-Zn complex is converted to the yellow azophenol within 3 msec, the mixing time of the stopped-flow instrument (Fig. 1A). Temperature jump studies show that over this pH range the rate constant of this rapid process varies from 100,000 to 50,000 sec^{-1}.‡ The remaining 3% of the total absorbance change occurs slowly, with a first-order rate constant of 0.4 sec^{-1}, reflecting a second process, kinetically distinct from the extremely rapid dissociation of the coordination complex itself. This residual slow change implies a relaxation process signaling a change in conformation and, hence, suggests the existence of different distinct conformations of yellow azocarboxypeptidase in solution.

Results from pH jumps from pH 6.0 to 8.5 differ markedly from those obtained when the pH is jumped from pH 8.5 to 6.0. Now only about 60% of the total absorbance at 510 nm is generated during the 3 msec mixing time of the instrument; the remaining 40% of the absorbance appears with a first-order rate constant of 5.0 sec^{-1} (Fig. 1B). Indeed, these data suggest that at pH 6, where the yellow species predominates, at least two protein populations exist: one readily forms the metal complex while the other does not. Thus, these experiments performed over a constant pH interval detect two populations through the effects of pH on

‡ The pH dependence of the extremely rapid rate was determined by temperature jump analysis and will be reported.

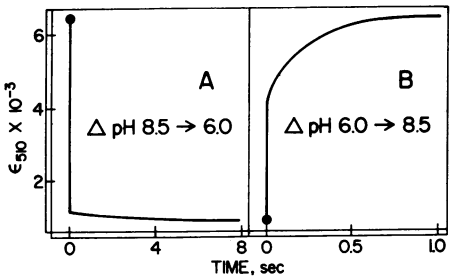

FIG. 1. Stopped-flow pH jump of azoTyr-248-carboxypeptidase A. (A) From pH 8.5 to 6.0. The circle (●), top left, represents the absorbance of the azoTyr-248-Zn complex at zero time; the vertical line, the rapid absorbance change on mixing. The exponential decrease in absorbance, at the bottom, is the slow process ($k = 0.4$ sec^{-1}). (B) From pH 6.0 to 8.5. The circle (●), bottom left, represents the absorbance of the azophenol at zero time; the vertical line, the absorbance change on mixing. The exponential increase in absorbance, at the top, is the slow process ($k = 5.0$ sec^{-1}). Final conditions: enzyme, 25 μM, 50 mM 2-(*N*-morpholino)ethanesulfonic acid (Mes) (pH 6.0) or 50 mM Tris (pH 8.5), 1 M NaCl, 25°.

the azoTyr-248-Zn complex itself, but they do not reveal the pH dependence, if any, of the equilibrium governing the relative proportions of the two populations.

The effect of pH on this equilibrium was investigated by varying the initial pH while keeping the final pH constant. Stopped-flow mixing of a series of enzyme solutions at pH 6.0, 6.6, 7.1, and 7.6 with buffer at pH 8.5 leads to the formation of the red azoTyr-248-Zn complex, monitored by the increase in absorbance at 510 nm. In all instances approximately 60% of the total change in absorbance occurs with a rate constant of about 10^5 sec^{-1} while the remaining 40% changes slowly, $k_{slow} = 5$ sec^{-1} (Table 1). Analogous results are obtained when the initial pH values are 6.4, 7.1, and 7.5 and the final pH is 8.0 (Table 2). Thus, the equilibrium is not dependent on pH since the same proportions are found even though the pH of the initial equilibrium solution varies from pH 6.0 to 7.6.

The simplest mechanism consistent with these data is:

$$E_r + H^+ \overset{K_1}{\leftrightharpoons} E_y \qquad (I)$$

$$E_y \overset{K_2}{\leftrightharpoons} E_y' \qquad (II) \qquad [1]$$

In Process I, E_r is the red coordination complex and E_y the yellow azophenol. In the isomerization, Process II, E_y' is a second yellow conformational state indistinguishable spectrally from E_y. It is obligatory that in only the E_y form can

Table 2. Amplitudes of fast and slow processes* (stopped-flow jumps to pH 8.0)

Initial pH	$\Delta A_{fast}:\Delta A_{slow}$	$k_{slow}(sec^{-1})$
6.4	61:39	2.1
7.1	54:46	2.1
7.5	56:44	2.1
Average	57:43	
Predicted†	60:40	

* Absorbance changes monitored spectrophotometrically at 510 nm. The initial enzyme in 5 mM Mes, 1.0 M NaCl was mixed at 25° with 0.1 M Tris, pH 8.0, 1.0 M NaCl.

† The ratio of amplitudes calculated from Eq. 2 where $pK_1 = 7.32$ and $K_2 = 1.11$. These values result from an analysis of a set of data obtained over the pH range 6.4 to 8.2.

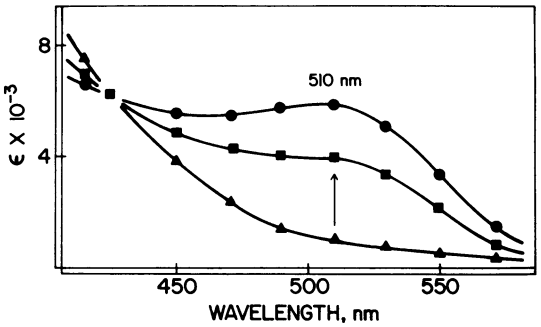

FIG. 2. Spectra of azoTyr-248-carboxypeptidase A, reconstructed from stopped-flow pH jump experiments from pH 6.5 to 8.2 performed at 20 nm intervals. The spectrum at zero time (▲) changes in 3 msec to the spectrum of 60% of the azoTyr-248-zinc complex (■) followed by a slower exponential change (k = 2.2 sec^{-1}) to finally reach the equilibrium spectrum (●) of azoTyr-248-Zn complex. Final conditions: enzyme, 25 μM, pH 8.2, 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes), 1 M NaCl, 25°.

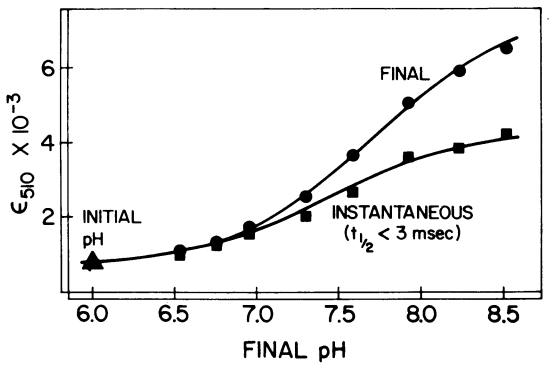

FIG. 3. Experimental and predicted pH dependence of fast and slow processes. Stopped-flow jumps of azoTyr-248-carboxypeptidase each from pH 6.0 to more alkaline pH values. The triangle (▲), bottom left, represents the absorbance of the azophenol at zero time of each experiment. For each final pH this absorbance (▲) increases within 3 msec to an intermediate absorbance (■), followed by a slower exponential increase to equilibrium (●). The predicted curves, superimposing the experimental data, were calculated from Eq. 2 where $E_y:E_y'$ = 53:47. Final conditions: enzyme, 25 μM, 1 M NaCl, 25°, buffers; 50 mM Mes (pH 6.5 and 6.7), 50 mM Hepes (pH 6.9 to 7.8), and 50 mM Tris (pH 8.2 and 8.5).

the azoTyr-248 moiety complex the zinc atom to form E_r. Since Process I in either direction is three to four orders of magnitude faster than is Process II, stopped-flow pH jump kinetics readily differentiate between them and allow each process to be considered separately, i.e., each can be treated in terms of relevant equilibrium constants. Thus, K_1, the equilibrium constant for Process I, is pH dependent, but K_2, that for Process II, is not. Even though both E_y and E_y' are yellow and, hence, can not be directly differentiated by their spectra, the amplitudes of these stopped-flow measurements are quantitative measures of their concentrations. Hence, the relaxation process becomes a precise gauge of their interconversion. For example, on lowering pH, E_r and E_y rapidly reequilibrate governed by the equilibrium constant, K_1. This process is coupled to the slow reequilibration of E_y and E_y', allowing further conversion of E_r to E_y. At pH 6.0 the ratio of $E_y:E_r$ is large; hence, on jumping from pH 8.5 to 6.0 virtually all of the color change occurs extremely rapidly, reflecting the disruption of the red azoTyr-Zn complex. In contrast, the pH jump from pH 6.0 to 8.5 reveals that at pH 6.0 E_y and E_y' represent about 60% and 40% of the total enzyme population, respectively. Importantly, this ratio remains constant on varying the initial pH while maintaining the final pH constant (Tables 1 and 2), indicative of the pH independence of the ratio of $E_y:E_y'$.

Discrimination of E_y and E_y' does not depend on the wavelength at which the observations are made.[§] The spectrum reconstructed from data obtained immediately after jumping pH from 6.4 to 8.0 and monitoring at 20 nm intervals between 410 and 570 nm (Fig. 2) is indeed that characteristic of the azoTyr-248-Zn coordination complex. The change in molar absorptivities attained immediately are only about 60% of those prevailing at equilibrium. However, the spectrum then slowly changes (k_{slow} = 2.1 sec^{-1}) to become identical with that at equilibrium. No additional transient processes are detected. Both initially and finally, the position of the maximum remains constant at 510 nm and a tight isosbestic point is observed at 427 nm, demonstrating that only two chromophoric species are present throughout. Since one of these is the red azoTyr-248-Zn complex, the spectra of the different yellow populations, discerned by their effects on the kinetics, must be identical.

These experiments indicate that the proposed mechanism describes the events observed. Hence, if the proportion of $E_y:E_y'$, i.e., K_2, is known, the results to be expected from the pH jump experiments in both the alkaline and acid directions over the pH interval from 6 to 8.5 can be calculated. The pH dependence of the amplitudes under these conditions can be formulated as:

$$[E_r]_{eq.} = \frac{[E_T]}{1 + a_H K_1^{-1}(1 + K_2^{-1})} \quad \text{(a)}$$

$$[E_r]_{fast} = \frac{[E_T]_{fast}}{1 + a_H K_1^{-1}} \quad \text{(b)} \qquad [2]$$

where $[E_r]_{eq.}$ is the concentration of E_r at equilibrium, $[E_r]_{fast}$ is the concentration immediately after the 3 msec mixing process, and a_H is the final hydrogen ion activity after mixing. In equation 2b, $[E_T]_{fast}$ is the sum of the concentration of E_r and E_y present initially, i.e., before the pH jump.

A program based on Eq. 2 was written for the Hewlett-Packard 9100 calculator. A series of ratios of the two conformers was postulated, and calculated curves[¶] were generated for jumping pH from 6.0 to different alkaline end points (Fig. 3). The best fit to the experimental data is obtained for a pK_1 = 7.45 and a ratio of $E_y:E_y'$ = 53:47. The same parameters were used to calculate the results expected for pH jumps from pH 8.5 to different acidic pH values (Fig. 4). Again, the theoretical curve and the experimental data coincide very closely. Thus, Eq. 2 is consistent with both present kinetic and previous spectral equilibrium data (5, 6) and thereby accounts for all observations (Tables 1 and 2, Figs. 1–4). Furthermore, an overall pK_{app} value of 7.7 was calculated from the kinetic data [$K_{app} = K_1(1 + K_2^{-1})^{-1}$] encompassing both reactions I and II. This value is identical

[§] Wavelengths must be chosen, of course, where there is a significant difference in the molar absorptivities of the E_y and E_r species to achieve such discrimination.

[¶] The concentrations of $[E_y]_{eq.}$, $[E_y']_{eq.}$, $[E_y]_{fast}$, and $[E_y']_{fast}$ were included in the calculation. At 510 nm in 1 M NaCl, 25°, the molar absorptivity values are 650, 7500, and 9500 cm^{-1} for the azophenol, azoTyr-Zn complex, and azophenolate species, respectively.

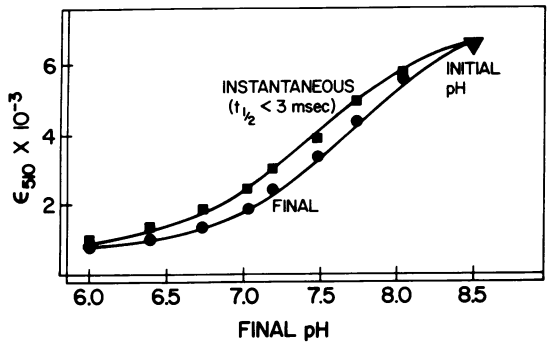

FIG. 4. Experimental and predicted pH dependence of fast and slow processes. Stopped-flow jumps of azoTyr-248-carboxypeptidase each from pH 6.0 to more acidic pH values. The triangle (▼), top right, represents the absorbance of the azoTyr-248-Zn complex at time zero of each experiment. For each final pH, this absorbance (▼) decreases within 3 msec to an intermediate absorbance (■), followed by a slower exponential decrease to equilibrium (●). The predicted curves were calculated from Eq. 2 where $E_y:E_y' = 53:47$. Final conditions: enzyme, 25 μM, 1 M NaCl 25°, buffers; 50 mM Mes (pH 6.0 to 6.7), 50 mM Hepes (pH 7.1 to 7.7), 50 mM Tris (pH 8.1).

to the $pK_{app} = 7.7$, derived from spectral equilibrium data (5, 6). We have considered a wide range of different models, but none of the functions generated from these has accounted for all observations. Though the excellent fit cannot ensure that Eq. 2 describes the events for the pH range 6.0 to 8.5 uniquely, it seems to approximate reality very closely. Additional processes could, of course, occur outside this pH range.

DISCUSSION

The present results demonstrate that in solutions of azocarboxypeptidase, the yellow azotyrosine exists over the pH range 6 to 8.5 in two pH independent, freely interconvertible conformations in a ratio of 53:47. Stopped-flow, pH jump experiments single out rate processes characteristic solely of these conformational states of the enzyme and differentiate their rates of interconversion from those which govern the formation of the red intramolecular chelate. It is important to note that the present approach clearly differentiates the influence of pH and other environmental parameters on the distribution of these conformations from their effect on catalysis. The exceedingly rapid rate observed, varying from 100,000 to 50,000 sec^{-1} over the pH range from 6 to 8.5, always accounts for the major fraction of the total absorbance change. This rapid process was not detected by Lipscomb and collaborators (14), who found only a single rate process, 6.1 sec^{-1} or 7.2 sec^{-1}, dependent on protein concentration, for a pH jump from 6.7 to 8.4. This rate is similar to 5 sec^{-1} observed in the present study, and characteristic of Process II (Eq. 1). The failure to detect the rapid process led to a number of explanations thought to be consistent with the existence of but a single slow rate (14). The fast rates detected show that the premise for those conjectures is not valid (9).

Based on the spectral properties of the azoenzyme (4–6) and the nitroenzyme (15) and on the kinetics of the crystalline enzyme (11), we pointed out previously that the conformation of carboxypeptidase in solution differs significantly from that in crystals. In the correlation of structural crystallographic detail with the functional properties of the enzyme, multiple inconsistencies have come to light, particularly when trying to account for the properties of various, chemically modified carboxypeptidases in solution (7). In particular, the spatial relationship of Tyr-248 to zinc has been unclear. X-ray crystallographic analysis (14, 16–18) first assigned Tyr-248 a unique conformation, in which it was separated from the zinc atom by 17 Å. However, after equilibrium studies on arsanilazocarboxypeptidase established that in solution Tyr-248 is coordinated to zinc (4–7), a reexamination of earlier x-ray data revealed a second conformation in the crystal, comprising 15–25% of the total, in which Tyr-248 is in close proximity to the zinc atom (19).

Our present findings form the basis for a general model that readily accounts for past results and predicts future findings concerning conformations of carboxypeptidase in the two physical states. The stopped-flow and temperature jump techniques here used visualize two predominant conformations of arsanilazocarboxypeptidase in solution. In one state the rapid interaction with zinc implies the ready access of azoTyr-248 to the zinc atom. In the other, azoTyr-248 forms the red complex at a rate three to four orders of magnitude slower and hence some change in conformation in the protein must occur to place the Tyr and the zinc atom in proper stereochemical positions for complex formation. These dynamic techniques demonstrate the nearly 1:1 ratio of two predominant azoTyr-248 solution conformations. This ratio contrasts markedly with two x-ray crystal conformations reported to exist in a ratio of approximately 1:4 (19). Qualitatively, it has been quite apparent that the conformation of the azoenzyme differs in the two physical states (4–6). The ratios of the solution conformations and crystal conformations can now be inspected and compared quantitatively for the first time.

As is apparent from the present data, solutions of azocarboxypeptidase are characterized by discrete rapidly equilibrating conformational states. In contrast, any static crystal form of carboxypeptidase could represent a departure from the dynamic solution equilibrium. The extremes to which ambient conditions, factors favoring crystallization and experimental procedures required for x-ray structure analysis, could shift this equilibrium are presently unknown, and the governing parameters now require experimental examination. Hence, the effects on conformational equilibria of isomorphous replacements, pH, salt, temperature, solvents, enzyme concentration, substrates, inhibitors, chemical modifications, and even environmental conditions, not previously considered relevant to enzymatic or crystallographic studies, must be explored systematically.

It is not known to what extent these parameters, singly or jointly, might, on crystallization, affect the stereochemical orientation of side chains or the flexibility of all or any part of the molecule to result in conformations which either deviate from or coincide with those in solution, as exemplified by the present azoTyr-248 case. In addition, since the azoprobe would seem limited largely to the recognition of azoTyr-248 conformers, the existence of additional conformational states must also be considered. Similarly varied and rapidly changing orientations between other amino acid side chains and components of the active center may likely occur, and will need to be discerned through other similarly specific monitors of conformational equilibria. In this view there would be no reason to single out the effect of substrate on the conformational change of crystals as being necessarily distinct from that of other environmental factors capable of altering such conformational equilibria by mechanisms including stabilization of "fluctuant configurations" (12). It seems clear that for correlations of structure with function in

particular, the tertiary structure of enzymes in crystals and in solution must be subdivided into levels of structure defined by the mutual orientation of and distance between motile functional groups, side chains and segments of the peptide chain.

It follows that crystals, constituted of conformations "selected" or "frozen" in this manner and static with respect to the free equilibration manifest in solution, could be expected to exhibit greatly reduced activities and markedly altered catalytic behavior, which indeed has been demonstrated for native carboxypeptidase α and γ crystals (11). Clearly, the correlation of structure and function leading to enzyme mechanisms requires identity of the systems from which the respective data are derived. We have previously emphasized that detailed kinetic analysis of substrate hydrolysis, catalyzed by different crystals, is a readily feasible means to assess and compare their functional properties (11). The present study suggests that these properties may be related closely to the motility of side chain structure and its rapid equilibration in solution. These substructural levels and their characteristics can now be examined quantitatively by stopped-flow methods and should be valuable guides to discern enzyme mechanisms and select crystals for x-ray structure analysis.

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