Kinetic Properties of Crystalline Enzymes. Carboxypeptidase A†

Curtis A. Spilburg,‡ J. L. Bethune, and Bert L. Vallee*

ABSTRACT: Spectrochemical probes have demonstrated that the conformations of carboxypeptidase A differ in solution and in the crystalline state. Detailed kinetic studies of carboxypeptidase A crystals and solutions now show that the physical state of the enzyme is also a critical parameter that affects this enzyme's function. Thus, for all substrates examined, crystallization of the enzyme markedly reduces catalytic efficiency, k_{cat}, from 20- to 1000-fold. In addition, substrate inhibition, apparent in solution for some di- and depsipeptides, is abolished with crystals, while longer substrates with normal kinetics in solution may exhibit activation with the crystals. The physical state of the enzyme also affects the mode of action of known modifiers of peptidase activity of the enzyme. In solution, addition of benzoylglycine or cinnamic acid markedly increases the rate of hydrolysis of CbzGly-Phe, but, with the crystalline enzyme, their addition hardly alters the activity. This is in accord with the weakening or absence of inhibitory enzyme–substrate binding modes. Kinetic studies on crystals were carried out over a range of enzyme concentrations, substrate concentrations, and crystal sizes, and in all instances the results are in good agreement with the theory developed by Katchalski for enzymes insolubilized by other means. Importantly, these kinetic parameters are determined under conditions which obviate artifacts due to diffusion limitation of substrates or products. The differences in the kinetic behavior of carboxypeptidase crystals, on the one hand, and of their solutions, on the other hand, bear importantly on efforts to interpret the function of the enzyme in structural terms. Hypothetical modes of substrate–enzyme interaction, generated by superimposing substrate models on the crystal structure of carboxypeptidase to stimulate kinetics in solution, have failed to detect all of these changes which affect inhibitory or activating binding modes.

Recent crystallographic studies have determined the static position of purported catalytically essential residues in carboxypeptidase (Quiocho and Lipscomb, 1971). To deduce possible mechanisms of action from such detailed structural information, models of substrates have been superimposed onto the three-dimensional structure in hypothetical productive and nonproductive binding modes (Lipscomb et al., 1968). However, these mechanistic conclusions are based on the kinetic and chemical properties of enzyme solutions, and it is assumed that these properties are conserved on crystallization (Lipscomb, 1972). This assumption is clearly critical when assigning a functional significance to specific residues discerned from the x-ray analysis of crystals.

We have previously shown that the detailed kinetics of carboxypeptidase A_α and A_γ solutions differ from those of the enzyme crystals (Spilburg, 1974; Spilburg et al., 1974). Thus, on comparing solutions and crystals, the catalytic rate constant, k_{cat}, is decreased 20- to 1000-fold for all substrates examined. In addition to this diminution of k_{cat}, crystallization of the enzyme strikingly alters productive and nonproductive modes of peptide and ester binding. Thus, for BzGly-Gly-L-Phe, normal kinetics in solution acquire substrate activation in crystals; for CbzGly-Gly-L-Phe, substrate inhibition is inverted to activation and, remarkably, the characteristically complex substrate inhibition of BzGly-OPhe in solution is replaced by substrate activation in the crystals. All these kinetic findings are completely consistent with previous chemical and spectral evidence for the existence of different conformations of the active center in the two physical states (Johansen and Vallee, 1971, 1973; Riordan and Muszynska, 1974).

Theory elaborated by Katchalski et al. (1971) and Bunting and Laidler (1972) has been successful in describing the kinetic behavior of insolubilized enzymes and is here extended to cross-linked crystals of carboxypeptidase. Kinetic studies were carried out over a range of enzyme concentrations, substrate concentrations, and crystal sizes, and in all instances the results are in good agreement with theory developed for enzymes insolubilized by other means. Importantly, kinetic parameters for the hydrolysis of substrates by crystals may be determined under conditions which obviate artifacts due to diffusion limitation of substrates or products. The present experiments not only serve to test and confirm that theory, but also provide a readily feasible means to assess and compare functional properties of enzyme crystals. This should prove a valuable guide to choices of crystals suitable for x-ray structure analysis, and such evaluations would seem indispensable for the design of mechanisms based on enzyme structure and function.

Materials and Methods

Carboxypeptidase A_α (Lot 73C-8083-9) and A_γ (Lot 2CA) were purchased as crystalline suspensions from Sigma and Worthington, respectively. The crystalline enzymes were washed three times with metal-free water and recrystallized according to the method of Cox et al. (1964). The peptidase and esterase turnover numbers for the enzymes in solution were 8000 ± 500 min^{-1} and 6500 ± 500 min^{-1}, respectively, when assayed under standard conditions with CbzGly-L-Phe and BzGly-OPhe.

Crystals used for kinetic analyses were prepared from solutions containing 6 mg/mL carboxypeptidase in 0.5 M NaCl, 0.02 M Veronal, pH 7.5. Small single crystals are formed in less than 5 h by rapid dialysis of this enzyme solution against buffer. Larger crystals can be prepared if the same stock pro-

† From the Biophysics Research Laboratory, Department of Biological Chemistry, Harvard Medical School, the Division of Medical Biology, Peter Bent Brigham Hospital, and Department of Radiology, Harvard Medical School, Boston, Massachusetts 02115. *Received September 13, 1976.* This work supported by Grant-in-Aid GM-15003 from the National Institutes of Health of the Department of Health, Education, and Welfare.

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tein solution is dialyzed overnight without stirring. Best results were obtained when the starting protein concentration was between 4 and 8 mg/mL.

Preparation of Cross-Linked Crystals. The α or γ crystalline suspensions were cross-linked with 1% glutaraldehyde for 90 min following the method of Quiocho and Richards (1966). The suspensions were then soaked overnight in 1.0 M NaCl, and the following day washed twice with 1.0 M NaCl to remove any native enzyme which might contaminate the cross-linked crystals. The crystals were resuspended in water and stored at 4 °C. When treated in this manner, the cross-linked crystals showed no loss of activity even after standing for 2 months at 4 °C.

Selection and Characterization of Crystals. When carboxypeptidase crystallizes, crystals of various sizes precipitate along with large amorphous masses. After cross-linking the crystal preparation, these large masses were removed, and the remaining single crystals were fractionated into two batches of uniform crystal size by repetitive application of the sedimentation procedure of Quiocho and Richards (1966). The first batch contained only those crystals which settled in 30 s. By examination with a hemocytometer and microscope, these large crystals were found to have an average length of 250 ± 100 μm and an average width of 53 ± 15 μm. The second batch contained very small crystals which remained suspended for 30 min. These crystals were used for kinetic measurements. The presence of diffusion limitation depends critically on the particle thickness (see below), a dimension which can be determined using scanning electron microscopy. Suspensions of small crystals were soaked in absolute ethanol to remove water, collected on a Millipore filter, and coated with gold–palladium in a Technics Hummer. Using calibrated polystyrene latex spheres (Fullam, Inc.), the average thickness was found to be 0.5 ± 0.2 μm. The average length and width were 3.0 ± 0.1 and 1.0 ± 0.4 μm, respectively. Photomicrographs of these crystals are shown in Figure 1.

Protein Concentration. Since cross-linked carboxypeptidase crystals are insoluble under standard conditions (see below), direct absorbance measurements cannot serve for the determination of protein concentration. Hence, the crystals were solubilized by heating in 0.1 M NaOH for 6 h at 70 °C and the protein concentration was determined using the method of Lowry et al. (1951), with native carboxypeptidase as the standard. Special care was taken to remove any buffer ions, e.g., Tris,[1] Mes, etc., which might interfere with color development (Peters and Fouts, 1969). In the range of protein concentration used, ~0.10 mg/mL, absorbance vs. protein concentration was linear.

Substrates and Inhibitors. The peptides, CbzGly-L-Phe, BzGly-L-Phe, CbzGly-Gly-L-Phe, and BzGly-Gly-L-Phe, and the ester BzGly-Gly-OPhe were provided by Dr. D. S. Auld. The sodium salt of the ester, BzGly-OPhe, was prepared as described elsewhere (McClure, 1966).

The inhibitors, β-phenylpropionic acid, indole-3-acetic acid, and carbobenzoxyglycine, were purchased from Aldrich and recrystallized before use. D,L-Benzylsuccinic acid was purchased from Burdick and Jackson Co. and the isomer was prepared as described by Byers and Wolfenden (1973). The protease inhibitor isolated from potatoes was a gift from Dr. Clarence Ryan.

Synthesis of Hexa- and Heptapeptides. Benzoylglycine

[1] Abbreviations used are: CMC, 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluenesulfonate; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; DMF, dimethylformamide.

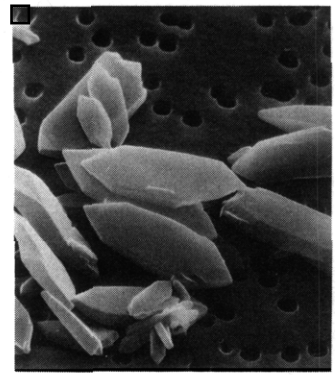

FIGURE 1: Electron micrograph of carboxypeptidase Aγ crystals collected on a Nucleopore filter (pore size = 1 μm) taken with a JEOL JSM-35 scanning electron microscope. The photographs were taken by Dr. Michael Gottesman, Department of Anatomy, Harvard Medical School.

(0.03 mol) and *N*-hydroxysuccinimide (0.03 mol) were dissolved in 50 mL of dioxane and cooled to 5 °C, and *N,N'*-dicyclohexylcarbodiimide (0.03 mol) was added with stirring. The solution was filtered the next day and 20 mL of water containing glycylglycine (0.035 mol) and $KHCO_3$ (0.07 mol) was added to the activated ester. After stirring for 4 h at room temperature, some solvent was removed under vacuum, and the solution was then acidified to pH 1.5 to precipitate the free acid. Solid $Bz(Gly)_3$ was collected and recrystallized from ethanol–water (mp 218–220). To prepare $Bz(Gly)_5$, the above procedure was repeated; however, the succinimide ester of $Bz(Gly)_3$ was prepared in DMF–dioxane. $Bz(Gly)_5$, mp 251–253 °C.

$Bz(Gly)_5$ (0.001 mol) was dissolved in a minimum amount of warm Me_2SO, cooled to 5 °C, and then added to a dioxane solution containing *N*-hydroxysuccinimide (0.001 mol) and *N,N'*-dicyclohexylcarbodiimide (0.001 mol). The next day, an aqueous solution containing $KHCO_3$ (0.0025 mol) and either Phe or GlyPhe (0.0012 mol) was added with stirring. After 4 h, all the solvent was removed under vacuum at 40 °C. The solid was collected and dissolved in water and adjusted to pH 7 by addition of concentrated base. The solution was filtered, the clear filtrate was acidified to pH 1.5, and the precipitate was collected, dried, and recrystallized from ethanol–water. ($Bz(Gly)_5$-L-Phe, mp 228–230 °C; $Bz(Gly)_6$-L-Phe, mp 248.5–250 °C).

Activity Measurements. Stirred peptidase assays were performed as described for enzyme solutions (Auld and Vallee, 1970). All steady-state kinetic profiles were determined in 1.0 M NaCl–0.05 M Tris, pH 7.5, at 25 ± 0.1 °C by a modification of the ninhydrin method using a Technicon AutoAnalyzer. The buffer had no effect on the activity measured.

The effect of stirring rate on enzymatic activity was determined at 1.0 mM CbzGly-L-Phe. The assay mixture was thermostatted at 0 °C and stirred with a magnetic stirring bar. A solenoid was placed on top of the stirring motor and the alternating current generated by the varying magnetic field was displayed on an oscilloscope (Telequipment S51B). The stirring rate was measured directly from photographs of the calibrated oscilloscope tracings. The rate of hydrolysis of CbzGly-L-Phe did not depend on the stirring rate, identical velocities being obtained at 1, 5, 10, or 30 revolutions per second. Thus, Nernst diffusion layer effects were not a factor in the subsequent analysis.

Esterase assays were performed at 25 °C by titration of the protons released on hydrolysis using 2 mM NaOH and a Radiometer titrator comprised of an ABU 12 autoburette coupled

TABLE I: Crystal Size as a Function of Settling Time.

Settling Time, (min)	Av Length (μm)	Av Width (μm)	Av Thickness (μm)
0.5	250 ± 100	53 ± 15	30 ± 10
>30	3.0 ± 1.0	1.0 ± 0.4	0.5 ± 0.2

to a TTT 11 autotitrator, TTA 31 titration assembly, and a PHM 28 pH meter. All assays were performed under a nitrogen atmosphere.

Modification with CMC. Crystals of carboxypeptidase were modified with 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate (CMC) in a manner similar to that described for the enzyme in solution (Riordan and Hayashida, 1970). Modification of crystals was carried out at 0 °C using 0.02 M CMC (Aldrich) in 0.05 M Mes–1.0 M NaCl buffer at pH 6.0. The reaction was stopped by dilution into the assay mixture containing 0.05 M Tris (pH 7.5)–1.0 M NaCl.

Results

Isolation and Characterization of Uniform Crystals. The original sedimentation procedure of Quiocho and Richards (1966) was employed repetitively to separate crystals of carboxypeptidase A_γ into two classes of fairly uniform size (Table I). The large standard deviation of any dimension indicates that this procedure results in a range of crystal sizes. All kinetic parameters reported in this work were determined with crystals with average dimensions, 3 × 5 × 0.5 μm (Figure 1). Crystals of carboxypeptidase A_α are parallelepipids and differ in shape from the "coffin-shaped" carboxypeptidase A_γ crystals (Figure 1). The kinetic behavior of both crystalline enzymes is identical, however.

Reproducibility of Assay Procedure. In our hands, the continuous-stirring method described under Methods has proven most suitable for kinetic analyses, obviating transport and packing problems involved in flow systems (Lilly et al., 1966). For statistical purposes, 20 assays were performed with uniform-sized crystals of average thickness, 0.5 μm, of several different preparations of carboxypeptidases A_α and A_γ. Both crystalline isomers hydrolyze 20 mM CbzGly-L-Phe with identical, highly reproducible turnover numbers, 210 min^{-1} ± 30.

Assessment of Diffusion Limitation. Due to the competing processes of diffusion, which supplies the interior of the insolubilized particle with substrate, and the catalytic reaction, which depletes the interior of substrate, a concentration gradient exists across the matrix. One important factor defining this gradient is the number of enzyme active centers occurring between the center of the particle and the surface. Based on this physical picture and the theoretical analysis of Katchalski et al. (1971), experimental tests may be devised for detection of diffusion limitation which would affect interpretations of the catalytic properties of such immobilized systems (see below). Thus, for an insolubilized particle of any size, the activity will always be proportional to the number of active centers when monitored at very high substrate concentrations. Such is not the case at low substrate concentrations. In this instance, for a large particle characterized by severe diffusion limitation, the observed activity will vary as the square root of the enzyme concentration within the matrix. Obviously, for small particles not subject to diffusion limitation, activity changes are always proportional to changes of the enzyme concentration when monitored at any substrate concentration. This different dependence on enzyme concentration, then, can serve to define the presence or absence of diffusion limitation. While the enzyme concentration of insolubilized enzymes bound covalently to a matrix is controlled readily to any desired level (Bunting and Laidler, 1972), that is not the case for crystals, where the effective enzyme concentration is fixed by the crystal density. However, in this instance, the enzyme active center concentration can be decreased to any desired level by employing a chemical modification, which inactivates the enzyme, allowing application of this criterion.

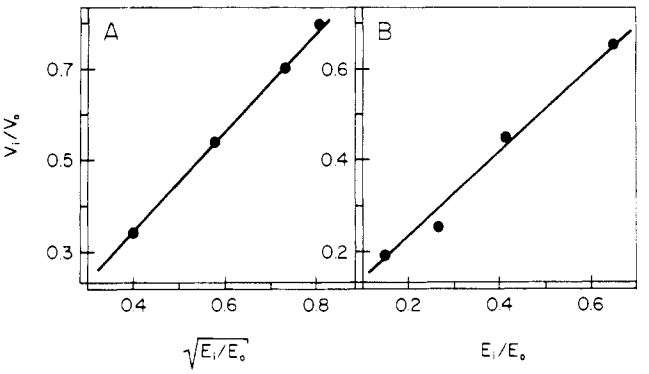

FIGURE 2: Activity as a function of enzyme concentration for two crystal sizes (A) 250 × 53 × 30 μm and (B) 3 × 1 × 0.5 μm.

Interaction of 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate (CMC) at pH 6.0 with carboxypeptidase in solution has been shown to cause a parallel loss of both peptidase and esterase activities, due to modification of a catalytically essential carboxyl group (Nau and Riordan, 1975). This procedure was employed with the two sizes of carboxypeptidase crystals to ascertain the extent of diffusion limitation occurring with each. For both crystal sizes, the active center concentration after a given exposure to CMC was determined by measurement of the activity at a high substrate concentration. Activities were then assessed at a low substrate concentration, 15-fold below K_M, and the dependence on active center concentration was determined. With the larger crystals, diffusion limitation is apparent, the velocity at the low substrate concentrations being a linear function of the *square root* of the active-center concentration (Figure 2A). No diffusion limitation is apparent with the smaller crystals since the activity is *directly proportional* to enzyme concentration (Figure 2B). In Figure 2A,B, the abscissa units are the relevant critical variables, i.e., in Figure 2A, the square root of concentration and, in 2B, the concentration itself.

Attainment of the Steady State. The size of the insolubilized particle also determines the time required to reach the steady-state substrate concentration within the crystal matrix. Using a limiting form of Barrer's analysis (1941), Laidler (1973) has shown that the time, Υ, required to attain 90% of the final substrate concentration (in the absence of reaction) at the center of the particle is given by

$$\Upsilon = 0.257(l^2/D_0')$$

where l is the crystal thickness and D_0' is the diffusion constant. Assuming a value for D_0' of 3×10^{-6} cm^2 s^{-1} (see below) and a crystal thickness of 0.5 μm, Υ is approximately 0.2 ms. All kinetic constants derived here for the enzyme crystals are then true steady-state parameters.

Hydrolysis of Di- and Depsipeptides. The double-reciprocal plot for the hydrolysis of CbzGly-L-Phe by the enzyme crystals

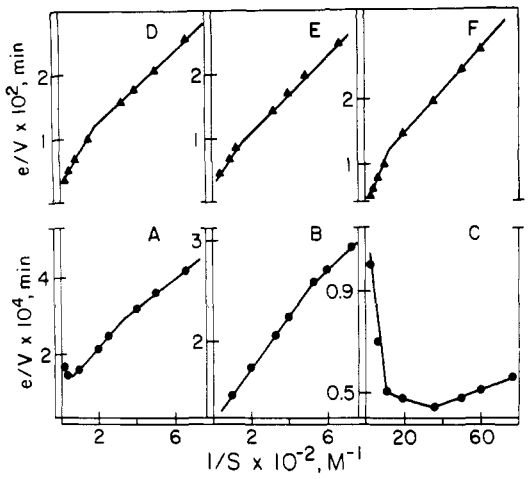

FIGURE 3: Lineweaver-Burk plots for the hydrolysis of CbzGly-L-Phe (left panel), BzGly-L-Phe (middle panel), and BzGly-OPhe (right panel) by carboxypeptidase solutions (A, B, and C) and crystals (D, E, and F).

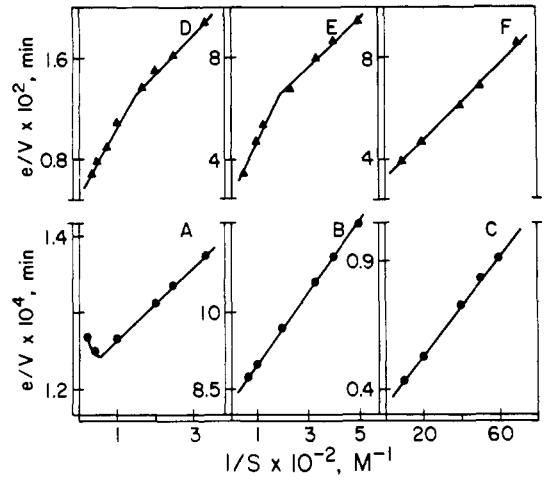

FIGURE 4: Lineweaver-Burk plots for the hydrolysis of CbzGly-Gly-L-Phe (left panel), BzGly-Gly-L-Phe (middle panel), and BzGly-Gly-OPhe (right panel) by carboxypeptidase solutions (A, B, and C) and crystals (D, E, and F).

is composed of two linear segments (Figure 3D). As S^{-1} approaches zero, there is a linear decrease in v^{-1}, but the slope changes at 6 mM, with substrate activation becoming apparent. Similar biphasic plots are found when the crystals hydrolyze CbzGly-L-Phe at pH 6.0 or 9.0. Importantly, even at 200 mM substrate, 15 times above the higher K_M value, activity continues to increase with increasing substrate concentration. Hydrolysis of this peptide by enzyme solutions is also characterized by substrate activation, but, in addition, above 10 mM substrate there is a third region, that of substrate inhibition (Figure 3A).

The Lineweaver-Burk plot for the hydrolysis of BzGly-L-Phe by carboxypeptidase crystals is also biphasic (Figure 3E). In the low substrate concentration region the plot is linear, while above 8 mM substrate activation is again observed. Similar behavior is observed for enzyme solutions, but the change in slope occurs at about fivefold lower substrate concentration (Figure 3B).

As with the dipeptides, the Lineweaver-Burk plot for hydrolysis of the ester, BzGly-OPhe, by carboxypeptidase crystals is characterized by substrate activation with the second linear region apparent above 0.6 mM substrate (Figure 3F). The kinetics of the enzyme solutions, studied over the same range of substrate concentrations, differ markedly from those of the crystals. Here, hydrolysis is characterized by complex substrate inhibition (Figure 3C).

Comparison of k_{cat} and K_M values, extrapolated from the linear regions in Figure 3, with those obtained in solution (Davies et al., 1968b) reveals that crystallization predominantly affects k_{cat}, the reduction ranging from 20- to 50-fold in both substrate concentration regions (Table II). The changes in the K_M values of these three substrates (Table II) likely reflect the elimination of the inhibitory substrate-binding modes. Thus, in the two physical states, both catalytic efficiency and at least some binding modes of the enzyme differ, though the kinetic anomalies preclude unambiguous analysis of the kinetic constants.

Hydrolysis of Tripeptides and Their Ester Analogues. The double-reciprocal plot for the hydrolysis of CbzGly-Gly-L-Phe by the crystals is biphasic (Figure 4D) with the change in slope occurring at 5 mM substrate. For the enzyme in solution (Figure 4A), the corresponding Lineweaver-Burk plot is linear up to 10 mM substrate, above which there is substrate inhibition. Again, as with BzGly-OPhe, substrate inhibition found for the enzyme solution is replaced by substrate activation in the crystals.

TABLE II: Kinetic Parameters of Peptide and Ester Hydrolysis.

Substrates	Solution k_{cat}	Solution K_M[a]	Crystals k_{cat}	Crystals K_M
CbzGly-L-Phe	10 000	6.0[b]	364	20.0
	5 500	2.0[c]	175	5.0
BzGly-L-Phe	8 700	2.7[b]	500	25.0
	5 500	0.8[c]	250	6.2
BzGly-OPhe	29 000	0.2	600	5.0[b]
			120	0.40[c]
CbzGly-Gly-L-Phe	8 000	0.25	200	12.0[b]
			120	4.0[c]
BzGly-Gly-L-Phe	1 200	0.80	43	10.0[b]
			22	2.3[c]
BzGly-Gly-OPhe	30 000	0.30	30	0.30

[a] M × 10³. [b] Values extrapolated from regions of high substrate concentrations. [c] Values extrapolated from regions of low substrate concentrations.

The double-reciprocal plot for the hydrolysis of BzGly-Gly-L-Phe can be divided into two linear segments (Figure 4E), with the change in slope occurring at 7 mM substrate. This is in marked contrast to the enzyme solutions, which exhibit normal Michaelis-Menten kinetics over the whole range of substrate concentration (Figure 4B).

The ester BzGly-Gly-OPhe is the only substrate examined which exhibits normal Michaelis-Menten kinetics with both crystals (Figure 4F) and solutions over the concentration range examined (Figure 4C).

The kinetic parameters extrapolated from the Lineweaver-Burk plots for the enzyme crystals and solutions (Auld and Holmquist, 1974; Auld and Vallee, 1970) are compared in Table II. Once again, the presence of altered modes of substrate binding in the two physical states precludes an unambiguous comparison of kinetic parameters for the tripeptides. However, the kinetics of the ester BzGly-Gly-OPhe are normal in both physical states. Hence, a direct comparison is possible. Here, the kinetic parameters clearly show that the effect of crystallization is entirely on k_{cat}, reducing it 1000-fold

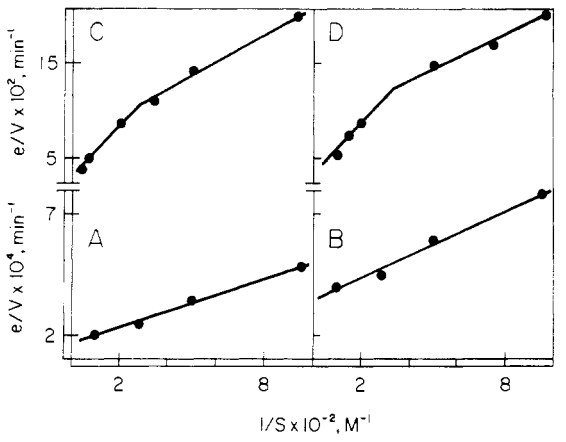

FIGURE 5: Lineweaver–Burk plots for the hydrolysis of Bz(Gly)$_5$-L-Phe (left panel) and Bz(Gly)$_6$-L-Phe (right panel) by carboxypeptidase solutions (A and B) and crystals (C and D).

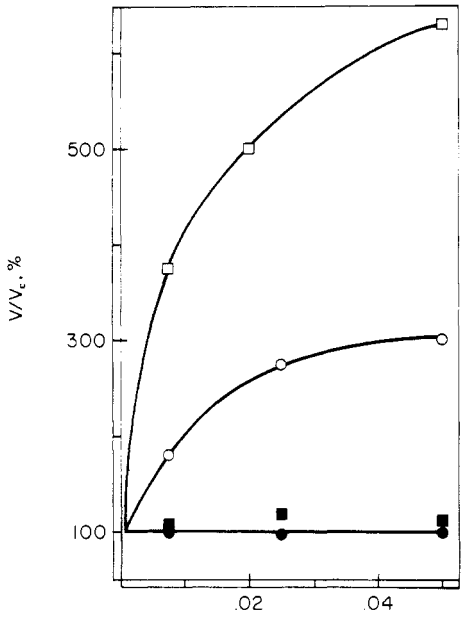

FIGURE 6: Effects of BzGly (○) and cinnamic acid (□) on hydrolysis of CbzGly-L-Phe by carboxypeptidase solution (open symbols) and crystals (solid symbols).

from 30 000 min^{-1} to 30 min^{-1}, while the value of K_M remains unchanged.

Hydrolysis of Hexa- and Heptapeptides. The double-reciprocal plots for the hydrolysis of Bz(Gly)$_5$-L-Phe and Bz(Gly)$_6$-L-Phe are shown in Figure 5. With these substrates, the kinetics of the solution enzyme are normal just as those for the corresponding tripeptide. However, the Lineweaver–Burk plots for the crystals are biphasic, with the change in slope occurring near 5 mM for both substrates.

Effect of Modifiers. It is well established that N-substituted products of hydrolysis of certain synthetic peptide substrates, such as BzGly, activate the hydrolysis of dipeptide substrates by the enzyme in solution (Davies et al., 1968b). Thus, as shown in Figure 6, BzGly enhances the activity of the enzyme in solution by 300%. However, no activation is observed with the crystalline enzyme. Other organic compounds that have similar effects are referred to collectively as modifiers, e.g., *trans*-cinnamic acid, which activates peptide hydrolysis in solution by 600%. Again, however, with the crystals, little or no activation is observed (Figure 6).

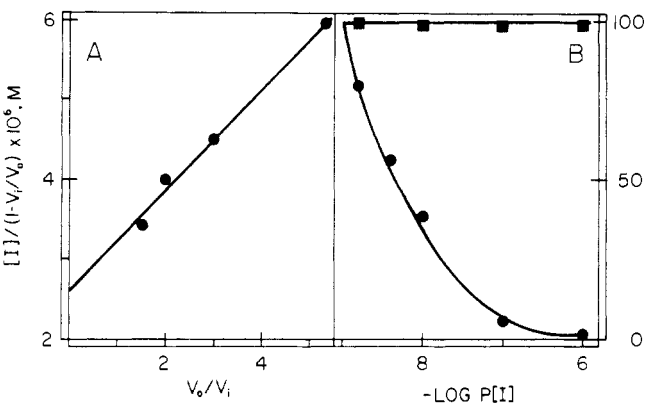

FIGURE 7: Interaction of inhibitors with carboxypeptidase crystals. (A) Henderson plot for the inhibition of the crystals by L-benzylsuccinate in 1.0 M NaCl, 0.05 M Mes, pH 5.5. (B) Interaction of the potato inhibitor with carboxypeptidase solutions (●) and crystals (■) in 1.0 M NaCl, 0.05 M Tris, pH 7.5.

TABLE III: Inhibition Constants for Peptidase Activity of Carboxypeptidase Solutions[a] and Crystals[b].

	K_I (Solution) M	K_I (Crystals) M
Carbobenzoxyglycine	2.9×10^{-2}	4.2×10^{-2}
Indole-3-acetic acid	1.7×10^{-4}	2.0×10^{-4}
β-Phenylpropionic acid	1.2×10^{-4}	3.0×10^{-4}

[a] Auld, D. S., and Vallee, B. L. (1970). [b] Inhibition constants determined between 5.0 and 1.0 mM CbzGly-Gly-L-Phe.

Inhibition Studies. The inhibition of the hydrolysis of CbzGly-Gly-L-Phe by CbzGly, indole-3-acetate, and β-phenylpropionate was studied over a substrate concentration range of 1–5 mM and plotted in Dixon coordinates. In both physical states, the values of K_I for the enzyme in solution and for the crystals are nearly the same (Table III). However, the interaction of the crystals with the large polypeptide inhibitor recently isolated from potatoes (Ryan et al., 1974) differs markedly from that found with the enzyme in solution. At 1×10^{-8} M inhibitor, in solution, the enzyme is 50% active. At this concentration, the enzyme crystals, however, retain full activity. Indeed, even when the inhibitor concentration is increased 100-fold, i.e., to 1×10^{-6} M (Figure 7), no inhibition of the crystals is observed where in solution the activity is <1%.

In solution, L-benzylsuccinate is the most potent low-molecular-weight inhibitor of carboxypeptidase, with a K_I of 2×10^{-8} at pH 5.5 in 1.0 M NaCl. For such strong binding, Henderson (1972) has derived dose–response relationships to determine the active-site concentration during the assay. Such experiments are best performed using a poor substrate, e.g., BzGly-Gly-L-Phe, where the assay system contains a high concentration of enzyme, e.g., ten times greater than the value of K_I. Thus, a significant fraction of the total inhibitor is bound to the enzyme. The dose–response plot for the effect of L-benzylsuccinate on the hydrolysis of BzGly-Gly-L-Phe by the crystals is shown in Figure 7. The intercept on the ordinate is equal to the active-site concentration, 2.4×10^{-6} M, in excellent agreement with the protein concentration, 1.7×10^{-6} M, determined directly by the Lowry method.

Discussion

The success of crystallographic analysis in the determination of protein structure has been accompanied by a growing con-

cern regarding the extrapolation of conclusions drawn from the unique structure observed in the crystal to the situation pertaining in solution. Since, at least in solution, the free-energy change of protein unfolding is almost zero, the structure of these macromolecules is thought to be motile; i.e., in solutions numerous readily interconvertible conformations exist simultaneously. Diazotized carboxypeptidase, uniquely modified at tyrosine-248, provides a classic example. The crystals of this enzyme are yellow, Tyr-248 being some 17 Å from the active-site zinc atom (Quiocho and Lipscomb, 1971). Solutions of this enzyme, however, are red, resultant from the formation of an intramolecular chelate between Tyr-248 and the active-site metal atom (Johansen and Vallee, 1971, 1973). Thus, a conformation present in solution either is not represented in the crystal or occurs only to a very minor extent. Moreover, the solution enzyme represents an equilibrium mixture of at least three conformations, demonstrated by stopped-flow pH jump and temperature-jump techniques (Harrison et al., 1975). Therefore, multiple conformations exist in solutions of this enzyme, and crystallization imposes a new set of forces on the molecules which not only alters these equilibria to exclude the conformation that predominates in solution but may even give priority to a new conformation not present in solution.

If such conformational changes modulate catalytic function, then assessment of enzymatic activity in the two physical states would be the most sensitive gauge of perturbation of the structure of the active site. Since ~50% of the total weight of protein crystals is solvent, and the contacts between different protein molecules are very few (Rupley, 1969), enzyme molecules located within the interior of the crystals are readily accessible to *small* molecules, such as substrates, by diffusion through these large pores. In this regard, Richards and coworkers (Doscher and Richards, 1963; Quiocho and Richards, 1964) demonstrated that by diffusing substrates into their interior, crystals of carboxypeptidase and ribonuclease S are enzymatically active. Their results indicated that the kinetic parameters for ribonuclease crystals were similar to, but not identical with, those for the enzyme in solution. On the other hand, they found marked differences between native carboxypeptidase in solution and its glutaraldehyde cross-linked crystals in regard to metal-stability constants and specific activities. However, at that time, there were no experimental means to determine unambiguously if kinetic similarities or differences were true properties of the enzymes in the crystalline state or resulted from the physicochemical properties of the total system, e.g., artifacts arising from the rate-limiting diffusion of substrate into and product out of the crystal matrix.

After the appearance of Richards' work, Katchalski et al. (1971) developed a quantitative theory of the kinetic properties of immobilized enzymes, an important part of which was the specification of experiments that could assess the effect of diffusion limitation on the observed catalytic parameters. We show here that this theory can be extended to determine true kinetic constants of carboxypeptidase crystals. Our experiments serve not only to test and confirm that theory, but also to assess and compare functional properties of the enzyme crystals.

Application of the analysis of Katchalski et al. (1971) requires that all of the active sites within the crystal matrix be accessible to substrates, and that the enzyme remain totally insoluble in the course of the assay. Even though substrate may readily enter the interstitial spaces, protein–protein contacts within the matrix might make most of the active sites inaccessible, which would lead to apparently diminished activity. Moreover, if the crystalline enzyme dissolves in the course of the assay, the activity observed cannot be employed unambiguously to determination of the kinetics of the crystals. Interaction of the enzyme crystals with tightly bound inhibitors of various sizes provides a means of determining if cross-linked carboxypeptidase crystals fulfill these requirements. Based on a dose–response method employing the *small,* tightly bound inhibitor, L-benzylsuccinate, the concentration of active sites can be determined kinetically (Figure 7). The correspondence between the concentration of active sites and that of the protein indicates that within the crystal all active sites are available to substrate.

The *large* macromolecular inhibitor of carboxypeptidase (mol wt ~4000), isolated from potatoes, has allowed assessment of the solubility of the crystals. Unlike the small organic inhibitors, such as L-benzylsuccinate, this polypeptide is excluded from the interior of the crystalline matrix. Thus, any inhibition observed with this agent is due to its interaction either with molecules on the crystalline surface or to enzyme which has dissolved. As shown in Figure 7, the potato inhibitor does not measurably inhibit the crystals, even at concentrations 100 times above the K_I observed in solution. This is in marked contrast to the action of small inhibitors, β-phenylpropionic acid, indole-3-acetic acid, and carbobenzoxyglycine, for which the values of K_I (Table III) are similar for both enzyme solutions and crystals.

These experiments collectively indicate that cross-linked crystals of carboxypeptidase have the same number of active sites as does an equivalent weight of enzyme in solution, that all these sites are available to substrate, and that the solubility of the crystals is below the level of detection. Therefore, the catalytic properties of these crystals can be analyzed based on the same considerations pertinent to other immobilized enzymes.

To characterize the activity of an enzyme in an essentially two-phase system—one phase in the present case being the enzyme crystals and the other the surrounding substrate solution—it is essential that the possible influence of diffusion limitation on the results be evaluated and experimental conditions chosen so as to obviate any effects resulting therefrom. Such effects manifest themselves largely at substrate concentrations below K_M, and Katchalski et al. (1971) have shown that, for films of insolubilized enzymes where the only relevant dimension is the film thickness, the extent of diffusion control of immobilized enzymes under such conditions (i.e., $[S] \ll K_M$) can be described quantitatively by the effectiveness factor, F

$$F = \frac{V}{V_0} = \frac{\tanh(\gamma l)}{\gamma l}$$

where V and V_0 are the observed activities for the insoluble system and for the same concentration of the enzyme free in solution, l is the thickness of the insolubilized particle, and γ is given by

$$\gamma = \frac{1}{2}\left[\left(\frac{k_{cat}}{K_M}\right)\left(\frac{[E]}{D_0'}\right)\right]^{1/2}$$

where k_{cat} and K_M are the pertinent kinetic parameters, [E] is the enzyme concentration, and D_0' the diffusion constant of substrate within the insolubilized system. A value of F equal to unity implies that there is no diffusion limitation.

To calculate F for the crystal-catalyzed reaction, the enzyme concentration within an insolubilized carboxypeptidase crystal, 25 mM, can be derived readily from its reported density of 1.17

g/cm^3 and 30% water content (Lipscomb et al., 1966). It is more difficult to estimate the value of D_0'. Bishop and Richards (1968) have shown that the composition of the crystal liquid may be considered identical to that of the bulk solvent. For small substrates, then, the value of D_0' will be determined only by simple area restriction due to solute size (Renkin, 1954).

$$\frac{D_0'}{D_0} = \left(1 - \frac{a}{r}\right)^2$$

where a is the solute radius and r the pore radius. For β-lactoglobulin, a protein of molecular weight similar to that of carboxypeptidase, Low et al. (1956) have shown that the crystal pore radius is 13 Å; the radius of CbzGly-L-Phe calculated from its molal volume in aqueous solution is 4.6 Å. Using these values, D_0' can be calculated to be 3×10^{-6} cm^2/s, or 40% of its value in free solution. The minimum value that F can attain will be that which is applicable when the enzyme in the crystalline state has the potential for activity identical to that in solution. Thus, use of k_{cat} and K_M for CbzGly-L-Phe hydrolysis derived from the low substrate concentration region for the enzyme in solution (Table II) results in an effectiveness factor calculated to be 0.93.[2] Therefore, assuming that no factor other than diffusion control is operative in reducing the apparent activity, k_{cat} for the crystals in the low substrate concentration region should be 5100 min^{-1}, rather than 175 min^{-1} as is observed (Table II, column 3). The relevant parameters from which to calculate F, then, are those for the crystals themselves and, on substituting the corresponding values for CbzGly-L-Phe hydrolysis by the crystals, F becomes 0.9997.

Solutions of the differential equations pertinent for high and low substrate concentrations (Dosher and Richards, 1963) provide the framework for an additional experiment to test the extent of diffusion limitation. In the physical situation pertinent to this problem, i.e., crystals of dimension $3 \times 1 \times 0.5$ μm, diffusion along the smallest dimension will be considered to control the whole system. Thus, the three-dimensional diffusional-reaction equations will be here reduced to the one-dimensional case, with a minuscule loss of accuracy and a large gain of clarity. When $[S_0] \ll K_M$, a pronounced substrate concentration gradient in the crystals characterizes the steady state. The concentration profile is given by the equation

$$[S] = [S_0](\cosh(2\gamma X)/\cosh(\gamma l))$$

where X is the structural coordinate and $[S] = [S_0]$ at $X = \pm(l/2)$ (Crank, 1975). Therefore, enzyme in the interior equilibrates with substrate at a lower concentration than does enzyme on the surface, and the observed activity is lower than that which would occur if the substrate concentration were everywhere the same, i.e., $[S_0]$. On the other hand, if $[S_0] \gg K_M$, virtually all the molecules within the crystal are saturated and there is no perceptible concentration gradient across the crystal, i.e.

$$[S] = [S_0] - \frac{k_{cat}E_0}{2D}\left(\frac{l^2}{4} - X^2\right)$$

For these two situations, the observed velocities will be,[3] for $[S_0] \ll K_M$

$$V = 2[S_0]\left(\frac{Dk_{cat}E_0}{K_M}\right)^{1/2} \tanh(\gamma l)$$

and for $[S_0] \gg K_M$

$$V = k_{cat}E_0 l$$

Therefore, if diffusion limitation be present, at low substrate concentration the velocity is proportional to the square root of the enzyme concentration, while at high substrate concentrations the velocity is directly proportional to the enzyme concentration.

On the other hand, if there be no diffusion limitation, then normal Michaelis-Menten kinetics apply, and activity is directly proportional to enzyme concentration at all substrate concentrations, i.e.

$$V = \frac{k_{cat}E_0[S]}{K_M + [S]}$$

The crystal size and the enzyme concentration within the matrix critically determine whether or not a crystal-catalyzed reaction is subject to diffusion control. As shown above, changing the enzyme concentration within an immobilized particle provides a convenient experimental test for the presence of such control. Such changes are readily attained for other immobilized systems by either covalently binding or physically entrapping different amounts of enzyme within the matrix (Bunting and Laidler, 1972). For crystals, the density fixes the maximum enzyme concentration; however, the enzyme concentration can be decreased by inactivating the crystals with a site-specific reagent. In the case of carboxypeptidase, such modification can be accomplished by use of CMC, allowing controlled changes in the enzyme concentration within the crystals.

Large crystals are subject to diffusion limitation, completely consistent with the findings for enzyme insolubilized by other means (Figure 2A) (Kasche et al., 1971). More importantly, the small (i.e., 0.5 μm) crystals (Figure 2B) are free of detectable diffusion control, since, here, activity is always directly proportional to enzyme concentration, in agreement with the previous calculation that $F = 0.9997$ (see above). Moreover, since K_M for the ester BzGly-Gly-OPhe is the same with both the solution and crystal enzymes (Table II), no effects of charge affect the assay results (Hornby and Lilly, 1968).

Collectively, these experiments indicate that for appropriate enzyme crystals kinetic parameters may be defined in as unambiguous a manner as for enzyme solutions. As shown here, catalysis by $3 \times 1 \times 0.5$ μm carboxypeptidase crystals will not be affected by diffusion limitation. Moreover, the absence of inhibition in the presence of the polypeptide inhibitor indicates that the surface of the crystal contributes negligibly to the observed activity. Therefore, any kinetic differences between the enzyme crystals and its solutions must be an inherent property of the enzyme in these physical states and cannot be

[2] In the case of the crystals actually employed, $0.5 \times 1 \times 3$ μm, application of the one-dimensional analysis actually yields too low an F value. Use of an equation pertinent to the actual experimental situation, i.e., three-dimensional analysis, results in an effectiveness factor of 0.96 (Bethune and Spilburg, in preparation).

[3] V_0, the activities observed for the solution enzyme under these conditions will be $(k_{cat}E_0[S_0]l)/K_M$ and k_cE_0l, respectively, thus yielding the activity ratios for $[S_0] \ll K_M$, $V/V_0 = \tanh(\gamma l)/\gamma l$ and for $[S_0] \gg K_M$, $V/V_0 = 1$, demonstrating that, at high substrate concentrations, any effects of diffusion limitation vanish. At low substrate concentrations, the activity is proportional to $(E_0)^{1/2}\tanh(\gamma l)$, where γ is itself proportional to $(E_0)^{1/2}$. However, for large crystals, γl will be large, and $\tanh(\gamma l)$ will be approximately equal to 1, being highly insensitive to any variation in the argument. Thus, the observed activity is proportional to $(E_0)^{1/2}$. Similar considerations apply in the three-dimensional case.

attributed to artifacts arising from diffusion limitation or surface phenomena.

Using these small crystals, we have confirmed the decrease in activity of carboxypeptidase in this physical state for a variety of peptide and ester substrates. However, activity measurements determined at one substrate concentration cannot differentiate changes in catalytic efficiency from those in substrate binding, and, moreover, cannot discriminate details of kinetic behavior, such as substrate activation or inhibition. Therefore, studies were performed over wide substrate concentration ranges for both the enzyme in the crystalline state and in solution, allowing kinetic detail to be observed in double-reciprocal plots. It is an important general feature that both physical states exhibit marked kinetic anomalies, apparent from the nonlinearity of these double-reciprocal profiles (Figures 3, 4, and 5). Multiple productive and nonproductive substrate binding modes have been postulated to account for the complex kinetic behavior exhibited by the solution enzyme. The crystals, however, exhibit a different pattern of kinetic anomalies, indicating that modes of binding must be altered in this physical state. For example, under no conditions do the crystals display substrate inhibition, apparent when the dipeptide, CbzGly-L-Phe (Figure 3D), and the ester, BzGly-OPhe (Figure 3F), are hydrolyzed by the enzyme in solution. Moreover, the extreme substrate inhibition found for this ester with the solution enzyme is inverted to substrate activation with the crystals. The physical state of the enzyme also affects the mode of action of known modifiers of peptidase activity of the enzyme. Thus, in solution, addition of BzGly or cinnamic acid markedly increases the rate of hydrolysis of CbzGly-L-Phe, attributed to release of substrate inhibition. Their addition hardly alters the activity of enzyme crystals, in accord with the weakening or absence of enzyme–substrate inhibitory binding modes (Figure 6).

The existence of substrate activation and inhibition observed with substituted dipeptides has previously been attributed to the fact that the binding sites of the solution enzyme are "designed" to accept larger substrates (Vallee et al., 1968). If the dimensions of the active center were to offer more potential points of contact than available in a given substrate, the binding of the substrate could result in multiple interaction, not necessarily consistent with a simple Michaelis–Menten mechanism. For the enzyme in solution, a model has been proposed which suggests that an increase in the length of the substrate should place more constraints on its manner of binding, and the enzymatic hydrolysis of such substrates should approach Michaelis–Menten kinetics (Vallee et al., 1968). As shown in Figure 4A–C, this hypothesis was confirmed by the linear kinetics found when tripeptides are hydrolyzed by the solution enzyme (Auld and Vallee, 1970).

This approach has been extended here to carboxypeptidase crystals to determine the nature of the peptide and ester binding sites in this physical state. As shown in Figure 4, increasing the length of the peptide substrate by one glycyl unit does not simplify the kinetics when these substrates are hydrolyzed by the enzyme crystals. Thus, both the tripeptides, BzGly-Gly-L-Phe and CbzGly-Gly-L-Phe, when hydrolyzed by the crystals, exhibit substrate activation, a mode not observed with enzyme solutions. Even more remarkably, an increase in the length of the substrates by three or four glycyl units to $Bz(Gly)_5$-L-Phe and $Bz(Gly)_6$-L-Phe still results in substrate activation (Figure 5) with crystals. It would appear, then, that the crystals have either an "expanded peptide binding site", such that more than one heptapeptide may be accommodated, or that a new binding site has been formed, allowing multiple binding and leading to the observed kinetic anomalies. For esters, on the other hand, increasing the substrate length from BzGly-OPhe to BzGly-Gly-OPhe results in normal kinetics for both enzyme solutions and crystals. The ester binding region then may be comparable in size in the two physical states.

The existence of such markedly different kinetic behavior between crystals and solutions has important implications for activity comparisons between the two physical states. Thus, crystals might appear to be as active as the solution enzyme if a substrate activation region for the crystals were compared to a substrate inhibition region for the solutions. The current data emphasize, then, the importance of comparing the kinetic behavior in both physical states over a wide range of substrate concentrations.

The alteration of these kinetic anomalies bears importantly on previous conclusions from the x-ray structure. From model-building studies, it was thought possible to account for substrate inhibition as well as for the normal kinetics found for longer substrates when hydrolyzed by enzyme solutions. However, as shown here, the crystals, in fact, do not share these kinetic features with the solution enzyme. Such inconsistencies raise serious questions with regard to the capability of crystallography to delineate binding modes, as well as to visualize possible mechanisms of action of carboxypeptidase.

Values of k_{cat} and K_M may be derived from the extrapolated linear regions of these double-reciprocal plots. For most substrates, the kinetic anomalies preclude a simple physical interpretation of k_{cat} and K_M since, in these instances, the constants likely reflect composite terms. However, for one ester, BzGly-Gly-OPhe, both solutions and crystals display normal Michaelis–Menten kinetics (Figure 4C,F). Here the kinetic parameters clearly show that the effect of crystallization is entirely on k_{cat}, reducing it 1000-fold from 30 000 min^{-1} to 30 min^{-1} (Table II), while K_M remains unchanged. While with other substrates such comparisons are more ambiguous owing to the kinetic anomalies, it is clear that the predominant effect is also on k_{cat}, with reductions ranging from 20- to 50-fold for all esters and peptides. Crystallization, then, predominantly lowers k_{cat}.

It is interesting to speculate on the physicochemical basis for this drastic reduction in catalytic efficiency for both esterase and peptidase activities. Dynamic studies have shown the existence of rapidly interconvertible structures of carboxypeptidase in solution, and the results support the view that enzymes can adopt multiple conformations. Crystallization, on the other hand, might single out a conformation in which side chains are altered so as not to permit the most efficient catalysis. In this regard, previous studies have shown that chemical modification of specific residues in the active site of carboxypeptidase can produce characteristic activity changes of the solution enzyme. Thus, nitration or coupling of Tyr-248 with a diazonium salt or modification of a single arginyl residue increases esterase activity and decreases peptidase activity. This differentiation between the two hydrolytic activities is not found on crystallization. Indeed, the decrease in both activities for the crystals is more akin to modification of Glu-270 or metal removal, but in these two instances the solution enzyme is inactivated completely. The activity changes found on crystallization are then completely different from those previously reported for specific chemical modification of essential residues in the active site, and it would seem unlikely that these changes are due to the alteration of a single residue.

Alternatively, flexibility of either single side chains or of parts of the molecule might be a critical feature of the hydro-

lytic mechanism. Moreover, from stopped-flow and temperature-jump studies, one such movement has been observed with carboxypeptidase in solution. On binding with the pseudo-substrate, Gly-L-Tyr,Tyr-248 was found to be displaced away from the catalytically essential zinc atom. Crystallization conditions, such as salt or pH, or even the crystal lattice itself, might place constraints on this kind of motility, resulting in a diminished value of k_{cat}. These results indicate, then, that by itself, x-ray crystallographic analysis may be unable to detect certain dynamic aspects of the mechanism of action, since the technique itself precludes such studies.

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