Kinetic Properties of Carboxypeptidase B in Solutions and Crystals†

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ABSTRACT: The correlation of the structure of crystalline enzymes with their activities in solution assumes that the catalytic properties are identical in the two physical states. The present data demonstrate that in bovine carboxypeptidase B they differ significantly. Normal Michaelis–Menten kinetics characterize the hydrolysis of several ester and peptide substrates in both physical states. Crystallization reduces k_{cat} 16- to 320-fold, while it affects K_M variably and less dramatically. Small molecules inhibit catalytic activity both in solution and in crystals, but the carboxypeptidase inhibitor from potatoes (molecular weight 4200) does not inhibit the crystals. The activities of bovine carboxypeptidases A and B toward identical substrates are more similar in their crystals than in their solutions. This suggests that, over and above the structural dissimilarity of their crystals, conformational differences may additionally determine the activities of the two enzymes in solution. The findings demonstrate that the catalytic properties of carboxypeptidase B depend critically on its physical state.

The structures of a large number of crystalline enzymes, determined by x-ray analysis, and their catalytic characteristics, examined in solution, have served to deduce mechanisms, even though the effect of the physical state on enzymatic function is generally unknown. Recent reviews (Weber, 1975; Blake, 1976) have pointed out the resultant complexities and their possible basis. The x-ray image of the structure, in effect, is a time-averaged picture which does not reveal the dynamics of catalysis. This static structure does not describe the conformational details of the constituent molecules whose structures may be different from or fluctuate about that of the average. Additionally, the process of crystallization itself could introduce new interactions that might modify the corresponding structures of these molecules in solution. In this regard, crystallization may restrain conformational fluctuations that could be basic for catalysis in solution. The relevance of crystal structures to the mechanisms of enzymes in solution, therefore, cannot be taken for granted (Spilburg et al., 1974, 1977).

In the course of a comprehensive survey to assess the effect of the physical state on the structure and function of enzymes, studies have been carried out in this laboratory to probe properties of carboxypeptidase A (CPD-A)[1] solutions and crystals. Kinetic (Spilburg et al., 1974, 1977), spectroscopic (Johansen and Vallee, 1971, 1973, 1975), and chemical-modification studies (Kagan and Vallee, 1969; Johansen et al., 1972; Riordan and Muszynska, 1974) all indicated significant differences between the enzymatic and conformational properties of those enzyme crystals and solutions.

To extend the insight thus gained, the kinetics of another enzyme, carboxypeptidase B, have been examined in both physical states. Carboxypeptidase B is a zinc exopeptidase (Cox et al., 1962; Wintersberger et al., 1965) which preferentially catalyzes the hydrolysis of substrates containing basic carboxyl-terminal amino acid residues (Folk, 1971). However, its specificity is broader than that of carboxypeptidase A (Wintersberger et al., 1962), since it also hydrolyzes a variety of synthetic hydrophobic substrates which normally serve to measure CPD-A activity (Reeck et al., 1971b). Further, and in contrast to carboxypeptidase A (Spilburg et al., 1974, 1977), the kinetics of carboxypeptidase B are normal both in solution and in the crystalline phase. The substrates that we have examined do not exhibit substrate activation or inhibition and, hence, the kinetic parameters characterizing carboxypeptidase B catalyzed hydrolysis can be unambiguously compared in the two states.

Materials and Methods

Bovine CPD-B was prepared as described by Reeck et al. (1971b) and further purified by affinity chromatography, employing as ligand L-benzyl succinate covalently coupled to Sepharose 4B (Peterson et al., 1976). Protein containing CPD-B activity was eluted in a single symmetrical peak with either 0.2 M arginine at pH 6.0 or with 0.5 M NaCl at pH 7.5 (0.05 M Tris·HCl). The purified enzyme has a k_{cat} of 3600 min^{-1} and a K_M of 0.23×10^{-3} M for BzGly-Arg. It was crystallized by dialyzing concentrated solutions (10–20 mg/mL) against 10 mM Tris-HCl, pH 7.5, for 2 days at 4 °C.

Preparation of Cross-Linked Crystals. CPD-B crystals in aqueous suspension were separated from the supernatant by centrifugation and resuspended in a solution containing 1% glutaraldehyde and 5 mM Mes, pH 6.6. The cross-linking reaction was stopped after 30 min by centrifuging the crystal suspension and washing of the crystals three times with 10 mM Tris·HCl, pH 7.5, to remove excess glutaraldehyde. The crystals were then soaked overnight in 1.0 M NaCl and washed twice the following day with 1.0 M NaCl to extract any soluble

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[1] Abbreviations used are: CPD-A, bovine carboxypeptidase A; CPB-B, bovine carboxypeptidase B; CPI, carboxypeptidase inhibitor from potatoes; Bz, benzoyl; Cbz, carbobenzoxy; OPhe, phenyllactate; OArg, argininic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.

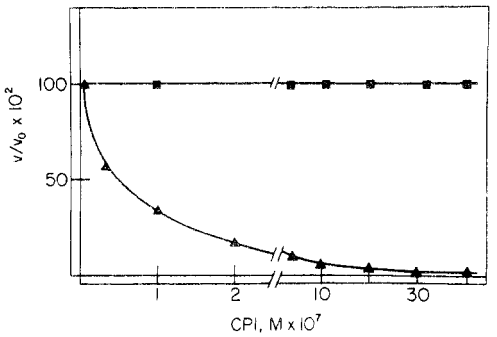

FIGURE 1: Inhibition of CPD-B crystal (■) and solution (▲) catalyzed hydrolysis of BzGly-Arg (10 mM) by potato inhibitor (CPI). Enzyme concentrations of solutions and crystal suspensions are 3.5×10^{-8} and 1.1×10^{-7} M, respectively. V_0 is the reaction velocity in the absence and V that in the presence of potato inhibitor at the concentration indicated. See Materials and Methods for assay conditions.

enzyme. Stock suspensions of cross-linked crystals were quite stable and showed no change in activity toward BzGly-Arg after 5 weeks at 4 °C. Microscopic examination of the crystalline enzyme revealed no differences in appearance before and after the cross-linking reaction. The crystals were well-shaped, flat needles, similar to those found by Wintersberger et al. (1962).

The dimensions of the crystals varied over a narrow range, i.e., $0.8 \pm 0.3 \times 1.0 \pm 0.5 \times 75 \pm 20$ μm. Hence, no fractionation according to size was required prior to carrying out kinetic experiments (Spilburg et al., 1977).

The protein concentration of cross-linked crystal suspensions was determined by the method of lowry et al. (1951) as described by Spilburg et al. (1977). The protein was solubilized by digestion at 70–90 °C in 0.15 M NaOH for 4 h.

BzGly-Arg, BzGly-OArg and CbzGly-Phe were purchased from Vega-Fox Biochemicals. CbzArg was obtained from Sigma Chemical Co. The inhibitor β-phenylpropionate, purchased from Aldrich Chemical Co., was recrystallized prior to use. BzGlyGly-Phe and BzGlyGly-OPhe were gifts of Dr. C. Spilburg and Dr. B. Holmquist, respectively. The macromolecular protease inhibitor isolated from Russet Burbank potatoes (Ryan, 1971) was provided by Dr. C. A. Ryan. To remove adventitious heavy metals, all solutions were extracted with 0.01% dithizone in carbon tetrachloride.

Activity Measurements. Peptidase activities were measured in solutions containing 0.10 M NaCl and 0.025 M Tris·HCl, pH 7.65, using a modified ninhydrin method and a Technicon Auto Analyzer (Auld and Vallee, 1970). Esterase activity was measured by titrating the protons released upon hydrolysis of ester substrates with 10 mM NaOH in a Radiometer titrator comprised of an ABU 12 autoburet coupled to a TTT 11 autotitrator, TTA 31 titration assembly, and a PHM 28 pH meter. To increase sensitivity to product formation, the Tris buffer concentration in esterase assays was 0.005 M. All assays were performed at 25 ± 0.01 °C. For solution assays, 10^{-5} M $ZnSO_4$ was added to prevent inactivation of CPD-B due to loss of metal. Assay mixtures containing crystalline CPD-B were stirred to maintain the homogeneity of the crystalline suspensions. Substrate concentrations at least threefold above and tenfold below K_M were employed to allow for valid comparisons between CPD-A and CPD-B catalyzed hydrolysis of the same substrates.

Results

Activity of CPD-B in the Crystalline State. The activity of crystalline CPD-B toward all substrates examined is 5% or less than that of the solution enzyme (Table I). The activity is reduced significantly toward standard basic substrates BzGly-Arg and BzGly-OArg, specific for CPD-B, and also toward hydrophobic substrates hydrolyzed by both CPD-A and CPD-B, i.e., CbzGly-Phe, BzGlyGly-Phe and BzGlyGly-OPhe.

TABLE I: Activity Ratios[a] for Crystals (V_c) and Solutions (V_s) of Carboxypeptidase B.

Substrate	V_c/V_s
BzGly-Arg	0.009
BzGly-OArg	0.002
CbzGly-Phe	0.02
BzGlyGly-Phe	0.05
BzGlyGly-OPhe	0.02

[a] Concentration of basic substrates was 1 mM, of hydrophobic substrates, 10 mM.

Solubility and Inhibition of Cross-Linked Crystals. Cross-linked crystals do not dissolve under conditions employed for these assays. This was demonstrated by suspending the crystals in buffer containing 0.10 M NaCl, 0.025 M Tris·HCl, pH 7.65, for periods up to 3 days. Upon separation by centrifugation, both crystals and the supernatant solution were analyzed for hydrolytic activity toward BzGly-Arg. The supernatant solution was completely inactive, while the crystals retained all activity.

The action of carboxypeptidase inhibitor (mol wt 4200) isolated from Russet Burbank potatoes (CPI) confirmed that cross-linked crystals of CPD-B are insoluble. While this inhibitor exhibits a K_I of 5×10^{-8} M in solution (Ryan et al., 1974) (Figure 1), it has no effect on the activity of crystalline suspensions (Figure 1). This not only indicates that no dissolved enzyme contaminates the crystal suspensions, but it also shows that the size of the inhibitor presumably accounts for the difference, since under identical conditions smaller inhibitors, e.g., CbzArg and β-phenylpropionate, do inhibit the crystals.

Kinetics of Peptide and Ester Hydrolysis. Activity measurements of enzyme solutions or crystals at a single substrate concentration (Table I) cannot distinguish between changes in catalytic efficiency, k_{cat}, and changes due to substrate binding, K_M. Therefore, the hydrolysis of several peptide and ester substrates by CPD-B in solution and in the crystalline state was compared over a wide concentration range. The kinetics of hydrolysis of both BzGly-Arg and BzGly-OArg, the conventional CPD-B substrates, are normal (Figure 2A,B,D,E). In solution, no inhibition is observed at high substrate concentrations, in contrast to findings with the porcine enzyme (Zisapel and Sokolovsky, 1972).

The lower activity toward BzGly-Arg of enzyme crystals relative to enzyme solutions is a consequence of changes in *both* K_M and k_{cat} (Table II). Their values in solution, 0.23×10^{-3} M and 3600 min^{-1}, respectively, agree well with previous reports of 0.15×10^{-3} M and 3300 min^{-1} (Reeck et al., 1971b). In contrast, changes in CPD-B activity toward BzGly-OArg subsequent to crystallization are largely due to a 275-fold reduction in k_{cat} (Table II). Again, the data in solution are in good agreement with previous reports (Reeck et al., 1971b).

Kinetics of CPD-B catalyzed hydrolysis of CbzGly-Phe, BzGlyGly-Phe, or BzGlyGly-OPhe, which are also substrates of CPD-A, are normal in both the solution and the crystalline state (e.g., Figure 2C,F). For CbzGly-Phe, the k_{cat} in either state is comparable to that for BzGly-Arg, although K_M is much higher in both physical states (Table II). In solution, CPD-B hydrolyzes BzGlyGly-Phe very slowly as a result both

TABLE II: Effect of Crystallization of CPD-B on Kinetic Parameters of Peptide and Ester Hydrolysis.

Substrates	Solution		Crystal		Ratio (crystal/solution)	
	k_{cat}[a]	K_M[b]	k_{cat}[a]	K_M[b]	k_{cat}[a]	K_M[b]
BzGly-Arg	3 600	0.23	220	8	0.06	33
BzGly-OArg	11 000	0.1	40	0.6	0.004	5.9
CbzGly-Phe	6 250	80	150	100	0.04	1.2
BzGlyGly-Phe	330	40	20	24	0.06	0.6
BzGlyGly-OPhe	55 000[c]	80[c]	170	3	0.003	0.04

[a] min^{-1}. [b] $M \times 10^3$. [c] Estimated from double-reciprocal plots.

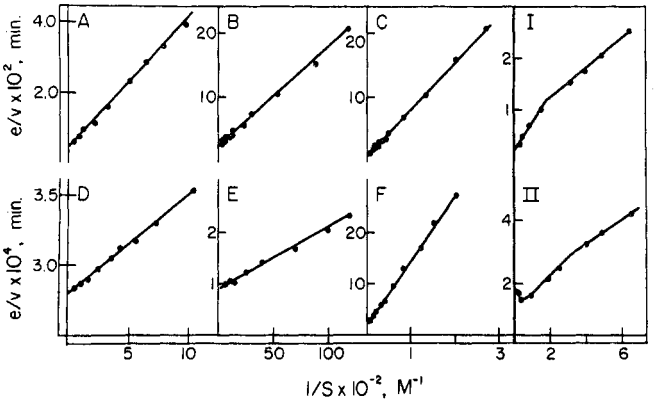

FIGURE 2: Double-reciprocal plots of the hydrolysis of BzGly-Arg (A, D), GzGly-OArg (B, E), and CbzGly-Phe (C, F) by carboxypeptidase B crystals (A-C) and solutions (D-F). I and II compare the carboxypeptidase A crystal- and solution-catalyzed hydrolysis of CbzGly-Phe, respectively (Spilburg et al., 1974, 1977).

of weak binding and of a low k_{cat}. However, while crystallization decreases k_{cat} 15- to 20-fold, K_M actually decreases with crystallization, i.e., binding becomes slightly stronger.

In solution, CPD-B binds BzGlyGly-OPhe, the ester homologue of BzGlyGly-Phe, so weakly that kinetic parameters cannot be determined accurately. However, over the substrate range which can be examined, double-reciprocal plots are linear and the best estimates of the kinetic constants are summarized in Table II. Measurements of kinetic parameters with the enzyme crystals actually proved more accurate than in solution, since crystallization increases the substrate affinity by approximately 25-fold while decreasing k_{cat} approximately 300-fold (Table II).

Discussion

The importance of an enzyme's physical state in determining its structure and activity has been discussed in recent reviews (Weber, 1975; Blake, 1976). Thus, changes in physical state could cause alterations both in the range of conformations available to a protein and in its mobility between several such conformations. Both structural and activity differences between CPD-A in solution and the crystalline state have been reported (Johansen and Vallee, 1971, 1973, 1975; Johansen et al., 1972; Riordan and Muszynska, 1974; Spilburg et al., 1974, 1977). However, it is difficult to quantitate activity differences between CPD-A crystals and solutions. Kinetic anomalies are typical of the hydrolysis of a variety of substrates; further, crystallization significantly alters the nature and details of these anomalies but does not abolish them. Thus, substrate inhibition, when observed in solution, is not apparent for the crystals where, instead, activation is enhanced or induced (Spilburg et al., 1974, 1977). However, for BzGlyGly-OPhe, the only instance so far where normal kinetics are observed in both states, k_{cat} is affected exclusively, whereas K_M remains invariant in both states (Spilburg et al., 1974, 1977). To further extend detailed examination of the consequences of crystallization on enzymatic catalysis, CPD-B, a closely related enzyme of broader specificity, has been examined.

Characterization of CPD-B Crystals. To interpret activity changes between enzyme solutions and crystals in terms of differences in enzyme structure between the two states, it is necessary to satisfy two requirements: (1) Enzyme solution must not contaminate the assays of crystals; (2) diffusion of substrate into or products from the crystals cannot affect or, in fact, represent the rate-determining step.

We have shown by two different means that cross-linked crystals of CPD-B are insoluble under the assay conditions. First, after stirring the crystals in assay buffer for 3 days, no enzyme activity could be detected in the supernatant buffer upon removal of the crystals. Second, the presence of dissolved enzyme was monitored using the potent macromolecular (mol wt 4200) inhibitor (CPI) isolated from potatoes (Ryan et al., 1974). If the inhibitor cannot enter the interior of the crystals, any inhibition resulting from addition of this agent to crystal suspensions would have to be due to interaction with dissolved enzyme or, possibly, with enzyme active centers exposed on the crystalline surface. Whereas the inhibition of CPD-B in solution is governed by a K_I of 5×10^{-8} M, the activity of crystal suspensions is not affected (Figure 1). Moreover, under identical conditions small inhibitors, such as CbzArg and β-phenylpropionate, inhibit hydrolytic activity of the crystals, demonstrating that they readily enter the crystal matrix. Together these results indicate that cross-linked crystals are not soluble and, further, that the activity observed is not solely the result of active centers exposed on the crystal surface.

Spilburg et al. (1977) have demonstrated that the effects of diffusion in crystals of CPD-A can be described by relations developed for enzymes immobilized in thin films (Katchalski et al., 1971). Though the equations likely overestimate the severity of diffusion limitation in the CPD-B crystals used here (two dimensions being small and approximately equal), application of such considerations indicates that diffusion processes cannot account for the reduction of activity observed. In the general case, diffusion limitation is importantly manifest at low substrate concentrations, i.e., at or below K_M. Under conditions where $[S] \ll K_M$, Sundaram et al. (1970), Katchalski et al. (1971) and Bunting and Laidler (1972) defined an effectiveness factor, F, for immobilized enzymes, where

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

and V and V_0 are the activities of equal concentrations of insolubilized and free solution enzyme, respectively. The thickness of the insolubilized particle is l and γ is given by

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

where k_{cat} and K_M are the pertinent kinetic parameters, [E] the enzyme concentration, and D_0' the diffusion coefficient of the substrate within the immobilized system. A value of $F = 1$ indicates that diffusion limitation is totally absent in the system.

To calculate F for a crystal-catalyzed reaction, the concentration of enzyme was determined, from the crystallographic unit cell dimensions (Schmid and Herriott, 1974), to be 14 mM. D_0' was estimated assuming that solvent structure in the crystal matrix is similar to the bulk solvent (Bishop and Richards, 1968) so that simple area restrictions due to size of solvent molecules relative to that of crystal pores modify the diffusion rate (Renkin, 1954). The pore radius was estimated to be 15 Å from reports of the crystal packing (Schmid and Herriott, 1974, 1976) and from pore-size determinations on β-lactoglobulin crystals, a protein of similar size (Low et al., 1956). The radius of BzGly-Arg was assumed to be about equal to that of CbzGly-Phe, i.e., 4.6 Å. Using these values, D_0' is estimated to be 3.6×10^{-6} cm^2/s or about 50% of its value in solution. If the kinetic parameters for the solution enzyme pertained in the crystalline states, the F value would be 0.7 for BzGly-Arg. At low substrate concentrations, a k_{cat} of 2500 min^{-1} would be expected if diffusion were tho only factor operating to reduce activity. Actually, a value of 220 min^{-1} is observed (Table II). Diffusion therefore cannot account for the observed decrease in activity. The relevant parameters are those characteristic of the enzyme in the crystalline state (Table II) and using these a F value of 0.999 is obtained, indicating that diffusion does not affect the kinetic parameters obtained for CPD-B crystals.

Effect of Crystallization on CPD-B. From specific-activity measurements it is apparent that CPD-B is a great deal less active in the crystalline than in the solution state (Table I). Since the reduction is not the result of diffusion limitation, it must reflect structural modifications that perturb inherent catalytic properties of the enzyme. These could be reflected in k_{cat}, the catalytic efficiency, and/or K_M, the substrate-binding affinity. Both parameters are obtained unambiguously, since normal Michaelis-Menten kinetics are observed with CPD-B in the solution and the crystalline state (Figure 2A–F).

As a result of crystallization, k_{cat} for CPD-B catalyzed hydrolysis of peptides is reduced by a rather constant (16- to 28-fold) factor (Table II). This is to be expected, since the same mechanism is presumed to operate in the hydrolysis of all peptide substrates by CPD-B. Such decreases, then, simply reflect a change in the enzyme's capacity to hydrolyze a peptide bond. Crystallization reduces k_{cat} for ester substrates by a much larger factor than that for peptide substrates (Table II). This difference in the effect of crystallization between ester and peptide substrates may indicate that different mechanisms are involved in CPD-B catalyzed hydrolysis of peptides and esters.

The structural bases for changes in k_{cat} when the physical state of the enzyme changes are not readily apparent and can only be surmised. Thus, modification of a single tyrosyl (Plummer and Lawson, 1966) or carboxyl (Plummer, 1971; Hass et al., 1972) group of CPD-B reduces both esterase and peptidase activities, reminiscent of the effects of crystallization. A conformation in the crystalline state precluding full participation of either of these residues might be responsible for the uniformly large changes in k_{cat}. This, in turn, could be a consequence of crystal packing forces causing the crystalline enzyme either to be in a different conformational state than the solution enzyme or to have a higher activation barrier for attaining a catalytically productive state in the crystalline phase.

The extent to which change of the physical state modifies the substrate binding area of CPD-B may at least be inferred qualitatively by examining the effect of crystallization on K_M values of various substrates. In contrast to the consistently large decreases in k_{cat} which are observed, crystallization affects substrate binding variably. Depending on the specific substrate examined, crystallization either increases, decreases, or has no effect on substrate binding affinity, indicating that the substrate-binding region of the enzyme must be structurally perturbed when the enzyme is crystallized.

Both binding affinity and catalytic efficiency of CPD-B, then, are affected by the physical state of the enzyme. These changes require that crystallization modifies the structural characteristics of the enzyme. In as much as the nature of these structural modifications is not apparent, the action of the enzyme in solution cannot be interpreted entirely in terms of the enzyme structure in the crystalline state.

Comparison of CPD-B and CPD-A Kinetics. Physical, primary sequence, and chemical-modification studies have demonstrated structural similarities and large areas of identity between bovine CPD-B and CPD-A (Cox et al., 1962; Wintersberger et al., 1965; Bradshaw et al., 1969; Plummer, 1969, 1971; Reeck et al., 1971a; Hass et al., 1972; Schmidt and Hirs, 1974; Titani et al., 1975). Homologous components in the substrate binding and catalytic sites have been identified chemically. Further, the structure of crystalline CPD-B (Schmid and Herriott, 1976) has extensive three-dimensional homology with CPD-A in the crystalline state.

In view of the structural homology reported, functional similarities between the two enzymes might be expected. The kinetics of CPD-B, however, are quite different from those of CPD-A. On the one hand, complex double-reciprocal plots are often observed with CPD-A in solution and/or the crystalline state (Figure 2, I and II). On the other hand, with CPD-B in both physical states the kinetics are normal even with those substrates which are characterized by anomolous kinetics when hydrolyzed by CPD-A (Figure 2C,E). CbzGly-Phe, for example, has a lower affinity for CPD-B than for CPD-A and, therefore, somewhat higher concentrations of substrate must be used to examine its hydrolysis by CPD-B than are required for CPD-A. It is clear, however, that over a range in substrate concentration approximately three- to fourfold above and tenfold below K_M there are no anomalies. This is in contrast to CPD-A where in the same range above and below apparent K_M anomalies are, indeed, evident. While substrate-binding models which include multiple overlapping binding sites are necessary to interpret CPD-A kinetics (Vallee et al., 1968), a simpler substrate binding model requiring no substrate activation mode pertains to CPD-B.

Differences of the kinetics between the crystals of the two enzymes may reasonably be related to differences in their three-dimensional structure in the crystalline state. Schmid and Herriott (1976) have suggested that a hydrophobic binding site provided by Tyr-198 in crystalline CPD-A (Lipscomb et al., 1970) is not present in crystalline CPD-B, since this residue is inverted and buried in the interior of CPD-B. The relevance of this observation to simpler kinetics of CPD-B solutions and crystals might be a basis for future investigations. Schmid and Herriott have also identified Asp-255 of CPD-B to be situated in a pocket homologous to the hydrophobic pocket of CPD-A (Lipscomb et al., 1970). Since this may be a component of the substrate-binding area of CPD-B, it could bear on the stronger

TABLE III: Ratios of Kinetic Parameters of CPD-B to Those of CPD-A in the Solution and in the Crystal State[a-c]

Substrate	Solution		Crystal	
	k_{cat}	K_M	k_{cat}	K_M
CbzGly-Phe	1.1	40	1.16	20
BzGlyGly-Phe	0.3	50	0.9	10
BzGlyGly-OPhe	1.7	270	5.6	10

[a] Ratios of parameters, CPD-B/CPD-A. [b] Since the substrate activation mode observed in most CPD-A kinetic profiles is absent in CPD-B kinetics, the low, unactivated region of CPD-A double-reciprocal plots is used for the purposes of quantitative comparison between kinetic parameters. [c] Kinetic parameters for CPD-A from Spilburg et al. (1974, 1977).

binding of ionic than of hydrophobic substrates to crystalline CPD-B (Table II). This amino acid substitution might then also account for the less firm binding of hydrophobic substrates to CPD-B compared with CPD-A crystals.

Much as substrate binding to the crystals of these two enzymes differ, there are also some similarities. Both hydrolyze three of the substrates examined with comparable catalytic efficiency (Table III). Additionally, the k_{cat} for hydrolysis of an active CPD-B substrate, BzGly-Arg by CPD-B crystals, is similar to the k_{cat} for hydrolysis of the similar CPD-A substrate, BzGly-Phe, by CPD-A crystals (Spilburg, 1974, 1977). From the similarities of the catalytic rate constants, k_{cat}, and the bonds cleaved, similar rate-determining steps for both enzymes could be inferred.

On changing the physical state of both enzymes, i.e., dissolving these crystals, the activities of CPD-A and CPD-B diverge. Table III shows that both the ratios of k_{cat}[CPD-B]/k_{cat}[CPD-A] and K_M[CPD-B]/K_M[CPD-A] vary between crystals and solutions. However, for each substrate examined the largest effect on going to the solution state is to increase the ratio of their K_Ms. This indicates that the substrate affinity of the two enzymes diverges more widely in solution than in the crystals. Apparently, in solution the enzyme structures are more dissimilar than in their crystals.

Dynamic studies (Harrison et al., 1975; Weber, 1975) indicate that enzymes in solution have multiple, rapidly interconverting conformations, as predicted by Linderstrøm-Lang (1952, 1959). The process of crystallization introduces interactions, e.g., crystal packing forces, the energetics of which may be comparable to those necessary to maintain the particular conformations themselves. Thus, the three-dimensional structures of enzymes in crystals and solutions may not always be identical. Indeed, different crystal forms may well comprise multiple and/or different populations of enzyme conformers. Thus, for example, in hexokinase different crystal forms exhibit variable catalytic properties. Indeed, a completely inactive native crystal form has been reported (Anderson et al., 1974). Clearly, any conclusions concerning structural bases for activity should ideally be based on measurements of structure and activity obtained on the same material. This would seem imperative when such data are to serve as the basis for defining a mechanism of action. Catalytic activity of enzymes is generally assessed on enzyme solutions, but at present comprehensive structural measurements cannot be performed in that physical state. At this point in time, determination of the activity of particular crystalline enzymes would seem to be the only feasible alternative approach for such comparisons. By this approach, it might become apparent at least whether or not the process of crystallization is associated with changes in activity. Toward this end, detailed kinetic studies on CPD-A have been performed in this laboratory (Spilburg et al., 1974, 1977) and are here compared with similar studies on CPD-B. In both enzymes, the predominant effect of crystallization is a consistently large reduction in catalytic efficiency, k_{cat}. This effect is not limited to these two enzymes. In glycogen phosphorylase (Kasvinsky and Madsen, 1976) crystallization also reduces k_{cat} dramatically. Yet we do not wish to imply that these reports indicate that large reductions in k_{cat} must necessarily accompany crystallization of enzymes, since Bayne and Ottesen (1976) have found that changes in the physical state of pig heart lactate dehydrogenase do not dramatically affect its activity. Similar observations have been reported in yet other systems (Rossi and Bernhard, 1970, Sawyer, 1972). It is not a foregone conclusion then that the most active conformations of an enzyme will predominate in both solutions and crystals or that crystals will always be inactive. No generalization regarding the effects of crystallization on enzyme function is evident as yet. Therefore, the activity of each enzyme and all of its crystal forms must be considered individually and without prejudice. Interpretation of three-dimensional structure, as currently determined largely by x-ray diffraction of crystals, in terms of catalytic mechanisms, must be firmly based on a detailed examination of the activity associated with the structure in the particular crystal form examined.

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Nanosecond Fluorescence Spectroscopy of Pyrenebutyrate–Anti-Pyrene Antibody Complexes†

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ABSTRACT: The utility of the long-lived fluorophore, pyrene, as a probe in nanosecond fluorescence depolarization measurements was investigated using pyrenebutyrate bound in the combining sites of rabbit antipyrenebutyrate immunoglobulin G. The time dependent anisotropy decay data points showed very little scatter in the time interval 0–350 ns, which is more than three times the comparable time interval observed with ϵ-1-dimethylamino-5-naphthalenesulfonyllysine (DNS-lysine) bound in the combining sites of anti-DNS antibodies [Holowka, D. A., and Cathou, R. E. (1976), *Biochemistry 15,* 3379]. Thus, the use of pyrene can significantly extend the range of macromolecular rotational correlation times that can be measured by the single photon technique. In the present investigation, we confirmed the presence of Fab segmental flexibility in immunoglobulin G molecules specific for a hapten different from DNS-lysine. We obtained a value of about 135 ns for the longer rotational correlation time which probably represents global rotation of the entire molecule. In the course of these experiments, we have also found that the combining sites of antipyrenebutyrate antibodies are, as expected, relatively nonpolar.

Nanosecond fluorescence spectroscopy is one of the most sensitive techniques currently available for the analysis of the conformation and dynamic properties of macromolecules. It has been successfully utilized to yield information on size, shape, and segmental flexibility (Yguerabide, 1972). Such studies of rotational motion require that the lifetime of the first excited singlet state of the fluorescent moiety be comparable to the rotational correlation times to be measured (Yguerabide, 1972). Substituted naphthalenesulfonates, such as DNS[1], which exhibit lifetimes of up to about 24 ns, depending on the

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[1] Abbreviations used: PBA, pyrenebutyric acid; PB-KLH, pyrenebutyryl-keyhold limpet hemocyanin; PB-HSA, pyrenebutyryl-human serum albumin; DNS, ϵ-1-dimethylamino-5-naphthalenesulfonyl; PBS, 0.02 M sodium phosphate–0.15 M NaCl buffer, pH 7.4.