Proc. Nat. Acad. Sci. USA
Vol. 71, No. 10, pp. 3922–3926, October 1974

The Physical State Dependence of Carboxypeptidase A_α and A_γ Kinetics

(enzyme kinetics/conformation/crystals/solution)

CURTIS A. SPILBURG, J. LEMUEL BETHUNE, AND BERT L. VALLEE*

Biophysics Research Laboratory, Department of Biological Chemistry, Harvard Medical School, and Division of Medical Biology, Peter Bent Brigham Hospital, Boston, Massachusetts 02115

Contributed by Bert L. Vallee, July 11, 1974

ABSTRACT **Spectrochemical probes have demonstrated that the conformations of carboxypeptidase A (EC 3.4.12.2) differ in solution and in the crystalline state. Detailed kinetic studies of carboxypeptidase A_α and A_γ crystals and solutions now show that the physical state of the enzyme is also a critical parameter that affects the function of the A_α and A_γ enzymes in the same manner. The kinetic profiles and the corresponding kinetic constants of substrate hydrolysis are, therefore, important functional indices of the known conformational differences of the enzyme in these two physical states. The complex kinetic behavior of this enzyme, however, precludes meaningful comparisons of activity measurements for crystals and solutions obtained at only one substrate concentration. Underlying differences in varying substrate-inhibiting or -activating binding modes can result in either high or low activity ratios, concealing the true, functional consequences of the change in physical state. Thus, for all substrates examined, crystallization of the enzyme markedly reduces catalytic efficiency, k_{cat}, from 20- to 1000-fold. Equally as important, the substrate inhibition, apparent in solution for some di- and depsipeptides, is abolished with crystals, while for longer substrates the normal solution kinetics may acquire activation with the crystals. Hypothetical modes of substrate–enzyme interaction, generated by superimposing substrate models on the crystal structure of carboxypeptidase to simulate kinetics in solution, have failed to detect both of these changes, which affect inhibitory or activating binding modes. The only structure of carboxypeptidase yet published and that of its functionally inert complex with the pseudosubstrate, glycyl-L-tyrosine, derive from a unique form of carboxypeptidase A_α crystals. These crystals differ from all others with regard both to their spectral properties and activity toward carbobenzoxyglycyl-L-phenylalanine, which is 30% of that in solution, though the significance of this value cannot be gauged without knowledge of the relevant kinetic constants. The rapidly accumulating evidence for functional and conformational differences between crystals and solutions and the recent stress on the nonproductive aspects of the carboxypeptidase A_α-glycyl-L-tyrosine complex, based on 30% site occupancy, suggest that the functional implications of its structural features require reevaluation.**

Numerous studies over the past 25 years have emphasized the remarkably complex kinetic behavior of carboxypeptidase A (EC 3.4.12.2; peptidyl-L-amino acid-hydrolase). It deviates from classical Michaelis–Menten kinetics when hydrolyzing di- and depsipeptides, particularly CbzGly-Phe, BzGly-Phe and BzGly-L-PhLac, commonly used because of their high turnover number (1). Hydrolysis of these substrates is distinguished by various combinations of substrate and product inhibition and/or activation, reflecting the topological organization of the active center and its modes of interaction with substrates (2, 3). A model for substrate and product binding has placed these findings on a common basis and suggested that the use of longer substrates would eliminate such kinetic anomalies (4, 5). The subsequent synthesis and kinetic analysis of the hydrolysis of tri- and tetrapeptides and their ester analogues verified this prediction, since these substrates are characterized by normal Michaelis–Menten kinetics (6–8). Importantly, all of these data and conclusions have been based on the behavior of the enzyme in solution.

In order to translate such functional data from solution kinetics into structural information, models of substrates have been superimposed onto the three-dimensional structure of the enzyme, as discerned from x-ray analysis of crystals, in hypothetical productive and nonproductive binding modes (9). This procedure has been thought to be a valid means of visualizing substrate binding directly and of identifying catalytic and mechanistic features of the active center (9). Throughout, such correlations presume that functional properties of carboxypeptidase A in solution can be interpreted in terms of its known crystal structure, based on the hypothesis that its conformations in the two states are identical (10).

However, studies using a number of spectrochemical probes have all shown that the conformations are different. Thus, either nitration of Tyr-248 or coupling it with diazoarsanilic acid reveals that, in solution, the location of this residue with respect to the zinc atom differs from that in the crystals (11–13). Moreover, even prior to the x-ray structure determination, the activity of enzyme crystals toward Cbz Gly-Phe was found to be much lower than that of its solutions, strongly suggesting the possibility of different conformations in the two physical states (14–16). Since explicit analyses of the detailed kinetics governing crystal-catalyzed hydrolysis have not been reported, we have now determined the kinetic parameters for crystals of carboxypeptidase A_α and A_γ acting on a series of substrates. The catalytic properties of these two forms are identical both in solution and in crystals.

On going from solutions to crystals of both the A_α and A_γ enzymes, the catalytic rate constant, k_{cat}, decreases markedly for all substrates examined. Moreover, crystallization of the enzyme can drastically alter the kinetic profile of any given substrate compared to that pertaining in solution under otherwise identical conditions. Thus, for BzGly-Gly-Phe, normal

Abbreviations: CbzGly-Phe, carbobenzoxyglycyl-L-phenylalanine; BzGly-Phe, benzoylglycyl-L-phenylalanine; BzGly-L-PhLac, benzoylglycyl-L-phenyllactate; CbzGly-Gly-Phe, carbobenzoxyglycylglycyl-L-phenylalanine; BzGly-Gly-L-PhLac, benzoylglycylglycyl-L-phenyllactate; Gly-L-Tyr, glycyl-L-tyrosine.

* Author to whom correspondence should be addressed.

TABLE 1. *Activity ratios* for crystals (V_c) and solutions (V_s) of carboxypeptidases A_α and A_γ*

Substrate	V_c/V_s
CbzGly-Phe	0.03
BzGly-Phe	0.03
BzGly-L-PhLac	0.01
CbzGly-Gly-Phe	0.05
BzGly-Gly-Phe	0.04
BzGly-Gly-L-PhLac	0.001

* Substrate concentrations: peptides (0.01 M), esters (0.001 M).

kinetics in solution acquire substrate activation in the crystals; for CbzGly-Gly-Phe, substrate inhibition is inverted to activation and, remarkably, the characteristically complex substrate inhibition of BzGly-L-PhLac in solution is replaced by substrate activation in the crystals. Thus, over and above the diminution of k_{cat}, crystallization of the enzyme strikingly alters productive and nonproductive peptide and ester binding to carboxypeptidases A_α and A_γ consistent with the spectral and chemical evidence for the existence of different conformations of the active center in the two physical states (11–13, 17). These kinetic data constitute, in fact, telling evidence of the functional consequences of such conformational differences, thereby making available novel indices for their detection. As a consequence, reliance on solution kinetic data to give functional significance to the crystal structure of carboxypeptidase cannot lead to valid mechanisms.

Thus far we have found only one substrate, BzGly-Gly-L-PhLac, which is hydrolyzed with normal kinetics by crystals and solution. It is, therefore, suitable to examine the effect of the physical state on structure-dependent function. This substrate is hydrolyzed at 1/1000th of the rate in solution, solely due to a decrease in k_{cat}; in contrast, K_M remains constant. A report of this work has been presented (18).

MATERIALS AND METHODS

Crystalline suspensions of several carboxypeptidase A_α (Sigma) and carboxypeptidase A_γ (Worthington) preparations were washed three times with metal-free water and recrystallized. CbzGly-Phe, BzGly-Phe, CbzGly-Gly-Phe, BzGly-Gly-Phe, and BzGly-Gly-L-PhLac were provided by Dr. D. S. Auld. Gly-Tyr (Mann) and benzoylglycine and glutaraldehyde (Aldrich) were used as received. Stock solutions were made up in 1.0 M NaCl–0.05 M Tris (pH 7.5) and extracted with dithizone in carbon tetrachloride. Enzyme crystal suspensions were crosslinked with 1% glutaraldehyde (15). After removal of residual, soluble enzyme by prolonged extraction with 1.0 M NaCl, the crystals were stored in water at 4°. Uniform crystals of appropriate dimensions were obtained by sedimentation (14, 15), selecting only those of 10 to 20 × 5 × 5 μm, such that diffusion of substrate is not rate limiting and specific activity does not depend on size (15). After the crystals were solubilized in 0.1 M NaOH at 70° for 6 hr, protein concentrations (to ±8%) were determined by the method of Lowry *et al.* (19), with native carboxypeptidase A as the standard. In our hands the continuous stirring method has proven most suitable for kinetic analyses, obviating transport and packing problems involved in flow systems (14, 15). The assays of esterase and peptidase activities have been described (6, 20). After sedimentation of crystals, the supernatant was found free of

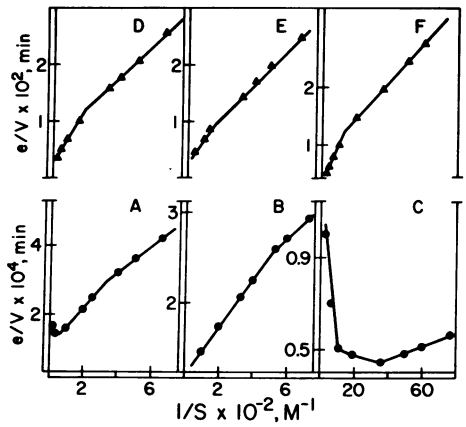

FIG. 1. Lineweaver–Burk plots for the hydrolysis of CbzGly-Phe (left panel), BzGly-Phe (middle panel), and BzGly-L-PhLac (right panel) by carboxypeptidase solutions (*A*, *B*, and *C*) and crystals (*D*, *E*, and *F*). The ordinate scales differ by 100, owing to the lower activity of the crystals.

enzymatic activity in all instances, demonstrating the absence of dissolved enzyme. In both physical states, specific activities were constant over a 12-fold enzyme concentration range. Crosslinking reduced activity 2-fold, as noted (15). For statistical purposes, 20 assays with uniform-sized crystals were carried out over a period of several months, with different preparations of carboxypeptidases A_α and A_γ. Both isomers hydrolyze Cbz-Gly-Phe with identical, highly reproducible turnover numbers, 210 min^{-1} ± 30. The results give confidence in the reproducibility of the method and the validity of deductions reached by comparing the functional properties of the enzyme in the two physical states.

RESULTS AND DISCUSSION

The activity of crystalline carboxypeptidases A_α and A_γ toward CbzGly-Phe is 30-fold lower than that of the enzyme in solution (Table 1), confirming previous observations (14, 15). Extension of such activity measurements to BzGly-Phe and BzGly-L-PhLac reveal similar 30- and 100-fold reductions, respectively. Indeed, for BzGly-Gly-L-PhLac, activity is reduced 1000-fold (Table 1). Thus, the effect of crystallization on activity is not confined to a single substrate or a single class of substrates.

However, activity measurements, determined at one substrate concentration, cannot differentiate changes in catalytic efficiency from those in substrate binding. The multiple-site model of substrate binding to carboxypeptidase (5) is based on the behavior of the enzyme in solution over a wide concentration range of CbzGly-Phe, BzGly-Phe, and BzGly-L-PhLac, all previously considered or used as standard substrates in the past. Their hydrolysis by the enzyme in solution is characterized by gross kinetic anomalies, apparent from the nonlinearity of double-reciprocal plots (Fig. 1*A–C*). The multiple-site model accounts for the anomalies by postulating multiple substrate-binding loci on the enzyme (Fig. 2). Both substrate activation and inhibition are apparent with CbzGly-Phe (Fig. 1*A*); BzGly-L-PhLac (Fig. 1*C*) shows extreme substrate inhibition, and BzGly-Phe (Fig. 1*B*) only substrate activation (1, 5). As revealed by the double-reciprocal plots, the changes in kinetic behavior occur within up to three regions of substrate concentration (Fig. 1). The kinetic param-

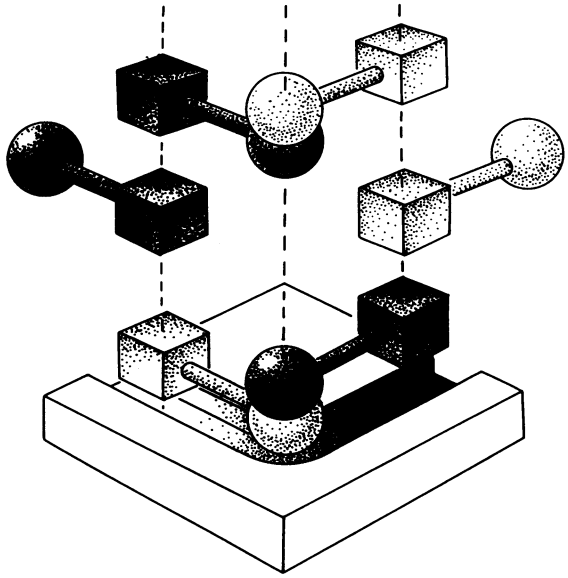

FIG. 2. Model portraying dual or multiple substrate-binding sites in solution. The rectangular area and groove schematically represent the enzyme surface and sites for substrate binding, respectively. Peptides (white symbols) and esters (black symbols) are thought to interact with the enzyme surface in regions characteristic of each, as indicated by the white and black areas of the groove; an area of overlap, representing one of the possibilities, is indicated by the coincidence of the symbols and the gray shading of the groove in the center. Some of the many alternative modes of possible substrate interactions are exemplified in projection. The representation of substrates is symbolic only and does not attempt to define the identity and number of substrate or protein residues (or groups) interacting, or the forces involved in such a scheme, e.g., conformational changes brought about by either inhibitors or substrates (4, 5).

eters derived by extrapolations from the linear segment corresponding to lowest substrate concentrations and from the activation region are summarized in the upper part of Table 2, columns 2 and 3. The regions of inhibition at high substrate are not readily analyzed numerically in this manner.

The kinetics of the enzyme crystals, studied over the same range of substrate concentrations, differ remarkably from those of the enzyme in solution. The hydrolysis of CbzGly-Phe (Fig. 1*D*) and BzGly-Phe (Fig. 1*E*) and that of BzGly-L-PhLac (Fig. 1*F*) catalyzed by carboxypeptidase A_α and A_γ crystals exhibit only substrate activation. Thus, throughout, crystallization eliminates the substrate inhibition observed in solution.

Comparison of k_{cat} and K_M values, extrapolated from the linear regions in Fig. 1*D–F*, with those obtained in solution (Table 2, columns 4 and 5) reveals that crystallization predominantly affects k_{cat}, the reduction ranging from 20- to 50-fold in both regions of substrate concentrations (Table 2, columns 2 and 4). The changes in the K_M values of these three substrates (Table 2, columns 3 and 5) likely reflect the elimination of the inhibitory substrate-binding modes to varying degrees and as a function of the range of substrate concentrations inspected. Thus, in the two physical states, both catalytic efficiency and at least some inhibitory binding modes of the enzyme differ, though the kinetic anomalies preclude the unambiguous analysis of the kinetic constants.

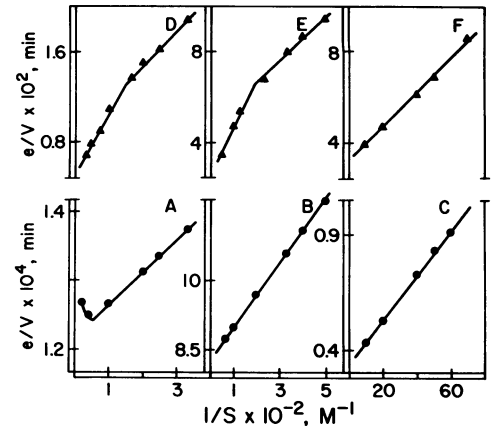

FIG. 3. Lineweaver–Burk plots for the hydrolysis of CbzGly-Gly-Phe (left panel), BzGly-Gly-Phe (middle panel), and BzGly-Gly-L-PhLac (right panel) by carboxypeptidase solutions (*A*, *B*, and *C*) and crystals (*D*, *E*, and *F*). The ordinate scales differ by 100 to accommodate the 50- to 1000-fold lower activity of the crystals.

The multiple binding site model (5) suggested that an increase in length of substrates would constrain the manner of their binding such that their hydrolysis would then approach Michaelis–Menten kinetics. In solution, this hypothesis has been verified for a series of tri- and tetrapeptide substrates with different blocking groups and their depsipeptide analogues, allowing the definitive delineation of kinetic parameters (6–8, 20). Thus, BzGly-Gly-Phe (Fig. 3*B*) and BzGly-Gly-L-PhLac (Fig. 3*C*) exhibit normal kinetics but differ from the above substrates only by the addition of another glycyl residue, and CbzGly-Gly-Phe (Fig. 3*A*) shows only substrate inhibition. The kinetic constants for these substrates, obtained under conditions identical to those in Fig. 1, are summarized in the lower part of Table 2, columns 2 and 3. The linearity of the double-reciprocal plots for BzGly-Gly-Phe and BzGly-Gly-L-PhLac readily allows determination of the kinetic constants, and their estimation for CbzGly-Gly-Phe is simplified greatly compared with those of its dipeptide homolog, CbzGly-Phe.

TABLE 2. *Kinetic parameters of peptide and ester hydrolysis*

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

* M × 10^3.

[a] and [b] refer to values extrapolated from regions of high and low substrate concentrations, respectively.

Crystals, however, hydrolyze BzGly-Gly-Phe with features typical of substrate activation (Fig. 3*E*). For CbzGly-Gly-Phe there is a dramatic reversal: substrate inhibition in solution is replaced by activation for crystals (Fig. 3*D*). Furthermore, this study also identifies BzGly-Gly-L-PhLac as the one substrate that is suitable for kinetic comparisons of crystals and solutions, since it is entirely devoid of anomalies in both physical states (Fig. 3*C* and *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 2, columns 2 and 4, bottom line). This reduction cannot be due to competitive substrate inhibition, since this would require a K_M lower by three orders of magnitude, while, in fact, K_M is unchanged (Table 2, columns 3 and 5, bottom line). Thus, crystallization abolishes the inhibitory binding modes whenever they are observed in solutions of all esters and peptides examined. Moreover, with crystals, all peptide substrates display activation, regardless of whether or not they do in solution. Thus, compared with solutions, the activity of carboxypeptidase crystals would be expected to increase if solely substrate binding were altered. Actually, in all instances, activity decreases due to the large decrease of k_{cat}. Again, for the two tripeptides and the ester analog, crystallization predominantly affects k_{cat}, the anomalies of peptide binding reflecting in K_M (Table 2).

The physical state of the enzyme also affects the mode of action of known modifiers of peptidase activity of the enzyme (21). Thus, in solution, addition of, e.g., benzoylglycine or cyclohexanol, markedly increases the rate of hydrolysis of CbzGly-Phe, attributed to prevention of substrate inhibition (5). Their addition hardly alters the activity of enzyme crystals, in accord with the weakening or absence of inhibitory enzyme–substrate binding modes.

The differences in the kinetic behavior of carboxypeptidase A_α and A_γ crystals on one hand, and of their solutions on the other, bear importantly on efforts to interpret the function of the enzyme in structural terms. X-ray structure analysis obviously can pertain directly only to its conformation(s) in the crystals. Similarly, the kinetics of the enzyme in solution can reflect only conformation(s) prevailing in that state. Although the determination of the total three-dimensional structure of carboxypeptidase in solution has not been feasible so far, the conformation(s) of the enzyme in the two physical states has been held either to be the same or not to differ significantly (9, 10). Based on this premise, the extensive kinetic information in solution has been applied directly to crystals (9, 10).

This assumption has important implications for the delineation of enzyme mechanisms in general and for that of carboxypeptidase in particular; its validity remains a serious, unresolved question. The state of knowledge continues to preclude matching of the three-dimensional crystal structure of carboxypeptidase with that in solution. However, as an alternative, Richards has successfully compared the functional properties of a number of enzymes, including carboxypeptidase A, in these two physical states (14–16, 22). The activity of carboxypeptidase A toward CbzGly-Phe is drastically reduced at all substrate concentrations studied. Further, ease of zinc removal, inhibition by β-phenylpropionate, degree of reversibility of denaturation, and the enzymatic consequences of organic and inorganic modifications were shown to be functions of the physical state, though the basis of these differences could not then be ascertained (13–15).

Our studies using Richards' procedures fully confirm these conclusions and bear out that "studies such as these can define the properties of enzyme in lattice crystals in the same manner as is normally done in solution" (15). Extending his approach, we have obtained kinetic profiles for six peptide and ester substrates over a broad range of concentrations and derived the kinetic parameters.

Of all substrates here examined, BzGly-Gly-L-PhLac is the only one that does not exhibit kinetic anomalies in either physical state. Hence, the effect of crystallization on k_{cat} and K_M can be evaluated unequivocally. In this instance the sole effect is on k_{cat}, the parameter also affected primarily in all other substrates (Table 2). It is an important, general feature of all these esters and peptides that substrate inhibition in solution is abolished in the crystals. As noted, moreover, in crystals, all peptides display substrate activation, regardless of whether or not they do in solution (Figs. 1 and 3).

In spite of existent data demonstrating differences in the rates at which crystals and solutions hydrolyze CbzGly-Phe and the questions raised regarding the structural significance of these differences (15), substrate model building experiments were performed based on solution kinetics (9, 23). Carboxypeptidasesubstrate complexes did not prove susceptible to direct crystallographic analysis; hence, such simulations with substrate models were substituted to localize substrate-binding sites to account for their catalytic, activating, and inhibiting modes (9, 23). For such purposes it was assumed that Gly-L-Tyr occupies the productive binding site, as deduced from crystallographic experiments (9, 23). This premise must now be viewed in the light of the fact that in those experiments, only one-third of the enzyme molecules were occupied by Gly-L-Tyr (23). Further, stress has now been placed on the nonproductive aspects of the Gly-L-Tyr active-site occupancy (24).

An interaction of CbzGly-Phe with Tyr-198 and Arg-71 was postulated to account for CbzGly-Phe inhibition (9, 23); as is apparent, this inhibition is not observed in the crystals (Fig. 1). It was pointed out (see above) that longer substrates are free of anomalies in solution (6–8). Model experiments with crystals accounted for this as well (9, 23); but again this is contrary to the present findings (Fig. 3). Model building experiments represent the only structural basis for observations regarding enzyme interactions with the ester, BzGly-L-PhLac (9); their significance must also be evaluated in the above context. Such inconsistencies raise questions regarding the value of this entire approach to identify productive and nonproductive substrate-binding modes when based on the crystal structure of the carboxypeptidase–Gly-L-Tyr complex.

Why would model building and inspection of the three-dimensional structure of carboxypeptidase fail to detect the substrate inhibition that is abolished in crystals? The power of x-ray crystallographic analysis resides in its capacity to visualize directly what must otherwise be inferred from more indirect approaches, such as kinetics. It may well be that the presumption of identical conformations in the two physical states (10) may have impeded recognition that they are different.

In this context it is remarkable that in the face of the accumulating experimental evidence for conformational (11–13, 17) and functional (14–16) differences between crystal and solutions of all carboxypeptidase forms, only those crystals used for x-ray analysis remain exceptions, both as regards

their properties (24, 25) and availability for study. It is curious that they alone should exhibit 30% of the activity of the enzyme in solution toward CbzGly-Phe (25). This differentiates them from all other crystal forms examined so far (12). Since conditions, experimental details, and results, e.g., kinetic profiles as well as k_{cat} and K_M values, have not been reported for the x-ray crystals (24, 25), such activities, dissimilar as compared with other crystals, may be apparent only.

The present kinetic data, in conjunction with earlier studies of dynamic, conformation-dependent spectral probes (11–13), are most consistent with the suggestion that the conformation of the active site of carboxypeptidase is a function of the physical state. Detailed kinetic analysis of substrate hydrolysis catalyzed by different crystals is a readily feasible means to assess and compare their functional properties. 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.

This work was supported by Grants-in-Aid GM-15003 and GM-02123; J.L.B. is the recipient of Career Development Award, GM-28081; and C.A.S. is the recipient of Postdoctoral Fellowship GM-54189, all from the National Institutes of Health, of the Department of Health, Education, and Welfare.

1. Davies, R. C., Riordan, J. F., Auld, D. S. & Vallee, B. L. (1968) *Biochemistry* **7**, 1090–1099.
2. Vallee, B. L. & Riordan, J. F. (1968) *Brookhaven Symp. Quant. Biol.* **21**, 91–119.
3. Vallee, B. L., Riordan, J. F., Auld, D. S. & Latt, S. A. (1970) *Phil. Trans. Roy. Soc. London Ser. B* **257**, 215–230.
4. Vallee, B. L. (1967) *Proc. VII Intern. Congr. Biochem. (Tokyo)*, 149–150.
5. Vallee, B. L., Riordan, J. F., Bethune, J. L., Coombs, T. L., Auld, D. S. & Sokolovsky, M. (1968) *Biochemistry* **7**, 3547–3556.
6. Auld, D. S. & Vallee, B. L. (1970) *Biochemistry* **9**, 602–609.
7. Auld, D. S. & Vallee, B. L. (1970) *Biochemistry* **9**, 4352–4359.
8. Auld, D. S. & Vallee, B. L. (1971) *Biochemistry* **10**, 2892–2897.
9. Lipscomb, W. N., Hartsuck, J. A., Reeke, G. N., Quiocho, F. A., Bethge, P. H., Ludwig, M. L., Steitz, T. A., Muirhead, H. & Coppola, J. C. (1968) *Brookhaven Symp. Quant. Biol.* **21**, 24–90.
10. Lipscomb, W. N. (1972) *Chem. Soc. Rev.* **1**, 319–336.
11. Johansen, J. T. & Vallee, B. L. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 2532–2535.
12. Johansen, J. T. & Vallee, B. L. (1973) *Proc. Nat. Acad. Sci. USA* **70**, 2006–2010.
13. Riordan, J. F. & Muszynska, G. (1974) *Biochem. Biophys. Res. Commun.* **57**, 447–451.
14. Quiocho, F. A. & Richards, F. M. (1964) *Proc. Nat. Acad. Sci. USA* **52**, 833–839.
15. Quiocho, F. A. & Richards, F. M. (1966) *Biochemistry* **5**, 4062–4076.
16. Quiocho, F. A., Bishop, W. H. & Richards, F. M. (1967) *Proc. Nat. Acad. Sci. USA* **57**, 525–537.
17. Shaklai, N., Zisapel, N. & Sokolovsky, M. (1973) *Proc. Nat. Acad. Sci. USA* **70**, 2025–2028.
18. Spilburg, C. (1974) *Fed. Proc.* **33**, 1529.
19. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275.
20. Auld, D. S. & Holmquist, B. (1974) *Biochemistry*, **13**, 4355–4361.
21. Davies, R. C., Auld, D. S. & Vallee, B. L. (1968) *Biochem. Biophys. Res. Commun.* **31**, 628–633.
22. Doscher, M. S. & Richards, F. M. (1963) *J. Biol. Chem.* **238**, 2399–2406.
23. Lipscomb, W. N., Reeke, G. N., Hartsuck, J. A., Quiocho, F. A. & Bethge, P. H. (1970) *Phil. Trans. Roy. Soc. London Ser. B* **257**, 177–214.
24. Lipscomb, W. N. (1973) *Proc. Nat. Acad. Sci. USA* **70**, 3797–3801.
25. Quiocho, F. A., McMurray, C. H. & Lipscomb, W. N. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 2850–2854.