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Torsional Strain Considerations in the Mechanism of the Proteolytic Enzymes, with Particular Application to Carboxypeptidase A

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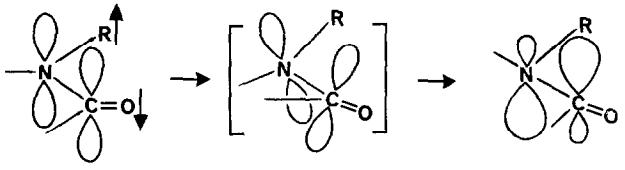
Torsional deformation of the peptide linkage by *anti* distortion of *cis* substituents (i.e., forcing groups attached to one side of an amide partial π bond out of plane in opposite directions) leads to rehybridization of the constituent atoms (nitrogen and carbonyl carbon) toward tetrahedral geometry. In consequence the partial π bond is uniquely activated toward *trans* (antarafacial) addition with defined steric orientation of addends. Application of these considerations to the known structure of an enzyme–substrate complex of carboxypeptidase A leads to a unique mechanistic hypothesis for proteolytic cleavage by this enzyme. Extant evidence concerning the mode of catalysis is considered in light of a mechanism involving electrostatically induced torsional activation of the scissile peptide bond, Lewis acid coordination of zinc to amide carbonyl, proton donation from Glu 270 to the amide nitrogen of the scissile bond, with concerted attack upon the amide carbonyl by solvent water.

The purpose of this article is to point out a hitherto unappreciated consequence of induced torsional strain in the amide functionality. While twisting of the >N—C(O)– pπ–pπ bond has been widely accepted as a plausible enzymic activation of the peptide linkage (*1–3*), a complete consideration of the chemical consequences of this molecular distortion has escaped scrutiny. We shall show that thermodynamic destabilization (*1–3*) of the amide functionality is not the only result of induced nonplanarity of the π system, but that according to the manner in which strain is introduced, certain mechanisms may be discounted relative to others for the critical addition step of transacylation. The concept that we here present, in conjunction particularly with the crystallographic structure of an enzyme–substrate complex, allows a definite exclusion of certain previously proposed mechanisms for the proteolytic enzyme carboxypeptidase A and suggests the true sequence of chemical events involved in its action.

Torsion vs rehybridization. We commence with an analysis of the response of the amide group to a forced distortion of its preferred planar configuration. In particular, consider the case in which the *cis* substituents of a partial π bond are drawn away from *opposite* faces of the nominal coplanar array of the functional group (see diagram). Such a distortion could easily be induced to a moderate extent by intramolecular forces involved in enzyme–substrate binding. The critical realization is that a pure torsional response (figure in brackets), with retention of sp^2 hybridization and a diminution of pπ–pπ overlap, is a naiveté (albeit a widely held one). In actuality, a modest *rehybridization* of the unsaturated atoms will occur ($sp^2 \rightarrow sp^{2-3}$). This has the primary effect of



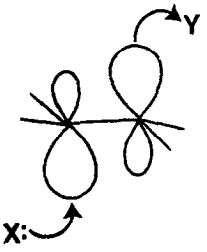
bringing the axes of the (predominately) p atomic orbitals back generally into parallel; the resultant gain in π overlap compensates for the expense of incorporation of some s character. Of particular relevance is the observation that it is orbital lobes on *opposite faces* of the partial π bond which are expanded in this mode of molecular distortion. It is the chemical consequences of this latter result that are significant and that will be elaborated upon subsequently.

Before an exploration of the stereochemical manifestations of the postulated rehybridizations, it is important to point out the factual foundation for the previously described geometrical readjustments. The effects described are an intrinsic property of $p\pi$–$p\pi$ bond; it has been unambiguously established for the olefinic linkage by *ab initio* molecular orbital calculations as well as by analysis of the vibrational force field for ethylene that rehybridization takes precedence over pure torsion (*4*, *5*). That this conclusion carries over to the amide partial π bond is documented by structural studies of strained lactams as well as by spectroscopic and theoretical examination of the amide linkage. Caprylolactam has a nonplanar *transoid* amide group in the crystal (*6*); both the carbonyl carbon as well as the amide nitrogen were revealed to have undergone modest rehybridization by X-ray crystallographic determination.[1] [The mode of deformation for this latter molecule—*syn* distortion of *trans* substituents—differs from the induced deformations considered elsewhere in this article, i.e., *anti* distortion of *cis* substituents. The stereochemical consequences of the different modes of deformation are in fact complementary (*4*); however, the same order of magnitude of rehybridization is to be anticipated in either case (*5*).] The question of the equilibrium geometry of a distorted amide may also be examined by analysis of the infrared vibration frequencies of suitable small molecules (e.g., formamide) (*7*) or by molecular orbital calculations (*8*, *9*). Force constants so obtained appear consistent with the hypotheses put forth in this article. From the latter it may be recognized that the carbonyl carbon of an amide should undergo substantially less rehybridization than the nitrogen upon "torsional" deformation. Nevertheless, such intramolecular readjustments as occur will be in the *directions* postulated; hence, the mechanistic inferences which follow are sustained.[2]

Enzymological consequences. We now turn to chemical implications of our conjectured rehybridization. Our only assumption is a simple and obvious one: For a concerted addition to such a strained double bond as we have described, attack will be upon the expanded atomic orbital lobes (i.e., maximum overlap considerations dictate the lowest energy reaction path). It follows that (a) *trans* (antarafacial) addition will occur

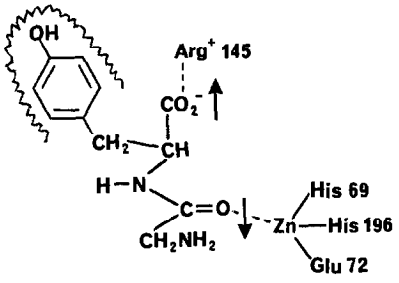
[1] Equivalent amounts of twisting plus out-of-plane bending are found for the nonplanar amide group.

[2] It should be appreciated how little energy is involved in rehybridization: For a 20° twist in ethylene (each *cis* substituent moved 10° out of original plane), the cost is ca. 2.5 kcal/mol (*5*). It should be less in amides because the vibrational frequencies are lower.



exclusively in the situation described previously and (b) a clear distinction can be made with regard to which face the respective addends (X, Y) will approach toward in the case of a polar π bond such as an amide (i.e., stereoselectivity will be coupled with regiospecificity). We shall indicate the significance of the latter point by analysis of a specific enzyme mechanism.

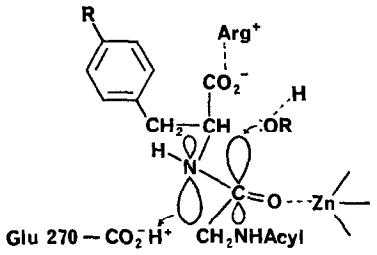
In spite of one of the most thorough kinetic and X-ray crystallographic examinations to date, the mechanism of carboxypeptidase A has remained substantially an enigma. We shall have reference to the structure of the enzyme complex with glycyl-L-tyrosine, following Lipscomb (*10–13*).[3] The disposition of substrate-binding groups at the active site allows a firm inference of torsional activation of the peptide linkage, as Lipscomb has pointed out (*10–13*). The relevant strain-inducing interactions are summarized as follows.



With the substrate tyrosyl aromatic ring fitted into the hydrophobic pocket of the enzyme and the amide carbonyl coordinated with the zinc atom at the active site, the electrostatic bond between the substrate tyrosyl carboxylate and Arg^+ 145 should clearly tend to twist the amide π bond in the manner depicted (arrows). Such a distortion may strongly be inferred from stereographic projections of the enzyme–substrate complex, although, as Lipscomb notes, such a conclusion follows from model building rather than the precise location of atoms by X-ray diffraction.

However, upon the assumption of the validity of this mode of peptide activation, the direction of *rehybridization* of the amide linkage may immediately be specified as depicted in the following diagram. This in turn suggests that an addition across the amide π bond (if that is indeed the mechanism) would be facilitated for approach of electrophile from below and nucleophile from above, as portrayed. The converse mode

[3] Vallee (*14*) has questioned the functional significance of the crystal structure of carboxypeptidase for mechanistic interpretation of reaction kinetics. We take the more charitable view that any catalytic activity at all within the crystal (3- to 100-fold reductions are common) indicates an intact and functioning active site (*15*).

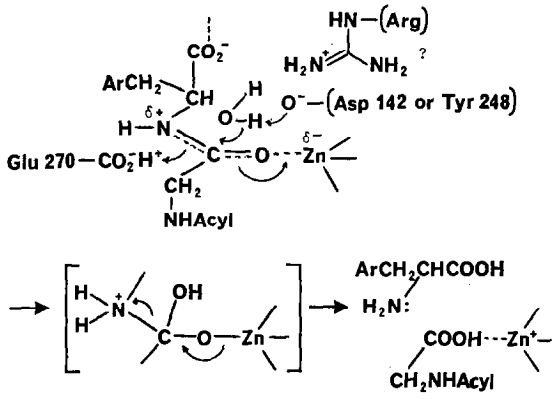


of addition should in fact be disfavored in a least motion concerted process. This mechanism differs profoundly from previous proposals based upon the crystal structure, which have uniformly invoked some form of nucleophilic participation (e.g., acyl or proton acceptance) by a carboxylate anion of glutamic acid 270 (*10–13*). However, by our analysis this latter residue is constrained to interact with the face of the amide on which the nitrogen p-lobe is expanded. We conclude that its most plausible role is that of *proton donor* (see diagram). Carrying through our analysis to its rational conclusion, approach of the nucleophile to the carbonyl carbon must be from the opposite (upper) face of the amide linkage, where the (weakly) expanded receptor p-lobe is to be found. The only sterically accessible enzyme functional group for the role of acyl acceptor is the phenolic hydroxyl of tyrosine 248. However, since this should lead to the formation of a detectable acyl-enzyme intermediate [which has never been observed; *O*-acetyl (*11*, *16*, *17*) and other functionalized Tyr 248 derivatives have been prepared and are catalytically active toward ester substrates (*18–21*)], we are led to propose a direct attack by water instead. We perceive in the vicinity of the Tyr 248 hydroxyl in the enzyme–substrate complex previously sparingly considered (*10–13*) guanidinium groups (Arg 71, 127) as well as an available carboxylate (Asp 142). We offer the aesthetic speculation that in the catalytic step a water molecule or a hydroxyl group is delivered to the amide carbonyl from what amounts to a water-binding and/or activating site comprised of these groups. For example, we suggest that in peptide hydrolysis a hydroxide may be yielded up from the solvation sphere of a guanidinium-carboxylate or -phenoxide ion pair, held against the appropriate face of the scissile amide bond. This aspect of the mechanism will be given further consideration later in conjunction with a discussion of pertinent experimental evidence. Our scheme therefore comprises the direct transfer of the acyl residue to solvent without the intermediacy of a covalent acyl-enzyme and, in fact, with only the transient existence of a tetrahedral intermediate (see diagram next page).[4]

It is appropriate to note that our proposal may be regarded as a specific example of the Pauling–Lienhard–Wolfenden generalization (*23*, *24*). We have prescribed the obligatory geometry of the transition state; the role of the enzyme is to direct the substrate to that conformation by virtue of its superior affinity for the distorted peptide (*25*, *26*). Given such an obvious clue as to the direction of the reaction coordinate, it is not difficult to implicate the additional chemical functionality at the active site.[5]

[4] Our scheme bears a limited similarity to a general mechanism tentatively put forward for esterase activity by Vallee (*22*).

[5] Lipscomb (*12*) acknowledges probable pyramidalization of the bound peptide linkage but does not pursue its consequences as we have demonstrated.



It is our contention that the aforedescribed mechanism is fully in accord with all available data on the catalytic action of carboxypeptidase (where such data are not selfcontradictory), and furthermore resolves certain unexplained features of its activity. In the following paragraphs we shall identify the particularly important evidence which substantiates the new mechanism.

The pH rate profile. Carboxypeptidase typically exhibits a rate maximum for peptide substrates at pH 7.3, with inflections at ca. pH 6.7 and 8.5 (*10, 13, 27*). More particularly, for the kinetically well-behaved substrate Cbz-Gly–Gly–L-Phe (*28–30*) a plot of $\log(k_{cat}/K_m)$ as a function of pH reveals ionizations with pK'_a of 6 and 9 as rate controlling (*29*). In the lower pH range, k_{cat} in fact is limiting, indicating a transformation in the enzyme–substrate complex. Since the attachment of H^+ to the *substrate* carboxylate anion would neutralize the electrostatic attraction for Arg^+ 145 and thus relax the torsional activation of the scissile peptide bond, we suggest that the apparent pK that restricts reaction on the acid side corresponds to the protonation of the terminal peptide carboxylate in the enzyme–substrate complex. The apparent increase by several pH units from the normal value for a –COOH group must be ascribed to the hydrophobic environment, for which there is precedent (see following paragraph). As an *alternative* we suggest for this protonation the deactivation of an enzymic H_2O-binding site (pK of 6.2 in uncomplexed enzyme), as will be developed subsequently.

Requiring even greater credulity is our proposal for the functionality which undergoes rate-limiting deprotonation with pK above 8.5. For the same substrate Cbz-Gly–Gly–L-Phe (*29*), the pK'_a of 9 is attributed to the K_m component. The indicated ionization of an *enzyme* group we think must be for none other than the *carboxyl* of Glu 270, previously implicated as the proton donor in the critical addition step of the peptide bond scission. This functional group at the active site in fact is situated within a cleft in the enzyme and is surrounded by hydrophobic groups (e.g., Ile 247, Leu 201, Leu 203). Under these circumstances such an anomalously high pK value for a –COOH group is at least plausible.[6] It might further be observed that there is no apparent k_{cat} limitation up to pH 10.5. We note that Glu 270 becomes largely covered and shielded from solvent by the substrate in complex with the enzyme (*10–13*). Hence, either this pK is shifted to yet higher values in the complex, or (more likely) the proton is kinetically

[6] We may cite an appropriate example: Transfer of acetic acid from H_2O to 82% dioxane–H_2O (30°) results in an increase of pK_{COOH} by 5.77 units (*30, 31*).

inaccessible to external bases. Quite possibly this involves substantial hydrogen bonding to the rehybridized amide nitrogen (which in turn contributes to substrate binding and therefore a K_m dependency).

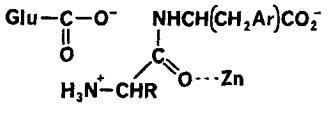
We should like to make further comment about this unusually nonacidic active site residue. We argue that not only is such a pK value sufficient but in fact it is necessary for effective catalysis. We have reference to Jencks' (*32*) recent realization of the requirements for general acid–base catalysis of complex reactions. It will be observed that the site of protonation of the substrate in our mechanism undergoes a large change in pK in the course of reaction (from amide nitrogen to carbinolamine) and that the pK of the catalyst is likely intermediate between such values (considering the nonpolar environment). With regard to the latter point, more important is the change in pK of the catalyst in the course of reaction. If one allows that in the incipient *enzyme–substrate* complex the carboxyl in question (Glu 270) is protonated, while in a conformationally shifted (and perhaps more solvated) *enzyme–product* complex (or, more accurately, enzyme–tetrahedral intermediate) more typical pK values occur, then a *thermodynamic driving force* for the critical proton transfer is apparent as the hydrolysis reaction proceeds. While the concept of a variable enzyme pK may seem heretical, it is complementary to the idea of a shifting substrate pK (*32*). We propose that an enhancement of the Brønsted acidity of neutral, proton-donating functionality attendant upon its transfer from lipophilic to polar environments *in the course* of an enzymic reaction constitutes a useful adjunct to conventional principles of acid–base catalysis.[7]

Role of zinc. Limited consideration has here been given to the complexed metal ion at the active site. Of course it is a binding site (amide carbonyl) and contributes to the torsional activation of the peptide bond as previously outlined. In this capacity it is also a *Lewis acid.* To the extent that it coordinates with carbonyl oxygen, it should enhance the electrophilicity of the amide carbon, as well as *increase* the double bond character of the $\rangle$C—N$\langle$ amide linkage. Consequently, the rehybridization which we have described may be more equally distributed between carbonyl and nitrogen atoms than in an uncomplexed amide. It is for this reason that we resist the temptation to estimate quantitatively the geometry of the transition state; evidence (force constants) from strained but uncomplexed amides may be of limited applicability to a peptide bound at the metalloenzyme active site.[2]

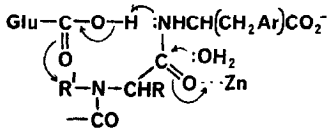
The dipeptide anomaly. The presence and integrity of the *second* peptide bond in substrates of carboxypeptidase has been found to be critical for rapid hydrolysis. Dipeptides bearing a free amino terminus react slowly unless that group is acylated (*33*). On the other hand, *N*-substitution on the second peptide bond (e.g., sarcosine) greatly decreases rates of hydrolysis (*34, 35*). Dipeptides and depsipeptides frequently show deviation from classical Michaelis–Menten kinetics (*28–30*). The crystallographic enzyme–substrate determination reveals that the carboxyl of Glu 270 is positioned proximate to the substrate functionality under consideration (*10–13*). Our interpretation is that dipeptides bearing a free (basic) amino terminus are unreactive because the latter functionality effectively deprotonates Glu 270; i.e., only if Glu 270 is catalytically active in its acid form does it become plausible that introduction of a spurious proton acceptor at the active site should inhibit reactivity. Alternative mechanistic proposals

[7] A corresponding rule involving base catalysis may be envisioned, as well as corollaries for charged functionality becoming neutral in the course of reaction.

(*10–13*) invoking nucleophilic participation by an ionized Glu 270 carboxylate may be discounted by this reasoning. On the other hand, it is reasonable that a catalytically beneficial hydrogen bond should exist between the *neutral* NH of reactive peptide substrates and the carbonyl of Glu 270–COOH (possibly via a water bridge). This might reasonably position the acid function for effective proton donation to the scissile peptide bond, as well as stabilize the carboxylate thus formed. Hence, substitution of the second amide >NH inhibits catalysis. These proposals are summarized in the diagrams given below. It is also possible that attractive forces between the enzyme and the second peptide linkage (and subsequent residues) of the substrate contribute to torsional activation of the scissile bond, as previously outlined.



Catalytically inactive



R' = H, catalytically active
R' = CH_3, catalytically inactive

Esterase activity, side-chain modification, and solvent deuterium isotope effects. Our mechanism rationalizes several hitherto unexplained characteristics of carboxypeptidase activity. These fall jointly under the categories enumerated and will be considered together for convenience. In summary, the facts are that several ester analogs (e.g., phenyllactates) are efficiently hydrolyzed (*10–13*). The high pH rate limitation is less evident. [This latter observation has been attributed to substrate inhibition (*20, 29*).] Derivatization of the side chain of Tyr 248 inhibits peptidase activity but does not prevent esterase activity (*16–21*). Likewise, modification of arginine residues at the active site by diacetyl (most likely Arg 71 or 127) abolished peptidase activity but enhanced esterase activity (*36*). Finally, no solvent isotope effect for peptidase activity has been found, but esterase activity exhibits a deuterium isotope effect of ca. two (*37*).

Since we regard both amide and ester hydrolyses as probably proceeding through the same mechanism,[8] these data allow a further refinement of our understanding of enzyme activity. We have identified the key prototropic step as transfer of the Glu 270 carboxylic acid proton to scissile amide nitrogen. The absence of a deuterium isotope effect suggests to us that this transfer is substantially complete in the transition state for peptide hydrolysis. On the other hand, with ester substrates the receptor atom (oxygen) is less basic and the transition state must occur earlier, resulting in conventional rate retardation for deuterium transfer. Presumably for ester hydrolysis nucleophilic attack by the acyl acceptor (water) is further advanced in the transition state;

[8] This is a matter of some contention (*38, 39*).

acid catalysis contributes proportionally less than torsion and hydration to the attainment of the activated complex.

The effect of side-chain modification may likewise be interpreted in terms of the intrinsic relative ease of ester vs amide hydrolysis. In either case by our model the acyl acceptor is a molecule of solvent water. As previously described, the major ambiguity in our mechanism is whether or how H_2O is activated for nucleophilic attack upon the scissile peptide bond. We propose that for susceptible *esters* minimal activation is necessary but, that for *peptides*, Tyr 248, Asp 142 plus either Arg 71 and/or Arg 127 in some manner cooperatively facilitate incipient or prior deprotonation of water so as to generate hydroxide as the actual nucleophile. Such activation may in one (less likely) extreme be visualized as base catalysis by Tyr 248 (present as an guanidinium–phenoxide ion pair). In such case the catalyst pK corresponding to acid quenching of the reaction (pH 6–7) may be conjointly due to protonation of this unusually acidic phenol. [Nitration studies allow an inference of a low pK for this tyrosine residue (*11*, *18*, *20*, *21*) although it is questionable that such acidity is attainable.] In the other extreme the polar environment of this region of the active site (i.e., two cationic arginine side chains, the tyrosyl residue, an aspartyl carboxylate, plus considerable exposure to solvent) may simply operate to orient a water molecule properly via hydrogen bonding and to provide a microregion of sufficient dielectric constant to support the charge generation in subsequent steps. It is this aspect of the mechanism of carboxypeptidase which in our opinion is most deserving of further experimental scrutiny.

Conclusion. While certain ambiguities remain in our mechanistic proposal, what we have attempted to show is that induced torsion in the amide bond will predispose that bond toward a particular mode of *trans* addition. The value of our postulate is that certain mechanisms may be excluded (or at least discounted) for enzymic reactions, if they do not exploit the stereodirective activation present in the complexed substrate. While this interpretational approach was illustrated for an exonuclease, we think it likely to be applicable to the endonucleases as well [particularly thermolysin (*40*), but also chymotrypsin, trypsin (*41*), pepsin, papain etc.]. Where an extended polymer is fitted to an enzyme cleft, we think it highly probable that a construable activating torsion might be induced at the catalytic site by nonbonded interactions between substrate and enzyme. Experiments designed to test the hypotheses put forth in this article are in progress.

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REFERENCES

1. R. LUMRY, "The Enzymes" (P. D. Boyer, H. Lardy, and K. Myrbäck, Eds.), 2nd ed., Vol. 1, p. 157. Academic Press, New York, 1959.
2. W. P. JENCKS, "Current Aspects of Biochemical Energetics" (N. O. Kaplan and E. P. Kennedy, Eds.), p. 273. Academic Press, New York, 1966.
3. W. P. JENCKS, "Catalysis in Chemistry and Enzymology," p. 282. McGraw-Hill, New York, 1969.

4. W. L. Mock, *Tetrahedron Lett.*, 475 (1972).
5. L. Radom, J. A. Pople, and W. L. Mock, *Tetrahedron Lett.*, 479 (1972).
6. F. K. Winkler and J. D. Dunitz, *J. Mol. Biol.*, **59**, 169 (1971).
7. S. T. King, *J. Phys. Chem.*, **75**, 405 (1971).
8. G. N. Ramachandran, A. V. Lakshminarayanan, and A. S. Kolaskar, *Biochim. Biophys. Acta*, **303**, 8 (1973).
9. G. N. Ramachandran and A. S. Kolaskar, *Biochim. Biophys. Acta*, **303**, 385 (1973).
10. J. A. Hartsuck and W. N. Lipscomb, "The Enzymes" (P. D. Boyer, Ed.), 3rd ed., Vol. 3, p. 1. Academic Press, New York, 1971.
11. F. A. Quiocho and W. N. Lipscomb, *Advan. Protein Chem.*, **25**, 1 (1971).
12. W. N. Lipscomb, *Proc. Nat. Acad. Sci. USA*, **70**, 3797 (1973).
13. W. N. Lipscomb, *Tetrahedron*, **30**, 1725 (1974).
14. C. A. Spilburg, J. L. Bethune, and B. L. Vallee, *Proc. Nat. Acad. Sci. USA*, **71**, 3922 (1974).
15. F. A. Quiocho, C. H. McMurray, and W. N. Lipscomb, *Proc. Nat. Acad. Sci. USA*, **69**, 2850 (1972).
16. J. F. Riordan and B. L. Vallee, *Biochemistry*, **2**, 1460 (1963).
17. R. T. Simpson, J. F. Riordan, and B. L. Vallee, *Biochemistry*, **2**, 616 (1963).
18. J. F. Riordan, M. Sokolovsky, and B. L. Vallee, *Biochemistry*, **6**, 358 (1967).
19. M. Sokolovsky and B. L. Vallee, *Biochemistry*, **6**, 700 (1967).
20. J. F. Riordan, M. Sokolovsky, and B. L. Vallee, *Biochemistry*, **6**, 3609 (1967).
21. J. T. Johansen and B. L. Vallee, *Proc. Nat. Acad. Sci. USA*, **68**, 2532 (1971).
22. B. L. Vallee, *Fed. Proc.*, **23**, 8 (1964).
23. G. E. Lienhard, I. I. Secemski, K. A. Koehler, and R. N. Lindquist, *Cold Spring Harbor Symp. Quant. Biol.*, **36**, 45 (1971).
24. R. Wolfenden, *Accounts Chem. Res.*, **5**, 10 (1972).
25. M. Caplow, *J. Amer. Chem. Soc.*, **91**, 3639 (1969).
26. W. P. Jencks, B. Schaffhausen, K. Tornheim, and H. White, *J. Amer. Chem. Soc.*, **93**, 3917 (1971).
27. H. Neurath and G. W. Schwert, *Chem. Rev.*, **46**, 69 (1950).
28. D. S. Auld and B. L. Vallee, *Biochemistry*, **9**, 602 (1970).
29. D. S. Auld and B. L. Vallee, *Biochemistry*, **9**, 4352 (1970).
30. D. S. Auld and B. L. Vallee, *Biochemistry*, **10**, 2892 (1971).
31. G. A. Rogers and T. C. Bruice, *J. Amer. Chem. Soc.*, **96**, 2473 (1974).
32. W. P. Jencks, *J. Amer. Chem. Soc.*, **94**, 4731 (1972).
33. K. Hofmann and M. Bergmann, *J. Biol. Chem.*, **134**, 225 (1940).
34. M. A. Stahmann, J. S. Fruton, and J. Bergmann, *J. Biol. Chem.*, **164**, 753 (1946).
35. E. L. Smith, *J. Biol. Chem.*, **175**, 39 (1948).
36. B. L. Vallee and J. F. Riordan, *Brookhaven Symp. Biol.*, **21**, 91 (1968).
37. B. L. Kaiser and E. T. Kaiser, *Proc. Nat. Acad. Sci. USA*, **64**, 36 (1969).
38. E. T. Kaiser and F. W. Carson, *Biochem. Biophys. Res. Commun.*, **18**, 457 (1965).
39. D. S. Auld and B. Holmquist, *Biochemistry*, **13**, 4355 (1974).
40. B. W. Matthews, P. M. Colman, J. N. Jansonius, K. Titani, K. A. Walsh, and H. Neurath, *Nature New Biol.*, **238**, 41 (1972).
41. R. Huber, W. Bode, D. Kukla, U. Kohl, and C. A. Ryan, *Biophys. Struct. Mechanism*, **1**, 189 (1975).