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Torsional-Strain Considerations in Enzymology

Some Applications to Proteases and Ensuing Mechanistic Consequences

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A stereoexplicit analysis of amide torsional strain reveals two distinct rehybridization modes, each of which correlates with a unique hydrolysis mechanism. These may be characterized as *anti* distortion of *cis* (*Z*) substitutents (yielding *trans* addition of HOR) and *syn* distortion of *trans* (*E*) substituents (correlating with *cis* addition). The latter mechanism is known to apply in the cases of the serine proteases, and supporting evidence for torsional activation is sought in crystallographic structures of trypsin with its inhibitors as well as from kinetic data. A contrast is made with the mechanism of carboxypeptidase A, which operates on the former principle. For this enzyme it is demonstrated that an unusual minor-protonation state is most probably the catalytically active species, a conclusion with some important ramifications. A general comparison of the torsional strain hypothesis is made with a recently propounded theory concerning stereoelectronic control of cleavage of hemiorthoamide tetrahedral intermediates. When these approaches to enzyme mechanism are used in tandem, a unique comprehension is achievable.

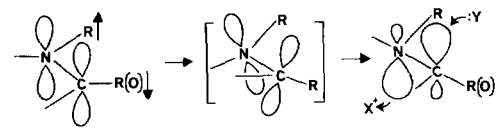
The concept of an induced activation of enzyme substrates, brought about by pre-equilibrium binding interactions, is not new in enzymology (*1*). However, the use of this principle for deducing specific enzyme mechanisms has received less attention than is merited. We have recently shown that detailed analysis of torsional-strain induced, stereoexplicit rehybridization of glycyltyrosine in complex with carboxypeptidase A, as inferred from crystallographic evidence, leads to a unique interpretation of the role of active-site residues in the catalytic steps of that enzyme (*2*). It is reasonable to inquire whether recognition of such a universal principle might also impose revision of mechanistic ideas about the serine proteases (trypsin, chymotrypsin, elastase). We here apply torsional-strain analysis to what is known of the reactivity as well as the structure of substrate and inhibitor complexes of these enzymes. Subsequently, we shall reconsider carboxypeptidase A to review the fundamental mechanistic contrast of these classes of proteases and to elaborate further on the perplexing pH–rate profile of this metalloenzyme. Finally, we show the congruence of our concepts with some recent insightful studies of stereoelectronic control in the breakdown of tetrahedral intermediates. Taken together, these theoretical approaches to the mechanism of induced amide hydrolysis provide the capability for uniquely defining enzyme mechanisms from relatively limited active-site structural information.

Torsion vs Rehybridization

Our approach focuses upon the manner in which an enzyme *activates* a peptide bond toward cleavage. We suggest that generally this may be accomplished by torsional

destabilization; i.e., the scissile >N–C(O)– linkage is rendered noncoplanar by nonbonded interactions between substrate and enzyme (*2*). This mode of activation has long been accepted as plausible *in principle*. What has hitherto been lacking is a full appreciation of the chemical consequences of such a distortion.

It must be realized that there are *two* kinds of torsional strain, each of which leads to a unique reaction coordinate. We approach the required analysis on an abstract level, since the phenomenon we are to describe is a general property of the $p\pi$–$p\pi$ bond and its applicability extends beyond peptide hydrolysis. A double bond (such as the partial π bond of an amide) may be torsionally deformed either by *anti* distortion of *cis* (*Z*) substituents or by *syn* distortion of *trans* (*E*) substituents (Scheme 1). In both of these



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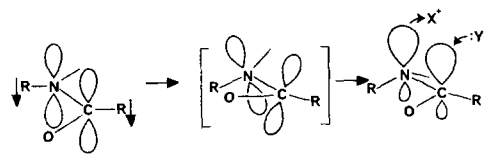


(2)

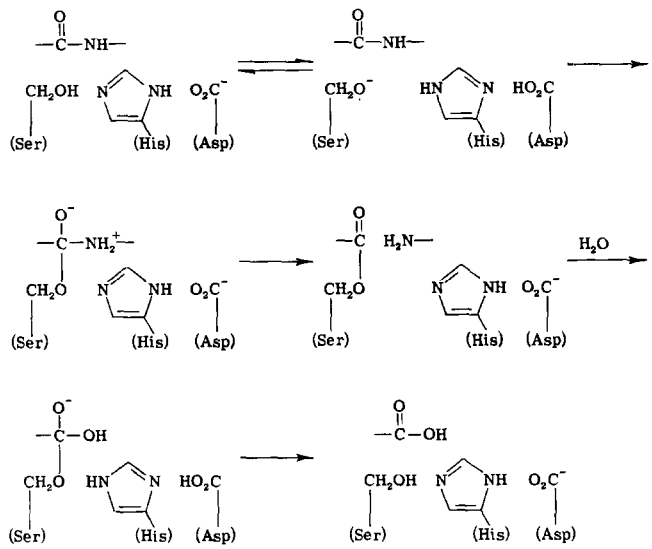
SCHEME 1

cases a pure rotational response (figure in brackets), with retention of sp^2 hybridization and a diminution of $p\pi$–$p\pi$ overlap, is *not* the ultimate response (*3*). In the former case, the resultant of drawing *cis* (*Z*) substituents away from opposite faces of the nominal coplanar array ((Eq. (1)) is a *rehybridization* of the constituent atoms ($sp^2 \rightarrow sp^{2-3}$). This has the primary effect of bringing the axes of the (predominantly) *p* atomic orbitals back generally into parallel; the resultant gain in π overlap compensates for the expense of incorporation of some *s* character. In consequence, orbital lobes on *opposite faces* of the partial π bond are expanded, and therefore addition to such a multiple bond would preferentially be *trans* (antiperiplanar), directed by maximum overlap considerations. Such an analysis applied to the crystallographic structure of a substrate complex of carboxypeptidase A has led to a novel formulation of the mode of proteolytic cleavage by that enzyme and may abrogate previous mechanistic proposals (*2*).

It now appears to us that torsional activation of the second kind (Scheme 1, Eq. (2)) may be applicable to the serine proteases. In the latter instance, the activating torsion is brought about by drawing *trans* (*E*) substituents away from the *same face* of the nominally coplanar $p\pi$–$p\pi$ bond. For this mode of distortion a particularly apt model exists in the case of the crystal structure of caprylolactam (*4*). The X-ray structure determination reveals a transoid amide group in which both nitrogen and carbonyl carbon have undergone partial pyramidalization in just the manner suggested. The stereochemical consequence of such rehybridization will be exclusive *cis* (synperiplanar) addition to the π bond, in contradistinction to the former case.

Enzymological Consequences

Our reason for suspecting that the latter mode of peptide activation might pertain to the serine proteases is based upon accumulated knowledge of the mechanism of these enzymes, summarized in Scheme 2 (*5–7*). Since the same imidazole group serves both as base (abstracting proton from nucleophilic serine hydroxyl) and as acid (delivering proton to scissile amide nitrogen), the net addition to the partial bond must be *cis*,[1] a conclusion substantiated by examination of crystallographic structure determinations



SCHEME 2

(see following). Therefore, if torsional activation of any kind is important in the mechanism of these enzymes, it must be of the latter kind [*syn* distortion of *trans* (*E*) substituents (Eq. (2), Scheme 1)].

Remarkable agreement with this proposal may be found in the crystal structure of the complexes of trypsin with its naturally occurring inhibitors (*8–10*). Bovine pancreatic trypsin inhibitor is a small protein with a region highly complementary to the active-site portion of trypsin. It contains a lysine residue (Lys-15) which fills the side chain pocket and presents a peptide linkage to the nucleophilic serine of trypsin. Two facts are relevant: (i) This amide group is transoid, and (ii) the carbonyl group (and presumably the nitrogen) is substantially pyramidalized in the complex (*8*, *9*). This latter geometrical distortion is a consequence of binding interactions in forming the complex, since the uncomplexed inhibitor does not exhibit such rehybridization. Furthermore, pyramidalization of the carbonyl may not be attributed to nucleophilic approach of enzymic HO_γ-Ser 195 (*11*, *12*), since it persists in the complex with anhydrotrypsin (a catalytically inactive form of the enzyme containing dehydroalanine, produced by elimination of the elements of water from the active site serine residue) (*13*). It has been

[1] This is true whether protonation is concerted with nucleophilic attack or subsequent thereto. Consideration of this aspect of the mechanism is deferred (last section). The residence of the bound proton between the His–Asp pair is formulated according to Ref. (*5*) and (*6*); see, however, Ref. (*7*).

suggested that the incipient proteolysis of the inhibitor complexed with trypsin does not proceed due to the extremely stable and rigid ternary structure of the complex. We concur in this assessment.

A possible implication of these observations is that this same mode of substrate deformation is integral to the mechanism of hydrolysis of the usual peptide substrates of trypsin and its congeners (*8*, *10*). We shall attempt to show how examination of this assumption allows a further refinement in understanding of the chemical principles governing action of the serine proteases. This insight is helpful in comprehending why both the acylation and the deacylation steps are accelerated enzymically. We shall consider these aspects in turn.

Acylation

In claiming significance for torsional activation, one must confront the fact that while trypsin and its congeners function chiefly as endopeptidases (wherein distortion of the scissile peptide linkage may be induced by nonbonded interactions with residues on either side of that bond), they also are catalytically effective for displacing ammonia from simple amides [i.e., acylphenylalaninamides, $RCONHCH(CH_2C_6H_5)CONH_2$, with chymotrypsin]. It follows that torsional activation may only be a contributing factor to the rate of hydrolysis of peptides, facilitating an otherwise already accelerated process. However, some evidence favoring substrate destabilization has been recorded.[2]

In the case if soybean trypsin inhibitor, it has been shown that formation of the stable complex (probably containing a covalent tetrahedral intermediate (*10*)), from an initial (noncovalent) inhibitor–enzyme complex is accelerated relative to k_{cat} for good substrates by a factor of approximately 100-fold (*15*, *16*). This is in accord with relief of the hypothesized strain, but its significance is questionable (see final section) and depends upon whether formation or decomposition of the quaternized amide carbonyl is normally rate determining in proteolysis (*8*, *14*).

Concerning the hydrolysis of good polypeptide substrates, in discussions of leaving-group specificities in the literature it has been pointed out that significant nonbonded interactions occur with the peptide residue being displaced in the acylenzyme-forming step for chymotrypsin (*17*). Of importance is the observation that the difference between amide substrates (e.g., $AcPheNH_2$) and peptides (e.g., $AcPhe–AlaNH_2$) appears not in K_m but rather in k_{cat} (which is enhanced for peptides). The inference is that a potentially greater binding interaction with the larger peptide is nullified by induction of strain in the substrate, which in turn expedites hydrolysis (*1*). The pertinent nonbonded interactions have in fact been identified by model building and in crystal structures of enzyme–inhibitor complexes, previously alluded to. In chymotrypsin they involve residues 42 (Cys), 57 (His), 192 (Met), 193 (Gly), and 195 (Ser) (*17*). Equivalent contacts plus additional interactions have been discerned in trypsin–inhibitor complexes (*10*). Similar studies with elastase and extended peptides may be interpreted in terms of substrate destabilization (*18*), which requires a reciprocal enzyme deformation, incidentally. The torsional-strain hypothesis represents a plausible mechanism by which the apparent peptide activation might be realized, although we have reservations on such inference.

[2] With physiological (amide) substrates, steps leading to enzyme acylation are rate determining (*14*)

Deacylation

The acylenzyme is rapidly hydrolyzed under conditions of normal proteolysis. It is our contention that the torsional activation hypothesis holds relevance for this step as well. While direct crystallographic evidence is lacking, it may reasonably be surmised by extrapolation from the inhibitor–trypsin tetrahedral-intermediate structures that the Ser-195 O_γ-ester obtained upon elimination of the departing amine will be noncoplanar. This follows from the assumption that the plane of the incipient carbonyl group [O=CR(OSer)] should bisect the $R'H_2N^+$—CR(OSer)—O^- angle of the dissociating tetrahedral intermediate as contained in the crystal structure of porcine trypsin with soybean inhibitor (*10*). Mentally performing this transformation upon the existing models reveals that the resultant ester (acylenzyme) will also be *torsionally activated* toward *cis* addition (e.g., by H_2O). As indicated in Scheme 2, by microscopic reversibility the same steps as in the acylation follow in reverse order. It might be noted that unnatural substrates, such as acetate esters, would not yield acylenzymes with rigidly oriented substrate binding. In consequence the intermediate would presumably not be torsionally activated and therefore might be expected to lead to detectable accumulation of acyl enzyme, as observed.[3]

The Question of Nonproductive Binding

It should be acknowledged that current mechanistic arguments for the serine proteases rely heavily for experimental support upon the structure of enzyme–inhibitor complexes. Inasmuch as these do not proceed readily on to hydrolyzed product, it is conceivable that they represent abortive binding of substrate to active site. Our hypothesis allows an inference that such is probably not the case. The strain activation present in the complexes correctly leads to the reaction coordinate. Conceivably, inhibitors might be found in which a substrate is deformed in such a way as to discourage transacylation; *anti* distortion of *cis* (*Z*) substitutents (as described previously) is one such possibility. In the present instance, failure to observe continuation of the hydrolysis mechanism with the trypsin inhibitors must be ascribed to other factors (tight binding of the potential fragments, exclusion of H_2O, etc.) as indicated by those responsible for the crystallographic evidence.

However, in the final assessment we would express some hesitation in accepting the conclusion of generic torsional activation in the hydrolysis mechanism of serine proteases. The preceding evidence is equivocal, both with respect to the kinetic data as well as regards the pertinence of the crystallographic results. Furthermore, there is a conflict to be resolved involving *additional* theoretical considerations, which suggest that potential rate acceleration from release of substrate strain might be partially neutralized by an unfavorable stereoelectronic factor (see final section).

Mechanism of Carboxypeptidase A

In an earlier article the mode of action of carboxypeptidase A was divulged (*2*). It is fundamentally different from that of the serine proteases and involves a direct *trans* addition of the elements of water across the scissile peptide linkage. This is a

[3] Consult Ref. (*1*), p. 224, for a succinct computation of the rate factors involved. Spectroscopic evidence for noncoplanarity (torsional activation) within the carboxylate of the acyl enzyme has been adduced (*19*).

necessary consequence of the mechanism of activation, which involves *anti* distortion of *cis* (*Z*) substituents (Eq.(1)). As was shown (*2*), the role of the enzyme active-site residues may be uniquely assigned, since the direction of approach of the H^+ and OH^- moieties is explicitly defined by the induced deformation of the substrate. *The proton donor can be no other than the side chain carboxyl of glutamic acid residue 270* (Glu-270). Most probably in the case of amide hydrolysis, a phenolate of tyrosine 248 (Tyr-248) functions as a base to abstract a proton from water, generating an OH^- nucleophile for synchronous quaternization of the substrate carbonyl (Fig. 1).[4]

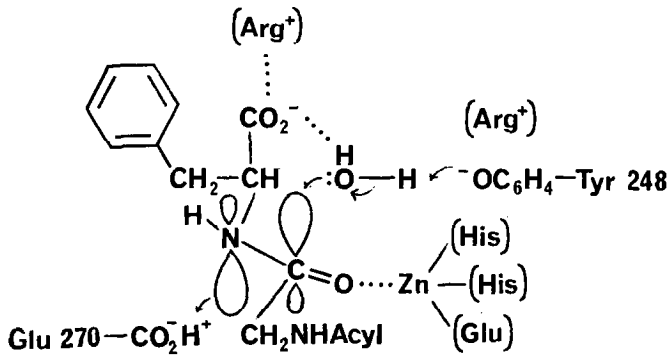


FIGURE 1

Active Site pK Values

While kinetic studies with carboxypeptidase are notoriously difficult (severe complications from product inhibition, etc.),[5] a general pH–rate profile for good peptide substrates unequivocally shows a rate maximum at neutral pH and limiting p*K* values near pH 6 and 9. Since the mechanism as revealed by torsional-strain analysis *requires* a protonated carboxyl on Glu-270 throughout its range of activity, we initially assigned to this residue the higher p*K* (= 9), an anomalously exhalted value for a carboxyl. As pointed out to us by W. W. Cleland, it is unnecessary to make this assumption.

The key realization is that the two ionizable active-site residues (most probably Glu-270 and Tyr-248) do not function completely independently but necessarily exhibit a reciprocal influence on total ionization of the protein. If we assign "normal" p*K* values to Glu-270-CO_2H (= 6) and Tyr-248-C_6H_4OH (= 9),[6] then indeed in the catalytically active pH range the predominant species will be Glu-270-CO_2^-...Tyr-248-C_6H_4OH. However, *this will not be the exclusive species present.* It is intuitively apparent (and may be rigorously demonstrated; see Appendix) that throughout the neutral pH range

[4] An alternative role for Tyr-248, that of acyl acceptor analogous to the active-site hydroxymethyl group of the serine proteases, may be discounted on the evidence that the acetyl-O_{Ph}-Tyr-248-modified enzyme is quite stable. Hence, the acyl enzyme intermediate in this putative mechanism should accumulate (the enzyme specificity is for the amino moiety, which is released); also see Ref. (*20*).

[5] Tight product binding is indicative of destabilization of bound substrate (*1*) and is also consistent with the minimal synthetase activity of carboxypeptidase (amide reformation; see, however, Ref. (*20*)).

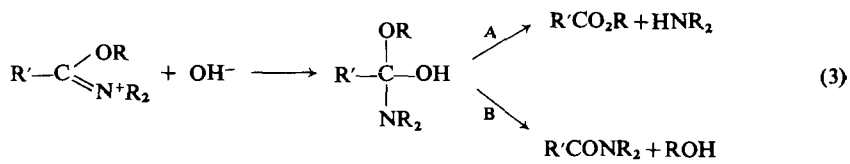
[6] These values represent small perturbations from typical carboxylates and phenols; however, there is independent evidence supporting such p*K*s for these active site residues of carboxypeptidase A (*21–25*).

there will be a small amount of the complementary pair, Glu-270-CO_2H...Tyr-248-$C_6H_4O^-$. Since the difference in the experimental pK values amounts to 3 units (pK 9 minus pK 6) it is a thermodynamic requirement that for every approximately 1000 "normal" ionization states there must be one in which *the reverse situation exists.* It is necessary and sufficient that this be the *catalytically efficacious species.* We need to point out that this interpretation is entirely consistent with the pH–rate determinations. The latter only defines the gross state of ionization; it is not possible to distinguish between the microscopic states described by any kinetic method involving pH variation (see Appendix).

The consequences of this realization may be briefly listed. Foremost is the intuitive appreciation of enzyme mechanism which ensues. Since the innately exothermic relay of a proton from Glu-270-CO_2H to Tyr-248-$C_6H_4O^-$ is coupled with the quaternization of the substrate carbonyl, the very great facility with which carboxypeptidase accomplishes hydrolysis is further understood (*2*). We suspect that this principle is broadly applicable in enzymology. Second, the interpretation applied to the rate parameters for such catalysis requires reconceptualization. In the example cited, where the catalytically active enzyme species represents *ca.* 1/1000 of the total, k_{cat} may in actuality be as much as 1000-fold greater than the kinetically measured value based on total enzyme. However, this conclusion depends on the relative affinities toward substrates of catalytically correctly and incorrectly protonated enzyme. The Appendix may be consulted for the appropriate governing equations.

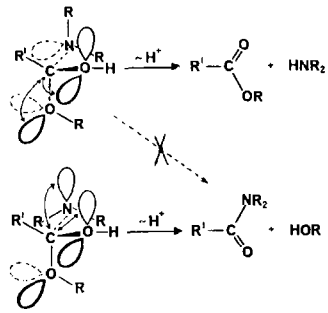
Stereoelectronic Control of Formation and Cleavage of Tetrahedral Intermediates

We should finally like to review briefly the work of Deslongchamps and co-workers (*26*, *27*) on the hydrolysis of imidate salts. We believe that the steroelectronic theory which they have developed is specifically applicable to peptide hydrolyses by enzymes. We suggest that their conclusions are not only congruent with our own, but they are complementary in the sense of illuminating different aspects of the same phenomenon. In general, imidate salts were observed to react with hydroxide to yield tetrahedral intermediates, which then partitioned between esters and amides (Eq. (3)). Division between paths A and B appeared to be largely dependent upon the *stereochemistry*



(3)

of the imidate salt. It had to be assumed (on the face of the evidence) that the tetrahedral intermediate breaks down to products instantaneously, before appreciable free rotation about C–O or C–N bonds can occur. By way of interpretation, it was postulated that ejection of a residue (HOR or HNR_2) critically requires the assistance of *two* antiperiplanar lone pair orbitals on the remaining heteroatoms of the incipient ester or amide (Scheme 3). In the event that only one pair was potentially available, facile decomposition was not observed. A large number of geometrically differently constrained imidates was examined, with uniform consistency with this theory.



SCHEME 3

It would indeed be surprising if enzyme hydrolyses did not obey this principle. We find the conformation of the tetrahedral intermediate crystallographically derived from the complex of trypsin with its inhibitors to be in concurrence. As most succinctly shown in the case of soybean inhibitor (with porcine trypsin (*10*)), the CH_2–O bond of Ser-195 lies in a plane bisecting the O–C–N angle of the intermediate (Fig. 2). The serine-O_γ lone pair is suitably oriented to operate in conjunction with the incipient carbonyl oxygen for expulsion of the amino residue. We note that the amino group itself has rotated out of position for potentially assisting reversion to amide; this is in concurrence with the torsional-activation hypothesis, and helps to explain the stability of the complex.

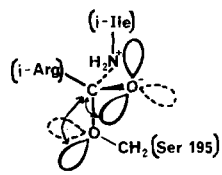


FIGURE 2

We suggest that this principle may be used in much the same way as we have previously demonstrated for the torsional-strain hypothesis. Proposed mechanisms based on crystallographic evidence must be consistent with the stereoelectronic requirements of the fundamental properties of the amide linkage, whether they be rehybridization propensities or secondary overlap considerations such as just described. Inconsistent mechanistic hypotheses must be discounted. In fact, these two properties are not mutually independent.[7] It will be observed that both theories specify rehybridization of the constituent atoms of the amide linkage. Torsional-strain analysis inquires of the least-motion reaction path based on the force field of the native amide, whereas the theory of Deslongchamps specifies the optimum geometry of the transition state (which necessarily resembles the tetrahedral intermediate). Both cases are connected by the

[7] We, at least, are acutely aware that these are intellectual constructs, which more or less accurately describe the chemical facts. If the interpretations put forth here are "better" than previous mechanistic ideas, it is merely that the perspective on the facts which we emphasize more coherently organizes the various phenomena to be explained.

central idea of a construable hybridization–overlap requirement. In a sense, they operate in tandem.

Finally, we would elaborate on the two mechanistic ideas we have presented for the purpose of rationalizing why torsional activation in the case of the serine proteases is not demonstrably more significant than it is. The fundamental realization is that accelerated *cis* addition by *syn* distortion of *trans* (*E*) substituents (Eq. (2)) opposes the antiperiplanar stereoelectronic facilitation previously described (Scheme 3, cf. Fig. 3).

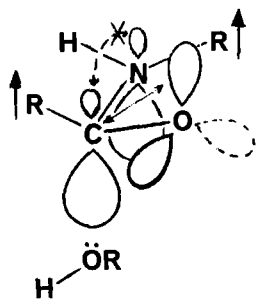


FIGURE 3

Rehybridization of the amide nitrogen in the direction forced by *syn* distortion *removes* one of the critical antiperiplanar lone pair interactions which, by microscopic reversibility, are crucial to facilitating quaternization of the carbonyl. Hence, only in the case of enzymes operating by *trans* addition (as in the case of carboxypeptidase A) can torsional strain effectively reinforce the required orbital interactions.[8] The mechanistic conclusion which follows from recognition of the *cis* addition sequence (Scheme 2) as well as the stereoelectronic principle in the serine proteases is that for unactivated substrates (i.e., unsubstituted amides) the nitrogen is first pyramidalized in one direction (lone pair *anti* to attacking Ser-O_γH), and then must invert prior to acceptance of a proton from the imidazole residue (His-57). In other words, the addition is clearly sequential rather than concerted, and the tetrahedral intermediate must indeed have a finite lifetime (*14*).[9]

APPENDIX

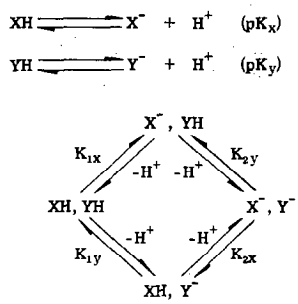
The following constitutes a demonstration of the kinetic equivalence of major- and minor-protonated forms for a simplified model of the enzymic process as alluded to earlier. This formulation follows that of Dixon and Webb (*28*).[10] It is assumed that there

[8] However, this statement does require a reservation. There is no evidence that a *syn* lone pair (such as produced by the distortions indicated by crystallographic evidence previously cited) cannot provide a similar, perhaps relatively diminished stabilization.

[9] Interpretation of the accelerated quaternization of the amide carbonyl in the case of soybean trypsin inhibitor (*15*, see discussion of *acylation*, earlier) is now feasible. Note that the relief of torsional strain upon covalent bonding to enzyme in this instance *overcomes* the negative stereoelectronic factor. The comparison with good substrates is delusive; the 100-fold differential in rates represents the net of a *larger* strain acceleration *minus* the facilitation attributable to a favorable antiperiplanar lone pair interaction (for which the absolute magnitudes may not be specified, of course).

[10] We particularly acknowledge Professor W. W. Cleland for drawing out attention to the concepts presented.

are two functional groups at the active site (XH and YH) which are ionizable (pK_x and pK_y), with maximum enzymic activity at intermediate pH. Scheme 4 summarizes



SCHEME 4

the postulated facts. When $pK_x < pK_y$, clearly X^-, YH will be the major species present at intermediate pH, with XH, Y^- available in minor amount. As Dixon and Webb show, the Michaelis pH functions (f, proportional to the reciprocals of the fractions of the amounts of the various ionization states) are as given in Eqs. (4)–(7).

$$f_{XH,\,YH} = 1 + \frac{K_{1x}}{(H^+)} + \frac{K_{1y}}{(H^+)} + \frac{K_{1x}K_{2y}}{(H^+)^2}, \tag{4}$$

$$f_{X^-,\,YH} = 1 + \frac{K_{1y}}{K_{1x}} + \frac{(H^+)}{K_{1x}} + \frac{K_{2y}}{(H^+)}, \tag{5}$$

$$f_{XH,\,Y^-} = 1 + \frac{K_{1x}}{K_{1y}} + \frac{(H^+)}{K_{1y}} + \frac{K_{1x}K_{2y}}{K_{1y}(H^+)}, \tag{6}$$

$$f_{X^-,\,Y^-} = 1 + \frac{(H^+)}{K_{2y}} + \frac{K_{1y}(H^+)}{K_{1x}K_{2y}} + \frac{(H^+)^2}{K_{1x}K_{2y}}. \tag{7}$$

It explicitly follows (by taking the ratio of Eqs. (5) and (6)) that the relative proportions of the intermediate ionization states (XH, Y^- and X^-, YH) are independent of pH and are proportional only to the difference in pK (Eq. (8)).

$$\frac{1/f_{X^-,\,YH}}{1/f_{XH,\,Y^-}} = \frac{K_{1x}}{K_{1y}}\left(= \frac{K_{2x}}{K_{2y}}\right). \tag{8}$$

Zeffren and Hall (*29*) generalize this scheme further. The simplifying assumption is made that the enzyme pK of XH is independent of the ionization state of YH and vice versa ($K_{1x} = K_{2x} = K_{ex}$, $K_{1y} = K_{2y} = K_{ey}$) (*30*), and an additional set of ionization constants is introduced, K_{esx} and K_{esy}, corresponding to the same transitions in the enzyme–substrate complex. It was then shown that the pH dependence of catalysis is described by the expressions given when X^-, YH is the catalytically active form of enzyme [Eqs. (9) and (10), wherein $k_{cat(opt)}$ and $K_{m(opt)}$ are the values of these parameters at the optimal pH; i.e., approximately midway between pK_x and pK_y].

$$k_{cat(X^-,\,YH)} = \frac{k_{cat(opt)}}{1 + (H^+)/K_{esx} + K_{esy}/(H^+) + K_{esy}/K_{esx}}, \tag{9}$$

$$K_{m(X^-,\,YH)} = K_{m(\mathrm{opt})}\left[\frac{1 + (H^+)/K_{ex} + K_{ey}/(H^+) + K_{ey}/K_{ex}}{1 + (H^+)/K_{esx} + K_{esy}/(H^+) + K_{esy}/K_{esx}}\right]. \tag{10}$$

An instructive comparison devolves from these terms. In this case (wherein the major-protonated form is the catalytically active species) K_{esy}/K_{esx} will be negligible and may be dropped (i.e., the trace amount of catalytically inactive form may be ignored). The expression for k_{cat} (Eq. (9)) then assumes its familiar form, yielding the typical trapezoidal pH profile upon logarithmic plot. However, in the instance that the minor form is the catalytic species, the derivation will yield an identical equation with K_{esy} and K_{esx} *reversed* (Eq. (11)). In the latter case,

$$k_{\mathrm{cat}(XH,\,Y^-)} = \frac{k_{\mathrm{cat(opt)}}}{1 + (H^+)/K_{esy} + K_{esx}/(H^+) + K_{esx}/K_{esy}}, \tag{11}$$

K_{esx}/K_{esy} will be significant and may well be dominant; i.e., $k_{\mathrm{cat(opt)}} \simeq [V_{\max}/(E)_0](K_{esx}/K_{esy})$. Here the plot of log reciprocal of rate function vs pH will have the identical shape as in the former case (hence being indistinguishable *vis a vis* pK_x and pK_y), only that it may be in theory vertically displaced by an amount corresponding to the ratio of acidity constants.

A similar analysis applies to K_m (Eq. (10)), with certain additional reservations. To the extent that ionization constants for free enzyme are unperturbed upon complexation ($K_e = K_{es}$), K_m should be unaffected in either case. However, it is intuitively likely that binding to the mechanistically "correctly protonated" form of enzyme should be stronger; in the instance in which the minor form is catalytically active and the major ("incorrectly protonated") form also binds but unproductively, a perturbation is introduced. Using the steady-state approximation for the minimal mechanism (Eq. (12)), in which ES′ represents nonproductive complexation with the unfavorably protonated form of enzyme (e.g., X^-, YH), the rate expression may be shown to be of the form given [Eq. (13), $K_m = (k_{-1} + k_{\mathrm{cat}})/k_1$] (*31*):

$$\mathrm{ES'} \overset{K_{s'}}{\rightleftharpoons} \mathrm{E} + \mathrm{S} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{ES} \xrightarrow{k_{\mathrm{cat}}} \mathrm{E} + \mathrm{P}, \tag{12}$$

$$v = \frac{k_{\mathrm{cat}}(\mathrm{E})_0(\mathrm{S})}{K_m + (K_m/K_{s'})(\mathrm{S}) + (\mathrm{S})}. \tag{13}$$

The latter expression (Eq. (13)) reduces to the usual Michaelis–Menten expression in the extreme that $K_{s'} \gg K_m$, corresponding to relatively weak affinity of the incorrectly protonated enzyme for substrate. However, in the instance that the incorrectly protonated form possesses comparable affinity to that of the catalytically active species, a substantial perturbation of the dependency of rate upon substrate may ensue, and application of the simpler rate expression will result in misleading rate parameters; i.e., "apparent $V_{\max}$" $= V_{\max}K_{s'}/(K_m + K_{s'})$, "apparent K_m" $= K_mK_{s'}/(K_m + K_{s'})$.

If any residual doubt exists as to the applicability of minor-protonation-state catalysis, one should consider abstractly a reversible enzymic reaction involving a bifunctional acid–base active site (of which there must be many examples). Should the major-

protonation state catalyze reaction in one direction, *necessarily* the minor state must catalyze the reaction in the opposite direction (microscopic reversibility).[11]

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[11] Serine proteases may be an example ($AspH + His \rightleftarrows Asp^- + HisH^+$), although the realization of minor-protonation-state catalysis renders moot those mechanistic arguments founded on favored protonation states (*5*, *6*). We also think that these considerations apply to the cysteine proteases (*32*). The phenomenon here characterized as minor-protonation-state catalysis encroaches on the concept of "negatively cooperative ligand binding" as described by Dixon and Tipton (*30*). The kinetic analysis abstracted in this appendix is vulnerable to criticism for its simplifying approximations. A more rigorous development may be patterned after the presentation of Ref. (*33*), pp. 142–162.