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Binding of Peptides to Elastase: Implications for the Mechanism of Substrate Hydrolysis†

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ABSTRACT: Equilibrium constants for the binding of several peptides to elastase have been measured and the free energies for enzyme-peptide complex formation calculated. The variation in binding energy with structure of the peptide points to several important aspects of the catalytic mechanism of the enzyme. The increased rate of hydrolysis noted for long peptides can now be shown to be due to a specific destabilization of the scissile amide bond in Michaelis complexes of the enzyme with long peptides. Stabilization of the transition state complex with long peptides exerts a smaller, but still appreciable, effect on the rate of substrate hydrolysis. Replacement of the substrate's planar scissile bond with tetrahedral groups results in at most a 2 kcal/mol increase in enzyme-peptide affinity. Strain in the enzyme-substrate complex is therefore unlikely to be the most important factor contributing to catalysis of hydrolysis. Large increases in enzyme-peptide binding, about 5 kcal/mol, are seen with peptides which form what would normally be considered a rather labile hemiacetal bond with the enzyme. The extra stability of these transition state analog complexes coincides quite well with that expected if covalent bond formation between the peptide and the enzyme involved no loss of entropy. Much of the catalytic power of elastase may therefore be due to nothing more complicated than its conversion of an entropically unfavorable bimolecular reaction to a unimolecular reaction by virtue of the formation of an enzyme-substrate complex.

Many enzymes which modify biological macromolecules are known to form significant enzyme-substrate contacts at points distant from the immediate site of chemical modification. Among the best characterized of these enzymes are those which catalyze the hydrolysis of polysaccharides and proteins. Extensive studies of enzyme-substrate interactions have been reported for lysozyme (Imoto *et al.,* 1972), pepsin (Fruton, 1971), carboxypeptidase A (Abramowitz *et al.,* 1967), papain (Schechter and Berger, 1968), subtilisin (Morihara *et al.* 1970; Kraut *et al.,* 1972), and chymotrypsin (Segal *et al.,* 1971). The forces involved in these interactions are rather easily studied through systematically varying the substrate. These systems have therefore been

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recommended as models in which the general problem of biological recognition might be studied (Berger *et al.*, 1969).

Recent studies of the interaction of one of the enzymes, porcine pancreatic elastase, with some model substrates have had as their goal the elucidation of the forces involved both in binding and hydrolysis of substrates. The efficiency of elastase-catalyzed hydrolysis of ester, amide, and peptide bonds has been shown to depend on the number of amino acid residues in the substrate (Atlas *et al.*, 1970; Gertler and Hoffman, 1970; Thompson and Blout, 1970). The increased efficiency of the enzyme observed with long substrates has been shown to be due, in large part, to an increased rate constant for the acylation reaction (Thompson and Blout, 1970, 1973a). For amide substrates this rate constant increases almost 100-fold on going from acetyl tri- to acetyl tetrapeptide amides. To account for this change, a specific interaction has been postulated between the P_5–P_4 peptide group and the S_5 and S_4 subsites of the enzyme's active center.[1]

It has been argued that the S_{54}–P_{54} enzyme–substrate contact is unlikely to directly influence the rate of the acylation reaction, since it is far removed from the scissile bond. The effect of this enzyme–substrate contact has therefore been proposed to be indirect, proceeding through an initial rearrangement of enzyme–substrate contacts in the S_1 subsite of the enzyme (Thompson and Blout, 1970, 1973a). This model predicts a rearrangement of the S_1–P_1 contact concomitant with the P_{54} peptide group binding to the S_{54} subsites. Since changes in the S_1–P_1 interaction energy might reasonably be expected to accompany a rearrangement of this enzyme–substrate contact, it was proposed to test the model by measuring the affinity for S_1 of various P_1 residues, both in the presence and absence of a P_{54} peptide group. The variation of enzyme–peptide affinity with the nature of the P_1 residue was thought also to have independent interest in that it might throw light on the mechanism of action of the enzyme.

To determine the energy of interaction of the P_1 residue and the S_1 subsite, it was necessary to use peptides which bind to the enzyme in a unique mode. I have, therefore, studied the binding energy of the peptides Ac-Pro-Ala-Pro-P_1 and Ac-Ala-Pro-P_1. The P_1 residue of these peptides is bound exclusively to the S_1 subsite by virtue of the proline residues inability to occupy the S_3 subsite (Thompson and Blout, 1973b).

I find that for certain P_1 residues the S_1–P_1 binding energies differ for the series Ac-Ala-Pro-P_1 and Ac-Pro-Ala-Pro-P_1, supporting the hypothesis that a rearrangement of the S_1–P_1 contact occurs upon binding of the P_{54} Ac-Pro group. This rearrangement destabilizes Michaelis complexes and analogs thereof, but stabilizes transition state analog complexes. The rate acceleration expected from these two effects can be calculated and is in good agreement with that observed experimentally, confirming the functional relationship between the rearrangement of the S_1–P_1 contact and the increased acylation rate constant.

[1] The nomenclature introduced by Schechter and Berger (1967) is used to facilitate discussion of the interactions between elastase and bound peptides. Amino acid residues and partial amino acid residues (*e.g.*, acetyl groups) of substrates are numbered P_1, P_2, P_3, etc., in the N-terminal direction, and P_1', P_2', etc., in the C-terminal direction from the scissile bond. The complementary subsites of the enzyme's active center are numbered S_1, S_2 and S_1', S_2', etc., in an analogous fashion. The binding mode of a peptide which occupies, for example, the S_4, S_3, S_2, and S_1 subsites of the enzyme will be denoted by the abbreviation S_{4321}.

Materials and Methods

Elastase catalyzed hydrolysis of substrates was followed in a pH-Stat as described previously (Thompson and Blout, 1973b). K_m was determined from Lineweaver–Burk plots. K_i for inhibitors of elastase was determined by inhibition of Ac-Pro-Ala-Pro-Ala-NH_2 hydrolysis and the use of Dixon plots. Inhibition was fully competitive within experimental error.

Thin-layer chromatography (tlc) of peptides, sources of synthetic intermediates and elastase, are all as described previously (Thompson and Blout, 1973b).

The preparations of Ac-Ala-Pro-OH, Ac-Ala-Pro-Ala-NH_2 (VII), Ac-Pro-Ala-Pro-OH, Ac-Pro-Ala-Pro-Ala-NH_2 (XVI), and Ac-Pro-Ala-Pro-Gly-NH_2 (XVIII) (Thompson and Blout, 1973a), Ac-Ala-Pro-alaninol (III), Ac-Ala-Pro-Aal[2] (IV), Ac-Pro-Ala-Pro-alaninol (XII), and Ac-Pro-Ala-Pro-Aal (XIII) (Thompson, 1973) have all been described previously.

Alanyl Methyl Ketone Hydrochloride. Carbobenzoxyalanyl chloromethyl ketone (450 mg, 1.75 mmol) (Thompson and Blout, 1973c) was dissolved in a mixture of *tert*-butyl alcohol (20 ml) and 1 M hydrochloric acid (2 ml) and hydrogenated for 5 hr at 20 psi with a 10% palladium–charcoal catalyst. The mixture was filtered through Celite and evaporated. The residue was crystallized from acetone to give 187 mg (87%). $[\alpha]^{25}D$ +96° (*c* 0.5, MeOH).

Acetylalanylprolinamide (*I*). Acetylalanylproline (150 mg, 0.66 mmol) (Thompson and Blout, 1973a) and *N*-methylmorpholine (0.972 ml, 0.66 mmol) were dissolved in acetonitrile (10 ml) and cooled to −20° in a Dry Ice–carbon tetrachloride bath. The mixture was stirred while isobutyl chloroformate (0.086 ml, 0.66 mmol) was added. After 10 min, ammonia gas was bubbled through the mixture for 5 min. The reaction was allowed to warm to room temperature over a period of 1 hr and stirred overnight. The solvent was removed *in vacuo;* the residue was dissolved in water and treated with Rexyn I-300 resin (Fisher). After filtration and evaporation of the water, the residue was crystallized from acetone, 115 mg (77%). mp 146–148°; $[\alpha]^{25}D$ −146° (*c* 3.0, 10^{-2} M aqueous $CaCl_2$). *Anal.* Calcd for $C_{10}H_{17}N_3O_3 \cdot 0.5H_2O$: C, 50.83; H, 7.68; N, 17.79. Found: C, 50.6; H, 7.6; N, 17.9.

Acetylprolylalanylprolinamide (*X*). Acetylprolylalanylproline (Thompson and Blout, 1973a) was reacted with ammonia under the conditions used to prepare Ac-Ala-Pro-NH_2. Acetylprolylalanylprolinamide was obtained in 60% yield as a hygroscopic solid. Single spot by tlc $R_{F\,II}$ 0.4. $R_{F\,VIII}$ 0.5. $[\alpha]^{25}D$ −203° (*c* 0.7, 10^{-2} M aqueous $CaCl_2$).

Acetylalanylprolinisopropylamide (*II*). Acetylalanylproline and isopropylamine were coupled by the procedure used to prepare Ac-Ala-Pro-NH_2, except that the amine was added as a liquid. After purification by treatment with resin, the product was stored under ether. Filtration of the insoluble gelatinous mass gave a 9% yield of material, single spot on tlc. $R_{F\,II}$ 0.8, $R_{F\,III}$ 0.7, $R_{F\,VIII}$ 0.7, $[\alpha]^{25}D$ −164° (*c* 0.3, 10^{-2} M aqueous $CaCl_2$). No attempt was made to recover more product from the ether. *Anal.* Calcd for $C_{13}H_{23}N_3O_3$: C, 57.97; H, 8.61; H, 15.60. Found: C, 58.3; H, 8.6; N, 16.0.

Acetylprolylalanylprolinisopropylamide (*XI*). Acetylprolylalanylproline and isopropylamine were coupled by the

[2] The abbreviation Aal is used to denote the aldehyde derived from alanine, *viz.*, 2 aminopropan-1-al.

procedure used to prepare Ac-Ala-Pro-NH_2, except that the isopropylamine was added as a liquid. Trituration under hexane gave a 72% yield of hygroscopic solid, single spot by tlc. $R_{F\,II}$ 0.5, $R_{F\,III}$ 0.6, $R_{F\,VIII}$ 0.7. $[\alpha]^{25}D$ −212° (*c* 0.42, 10^{-2} M aqueous $CaCl_2$). *Anal.* Calcd for $C_{18}H_{30}N_4O_4 \cdot 0.5\ H_2O$: C, 54.94; H, 8.45; N, 14.24. Found: C, 55.4; H, 8.4; N, 14.7.

Acetylalanylprolylalanyl Methyl Ketone (*V*). Acetylalanylproline (148 mg, 0.65 mmol) and *N*-methylmorpholine (0.072 ml, 0.65 mmol) were dissolved in acetonitrile (10 ml) and cooled to −20° in a Dry Ice–carbon tetrachloride bath. The mixture was stirred while isobutyl chloroformate (0.08 ml, 0.65 mmol) was added. After 10 min, a solution of alanyl methyl ketone hydrochloride (81 mg, 0.65 mmol) in 1 ml of *N,N*-dimethylformamide was added, followed immediately by *N*-methylmorpholine (0.072 ml, 0.65 mmol). The reaction mixture was allowed to warm to room temperature over a period of 1 hr and stirred overnight at room temperature. After evaporation of the solvent, the product was purified by silica gel column chromatography in chloroform–10% methanol. Fractions containing material $R_{F\,VIII}$ 0.7 were collected, evaporated, and the residue was crystallized from acetone–ether in 30% yield. $[\alpha]^{25}D$ −199° (*c* 0.4, 10^{-2} M aqueous $CaCl_2$). *Anal.* Calcd for $C_{14}H_{23}N_3O_4$: C, 56.55; H, 7.80; N, 14.13. Found: C, 56.9; H, 7.7; N, 14.1.

The optical integrity of the asymmetric center adjacent to the ketone carbonyl was demonstrated by observing the slow racemization of that center under conditions more favorable to racemization than any encountered in the preparation and purification of the peptide. In 0.02 M aqueous NaOH the product ($[\alpha]^{25}D$ − 199°) gave rise to a new, chromatographically identical material $[\alpha]^{25}D$ −173°, with a half-life of about 10 min.

Acetylprolylalanylprolylalanyl Methyl Ketone (*XIV*). Acetylprolylalanylproline and alanyl methyl ketone hydrochloride were coupled by the procedure used to prepare Ac-Ala-Pro-Ala methyl ketone. The product was obtained in 54% yield by silica gel chromatography in chloroform–10% methanol and crystallization from ether. Single spot on tlc. $R_{F\,VIII}$ 0.7. $[\alpha]^{25}D$ −253° (*c* 0.4, 10^{-2} aqueous $CaCl_2$). *Anal.* Calcd for $C_{19}H_{30}N_4O_5$: C, 57.85; H, 7.67; N, 14.20. Found: C, 58.0; H, 7.5; N, 14.1.

Slow racemization to a new material $[\alpha]D$ −225° was demonstrated under the same conditions described above for Ac-Ala-Pro-Ala methyl ketone.

Acetylalanylprolylalanine (*VI*). Acetylalanylproline (160 mg, 0.70 mmol) and alanine benzyl ester hydrochloride (151 mg, 0.70 mmol) were coupled by the procedure used to prepare Ac-Ala-Pro-NH_2, but purified as follows. The crude product was dissolved in chloroform and extracted twice with 0.1 M hydrochloric acid, twice with 5% aqueous sodium bicarbonate, and once with water. The chloroform solution was dried and evaporated to give a product which formed a gel under ether. Single spot by tlc. $R_{F\,VIII}$ 0.7.

Acetylalanylprolylalanine benzyl ester was dissolved in *tert*-butyl alcohol and hydrogenated for 12 hr at 20 psi with 10% palladium–charcoal. The solution was filtered through Celite and evaporated. The residue crystallized from ethyl acetate, 60 mg (35%). Single spot by tlc. $R_{F\,II}$ 0.5, $R_{F\,III}$ 0.1.

Acetylprolylalanylprolylalanine (*XV*). Acetylprolylalanylproline was coupled to alanine benzyl ester hydrochloride and the product hydrogenated by the procedure used to prepare Ac-Ala-Pro-Ala-OH. *Anal.* Calcd for $C_{18}H_{28}N_4O_6 \cdot H_2O$: C, 52.16; H, 7.3. Found: C, 52.2; N, 7.3.

Acetylalanylprolylglycinamide (*IX*). Acetylalanylproline and glycine amide hydrochloride were coupled by the procedure used to prepare Ac-Ala-Pro-NH_2. The product obtained in 75% yield by trituration under ethyl acetate showed a single spot on tlc. $R_{F\,II}$ 0.5, mp 151–153°. *Anal.* Calcd for $C_{12}H_{20}N_4O_4$: C, 50.69; H, 7.09; N, 19.71. Found: C, 50.6; H, 6.9; N, 19.3.

The Binding Mode of the Peptides Ac-Ala-Pro-X and Ac-Pro-Ala-Pro-X.

Assignment of the S_{321} binding mode to the Ala-Pro-X sequence in the peptides Ac-Ala-Pro-X and Ac-Pro-Ala-Pro-X is based primarily on our knowledge of the topography of the active center of elastase. The S_3 subsite of the active center is known to exclude proline, but not alanine, residues of bound peptides (Thompson and Blout, 1973b). Although no direct evidence is presently available, it would appear likely that the S_1 subsite will have similar properties, since this subsite binds amino acids with large side chains less well than alanine (Thompson and Blout, 1973a). Furthermore, enclosing the C_α and N atoms of residue P_1 in a five-membered ring would require a significant distortion of the conformation of bound P_2 and P_1 residues proposed by Shotton *et al.* (1972). In contrast to the S_3 and S_1 subsites, S_4 and S_2 will accept alanine and proline residues equally well (Thompson and Blout, 1973b). Little enzyme substrate interaction occurs beyond the S_5 subsite of the enzyme (Thompson and Blout, 1973a). It follows from these data that the binding mode in which the Ala-Pro-X sequence fills the S_{321} subsites is probably the only one which allows full contact between the peptides Ac-Ala-Pro-X and Ac-Pro-Ala-Pro-X and the enzyme's active center.

For particular compounds additional evidence supporting the above binding mode is as follows: (1) Ac-Ala-Ala-OH has been tentatively shown to bind to elastase in the S_{432} mode (Shotton *et al.*, 1972). The substitution of proline for alanine residues in the S_2 subsite, or an amide group for a C terminal carboxylate function, hardly affects the inhibition constant, K_i, of acetyl dipeptides (Thompson and Blout, 1973a, and unpublished data). Hence it is likely that the major binding mode of the Ac-Ala-Pro sequence in Ac-Ala-Pro-OH, and Ac-Ala-Pro-NH_2 in particular, and Ac-Ala-Pro-X in general, is S_{432}. (2) The high k_{cat} of amide cleavage from Ac-Pro-Ala-Pro-Ala-NH_2 (Thompson and Blout, 1970) supports primary binding of this peptide in the productive mode, by definition S_{54321}. The difference Fourier map of a complex of elastase and an analogous peptide, Ac-Pro-Ala-Pro-Ala-OH (Shotton *et al.*, 1972), is consistent with such a unique binding mode.

This combination of general and particular evidence makes it likely that the only strong binding mode of each of the peptides in Tables I and II is that in which the Ala-Pro-X sequence occupies the S_{321} subsites of the enzyme's active center. The group X will henceforth be referred to as P_1, since it will occupy the S_1 subsite.

Results

A. Interdependence of the S_{54}–P_{54} and S_1–P_1 Enzyme–Substrate Contacts. In Tables I and II, respectively, are listed the dissociation constants for complexes of elastase with Ac-Ala-Pro-P_1 and Ac-Pro-Ala-Pro-P_1. Most of these constants have been measured as inhibition constants (K_i)

TABLE I: Effect of Various P_1 Residues on the Dissociation Constant of Complexes of Elastase with Ac-Ala-Pro-P_1 Peptides.

Inhibitor		K_i(mM)[a]
P_4 P_3 P_2 P_1		
Ac-Ala-Pro-NH_2	(I)	64
Ac-Ala-Pro-NH-$CH(CH_3)_2$	(II)	1.25
Ac-Ala-Pro-NH-$CH(CH_3)CH_2OH$	(III)	7.0
Ac-Ala-Pro-NH-$CH(CH_3)CHO$	(IV)	0.062
Ac-Ala-Pro-NH-$CH(CH_3)COCH_3$	(V)	4.8
Ac-Ala-Pro-NH-$CH(CH_3)COOH$	(VI)	1.2
Ac-Ala-Pro-NH-$CH(CH_3)CONH_2$	(VII)	K_m = 4.2
Ac-Gly-Pro-NH-$CH(CH_3)CONH_2$	(VIII)	K_m = 33
Ac-Ala-Pro-NH-CH_2CONH_2	(IX)	45

[a] pH 9.0, 37°, 10^{-2} M aqueous $CaCl_2$.

TABLE II: Effect of Various P_1 Residues on the Dissociation Constant of Complexes of Elastase with Ac-Pro-Ala-Pro-P_1 Peptides.

Inhibitor		K_i(mM)[a]
P_5 P_4 P_3 P_2 P_1		
Ac-Pro-Ala-Pro-NH_2	(X)	3.2
Ac-Pro-Ala-Pro-$NHCH(CH_3)_2$	(XI)	0.074
Ac-Pro-Ala-Pro-$NHCH(CH_3)CH_2OH$	(XII)	0.6
Ac-Pro-Ala-Pro-$NHCH(CH_3)CHO$	(XIII)	0.0008
Ac-Pro-Ala-Pro-$NHCH(CH_3)COCH_3$	(XIV)	1.5
Ac-Pro-Ala-Pro-$NHCH(CH_3)COOH$	(XV)	4.3
Ac-Pro-Ala-Pro-$NHCH(CH_3)CONH_2$	(XVI)	K_m = 3.9
Ac-Pro-Gly-Pro-$NHCH(CH_3)CONH_2$	(XVII)	K_m = 43
Ac-Pro-Ala-Pro-$NHCH_2CONH_2$	(XVIII)	K_m = 22

[a] pH 9.0, 37°, 10^{-2} M aqueous $CaCl_2$.

but those of substrates are Michaelis constants (K_m).[3] As argued above, each peptide, by virtue of its sequence, will have a single strong binding mode, and each K_i and K_m value will therefore refer to a unique enzyme–peptide complex (Thompson and Blout, 1973b). The free energy of formation of each enzyme–peptide complex from the enzyme and peptide, ΔF_{EP}, may therefore be calculated from K_i or K_m using the equation.

$$\Delta F_{EP} = -RT \ln (1/K)$$

The values of ΔF_{EP} obtained allow us to calculate a quantity, ΔF_{P_1}, characteristic of the S_1–P_1 interaction.[4]

$$\Delta F_{P_1}(AP) = \Delta F_{EP}(Ac\text{-}Ala\text{-}Pro\text{-}P_1) - \Delta F_{EP}(Ac\text{-}Ala\text{-}Pro\text{-}NH_2)$$

$$\Delta F_{P_1}(PAP) = \Delta F_{EP}(Ac\text{-}Pro\text{-}Ala\text{-}Pro\text{-}P_1) - \Delta F_{EP}(Ac\text{-}Pro\text{-}Ala\text{-}Pro\text{-}NH_2)$$

If

$$\Delta F_{P_1}(AP) = \Delta F_{P_1}(PAP)$$

the most reasonable conclusion to be drawn is that the S_1–P_1 interaction is very similar in the two series of peptides, Ac-Ala-Pro-P_1 and Ac-Pro-Ala-Pro-P_1. Where

$$\Delta F_{P_1}(AP) \neq \Delta F_{P_1}(PAP)$$

however, we can infer that changes have occurred in the S_1–P_1 contact in response to the Ac-Pro group binding to the S_{54} subsites.

In Table III are listed the values of ΔF_{P_1} derived from the enzyme–peptide dissociation constants in Tables I and II. Examination of these values of $\Delta F_{P_1}(AP)$ and $\Delta F_{P_1}(PAP)$ shows that for P_1 isopropylamide and P_1 alaninol groups $\Delta F_{P_1}(AP)$ is very nearly equal to $\Delta F_{P_1}(PAP)$.

TABLE III: The Free Energy of Binding of P_1 to S_1, with Respect to P_1 = NH_2 in the Series Ac-Ala-Pro-P_1 and Ac-Pro-Ala-Pro-P_1.

P_1	$\Delta F_{P_1}(AP)$ (kcal/mol)	$\Delta F_{P_1}(PAP)$ (kcal/mol)	$\Delta F_{P_1}(PAP) - F_{P_1}(AP)$ (kcal/mol)
$NH\text{-}CH(CH_3)_2$	−2.4	−2.3	+0.1
$NH\text{-}CH(CH_3)CH_2OH$	−1.4	−1.0	+0.3
$NH\text{-}CH(CH_3)CHO$	−4.3	−5.1	−0.8
$NH\text{-}CH(CH_3)COCH_3$	−1.6	−0.5	+1.1
$NH\text{-}CH(CH_3)COO^-$	−2.5	+0.2	+2.6
$NH\text{-}CH(CH_3)CONH_2$	−1.7	+0.1	+1.8
$NH\text{-}CH_2CONH_2$	−0.2	+1.2	+1.4

For these P_1 groups, therefore, the S_1–P_1 interaction is unaffected by the presence of the Ac-Pro group in the S_{54} subsites.

In contrast to this result, the values of $\Delta F_{P_1}(AP)$ and $\Delta F_{P_1}(PAP)$ are not equal for P_1 alaninal, alanyl methyl ketone, alanine, alaninamide, or glycinamide groups. In these cases the binding energy characteristic of the S_1–P_1 contact, and therefore the nature of the S_1–P_1 contact itself, appears to depend on whether a P_4 Ac or P_{54} Ac-Pro group binds to the S_{54} subsites of the enzyme. In these peptides the S_1–P_1 contact clearly changes in response to the changed S_{54}–P_{54} enzyme–substrate contact.

The S_{54}–P_{54} and S_1–P_1 enzyme–substrate contacts can, therefore, be shown to be interdependent. Below we consider how the interaction between the contacts can weaken or strengthen enzyme–peptide binding.

B. Effect of the S_{54}–P_{54}—S_1–P_1 Interaction on the Enzyme–Peptide Binding Energy and the Rate of Substrate Hydrolysis. Interaction between the S_{54}–P_{54} and S_1–P_1 contacts could either stabilize or destabilize an enzyme–peptide complex. If there were no interaction, the binding energy of each peptide would presumably be the sum of the binding energies of its parts; *e.g.*, for Ac-Pro-Ala-Pro-Ala-NH_2 the expected binding energy in the absence of the S_{54}–P_{54}—S_1–P_1 interaction would be the binding energy of Ac-Ala-Pro-Ala-NH_2 (VII) (−3.4 kcal/mol) plus the free energy change characteristic of a P_4 Ac → P_{54}Ac-Pro substitution. This latter term may be calculated

[3] Since the acylation step is the rate-determining step of hydrolysis for amide substrates of elastase, $K_m = K_s$.

[4] In the event that all enzyme–peptide contacts outside the S_1 subsite are similar for the peptides $P_1 = P_1$ and $P_1 = NH_2$, ΔF_{P_1} corresponds simply to the S_1–P_1 binding energy. If, on the other hand, enzyme–peptide contacts outside the S_1 subsite change as a consequence of the change in P_1, these changes will also be reflected in ΔF_{P_1}. In this case, ΔF_{P_1} is only a minimum measure of the S_1–P_1 binding energy but will still be characteristic of a given S_1–P_1 interaction.

as −1.8 kcal/mol, comparing the binding energies of Ac-Pro-Ala-Pro-NH_2 (X) (−3.5 kcal/mol) and Ac-Ala-Pro-NH_2 (I) (−1.7 kcal/mol) which, for present purposes, we may define as having no P_1 residue and, therefore, no S_{54}-P_{54}—S_1-P_1 interaction. The expected binding energy of Ac-Pro-Ala-Pro-Ala-NH_2 is therefore (−3.4) + (−1.8) = −5.2 kcal/mol, 1.8 kcal/mol in excess of the experimentally observed value of ΔF_{EP} (−3.4 kcal/mol). The difference of 1.8 kcal/mol we ascribe to the factor not considered in the calculation, the interaction between the Ac-Pro group added to S_{54} and the Ala-NH_2 group in S_1. For P_1 Ala-NH_2 groups, therefore, the contact-contact interaction destabilizes by 1.8 kcal/mol the enzyme-peptide complex, which in this case is a Michaelis complex and the ground state for the acylation step of the catalytic process. For P_1 Gly-NH_2 groups (peptides IX and XVIII) a similar calculation shows that the enzyme-peptide complex is destabilized by 1.4 kcal/mol. The slight difference between the figures for P_1 Ala-NH_2 and Gly-NH_2 may be a result of the greater flexibility of the glycine peptides and the consequent ease with which they may avoid unfavorable enzyme-substrate contacts.

The same line of reasoning may be applied to determine the energy involved in the S_{54}-P_{54}—S_1-P_1 interaction for any of the P_1 groups in Tables I and II. In particular, it may be applied to the complexes of elastase with peptide aldehydes, which are good analogs of the acylation reaction transition state complex (Thompson, 1973). In the absence of the S_{54}-P_{54}—S_1-P_1 interaction, the binding energy of Ac-Pro-Ala-Pro-Aal (XIII) would be −7.8 kcal/mol; *i.e.*, 1.8 kcal/mol more favorable than that of Ac-Ala-Pro-Aal (IV) (−6.0 kcal/mol). The observed binding energy of −8.7 kcal/mol is 0.9 kcal/mol more favorable than that calculated. Thus, the S_{54}-P_{54}—S_1-P_1 interaction appears to stabilize the transition state analog complex by 0.9 kcal/mol and, presumably, will stabilize the true transition state complex at least as much.

The S_{54}-P_{54}—S_1-P_1 interaction, in destabilizing the ground state and stabilizing the transition state complex as described above, will reduce the free energy of activation for the acylation reaction by at least 1.8 + 0.9 = 2.7 kcal/mol. An independent estimate of the effect of the S_{54}-P_{54}—S_1-P_1 interaction on the free energy of activation can be obtained by observing the effect of the P_4 Ac → P_{54} Ac-Pro substitution on the rate of the acylation reaction. A 94-fold rate increase is observed on going from Ac-Ala-Pro-Ala-NH_2 (0.9 sec^{-1}) to Ac-Pro-Ala-Pro-Ala-NH_2 (8.5 sec^{-1}) (Thompson and Blout, 1973a), which will correspond, according to transition state theory, to a decrease in the free energy of activation of 2.8 kcal/mol. The close agreement between this latter estimate of the changed free energy of activation and that derived above using enzyme-peptide binding energies supports the hypothesis that the interaction between the S_{54}-P_{54} and S_1-P_1 contacts is directly responsible for the increased rate of reaction.

Discussion

Mechanism of the S_{54}-P_{54}—S_1-P_1 Interaction. The relationship, demonstrated above, between the S_{54}-P_{54} contacts rearrangement of the S_1-P_1 contact and the S_{54}-P_{54} induced increase in the acylation rate constant has the important corollary that studies of either phenomenon can be used to deduce their common mechanism. A previous study of the relationship between the S_{54}-P_{54} contact and the rate of substrate hydrolysis (Thompson and Blout, 1973c) has demonstrated that the single most important factor in increasing the rate of hydrolysis was the enzyme's interaction with the substrates P_4 α-amino group. Typically, this group was found to increase the acylation rate constant about tenfold. Since a close relationship has now been shown between the increased acylation rate constant and the rearrangement of the S_1-P_1 enzyme-substrate contact, this would suggest that the S_{54} subsites' interaction with the P_4 α-amino group is also the single most important factor in rearranging the S_1-P_1 contact.

Previous studies dealing with the rates of substrate hydrolysis by elastase have indicated that the S_1-P_1 contact changes in response to substrate chain length. The present study focuses attention on the rather limited area involved in these changes. The values of ΔF_{P_1}(AP) and ΔF_{P_1}(PAP), listed in Table III, indicate that the S_{54}-P_{54} contacts rearrangement of the S_1-P_1 contact is negligible for those P_1 groups lacking a carbonyl group (P_1 isopropylamide and alaninol) and is strong only for P_1 groups having this substrate-like feature (P_1 alaninal, alanyl methyl ketone, alanine, alaninamide, and glycinamide). This behavior is not consistent with a wholesale displacement of the peptide within the active center of the enzyme but is consistent with a specific reorganization of the carbonyl binding area of the S_1 subsite following binding of the Ac-Pro group to the S_{54} subsites. This result supports and amplifies the proposal made earlier (Thompson and Blout, 1970, 1973a) that a conformational change in the enzyme is involved in the S_{54}-P_{54} induced increase in the rate constant for the acylation reaction.

The simplest hypothesis consistent with the observed binding energies of peptides to elastase is that the S_{54}-P_{54}—S_1-P_1 interaction documented above is mediated by the enzyme's changing from one conformation, in which S_1 can accommodate planar or tetrahedral groups but S_{54} cannot properly bind P_{54}, to another in which the S_{54}-P_{54} contact is fully formed but S_1 can bind only tetrahedral P_1 groups. A peptide with both a P_{54} group and a planar P_1 group will find neither conformation an ideal fit and will, therefore, have its binding energy reduced by the S_{54}-P_{54}—S_1-P_1 interaction energy. The potential binding energy of such a peptide can only be fully realized by residue P_1 assuming a tetrahedral structure.

Mechanism of Catalysis. Variations within the two series of peptides studied here (Tables I and II) are confined for the most part to the P_1 residue. Each series may therefore be thought of as specifically probing the interactions between the catalytic residues of the enzyme and the scissile bond of the substrate. By comparing the free energy of binding of these peptides, it should therefore be possible to learn something of the nature of the forces involved in bond cleavage.

(a) DESTABILIZATION OF THE GROUND STATE COMPLEX. The noncovalent interactions between an enzyme and its substrate are likely to be weaker in the ground state than in the transition state complex even in enzymes manifesting covalent catalysis. The binding energy of a substrate may therefore be expected to be significantly lower than that of a substrate analog which mimics the noncovalent interactions of the transition state. Elastase, for example, binds the substrate Ac-Pro-Ala-Pro-Ala-NH_2 (XVI, ΔF_{EP} −3.4 kcal/mol) less tightly than the substrate analogs Ac-Pro-Ala-Pro isopropylamide (XI, ΔF_{EP} −5.9 kcal/mol) and Ac-Pro-Ala-Pro alaninol (XII, ΔF_{EP} −4.6 kcal/mol), both of which mimic the tetrahedral nature of the transition

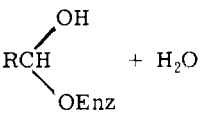
state. It is apparent from these results that about 2 kcal/mol is potentially available to reduce the free energy of activation for hydrolysis as a result of the ill-fit, or strain, existing in the ground state enzyme–substrate complex.[4]

By comparison very little strain exists in the enzyme's complex with the substrate Ac-Ala-Pro-Ala-NH_2, since the binding energies of the substrate (−3.4 kcal/mol) and the analogous isopropylamide (II, −4.1 kcal/mol) and alaninol (III, −3.1 kcal/mol) are very similar. Destabilization of the Michaelis complex therefore appears to be almost entirely a consequence of forming the S_{54}–P_{54} contact and results from the S_{54}–P_{54}···S_1–P_1 interaction discussed above. The induction of this strain probably represents the primary contribution of the S_{54}–P_{54}—S_1–P_1 interaction to facilitating substrate hydrolysis.

Although a reduction of 2 kcal/mol in the free energy of activation is not inconsiderable and is extremely important when discussing the S_{54}–P_{54}—S_1–P_1 interaction, it is equivalent to only a 40-fold increase in the rate of hydrolysis. It cannot, therefore, be the major factor determining the catalytic power of elastase.

(b) STABILIZATION OF THE TRANSISTION STATE COMPLEX. An indication of other factors which facilitate substrate hydrolysis may be found in the great stability of the enzyme's complexes with the peptide aldehydes XIII and IV. These aldehydes have been postulated to form hemiacetals with Ser-188 of the enzyme (Thompson, 1973). Since, by analogy with acetaldehyde, the peptide aldehydes will be at least 50% hydrated in aqueous solution, the overall reaction may be written.

$$RCH(OH)_2 + EnzOH \rightleftharpoons RCH(OH)_2 \cdot EnzOH \rightleftharpoons$$



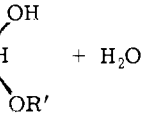
$$+ H_2O$$

The observed free energy for aldehyde binding in the case of Ac-Pro-Ala-Pro-Aal (XIII), ΔF_{EP}, is −8.7 kcal/mol. Since only 50% of the aldehyde is present in the hydrated form, the standard free energy change for the above reaction will therefore be (−8.7) + (−0.4) = −9.1 kcal/mol.

The free energy change for the first partial reaction, in which only noncovalent bonds are formed, may be estimated from the binding energies of the peptides Ac-Pro-Ala-Pro isopropylamide (XI, −5.9 kcal/mol) and Ac-Pro-Ala-Pro alaninol (XII, −4.6 kcal/mol) to be about −5 kcal/mol. The free energy change for the second partial reaction, involving covalent bond formation, is therefore between −3 and −4.5 kcal/mol.

For comparison, the hemiacetal of XIII with *N*-acetylserinamide, which is a rather acidic alcohol (pK_a = 13.6), would be expected from the data of Sander and Jencks (1968) to have a formation constant no greater than that of the aldehyde hydrate (standard state of water 1 M). Thus, the standard free energy for the reaction

$$RCH(OH)_2 + R'OH \rightleftharpoons$$



$$+ H_2O$$

where R'OH is *N*-acetylserinamide, will be at least +2 kcal/mol. The covalent bond between the enzyme and Ac-Pro-Ala-Pro-Aal would appear to be about 6 kcal/mol ((−9.1) - (−5) - (+2)) stabler than the analogous bond between the aldehyde and *N*-acetylserinamide.

We might consider why the bond between the peptide aldehyde and Ser-188 should be so much stronger than the same bond to a simple alcohol. The stability could be due to some special feature of the enzyme, *e.g.*, an arrangement of hydrogen bonding and charged groups calculated to interact optimally with the new tetrahedral arrangement of atoms and thereby lower the enthalpy of formation of the hemiacetal bond. In this case, however, it would be surprising that other tetrahedral groups, *e.g.*, P_1 alaninol residues, are not bound more tightly to the enzyme.

A more reasonable hypothesis is that the enzyme–aldehyde bond has a peculiarly favorable entropy of formation, since its formation is virtually an intramolecular reaction within the enzyme–peptide complex. Measurements of the entropy of formation of tetrahedral addition complexes of aldehydes have given values in the vicinity of −24 eu/mol (standard state of water 1 M, Schaleger and Long, 1963). Thus, if preliminary noncovalent complex formation entirely obviated the need for a further decrease in entropy during covalent bond formation, the bond formed would appear stabler by about 24 eu or 8 kcal/mol. This effect alone could account for the stability of the hemiacetal bond. The hypothesis that bond formation within the enzyme–aldehyde complex could occur with a negligible loss of entropy is entirely consistent with the picture of a relatively rigid enzyme–ligand complex that has emerged from X-ray diffraction studies of several enzymes.

What are the implications of the above argument for the catalytic mechanism of the enzyme? As pointed out previously (Thompson, 1973), there are distinct similarities between the hemiacetal form of the enzyme–aldehyde complex and the tetrahedral transition state complex. If the hemiacetal bond in the former is stable and has a negligible entropy of formation, it would seem reasonable that the enzyme–substrate bond in the latter complex is also abnormally stable and will be formed in an entropically favored reaction. The acylation reaction within the enzyme–substrate complex may therefore occur with an entropy of activation reduced by about 18 eu (6 kcal/mol) and, other things being equal, would proceed more than 10^4-fold faster than an analogous bimolecular reaction where a ΔS^* of approximately −24 eu would be anticipated. It would seem a reasonable hypothesis, therefore, that a large part of the catalytic power of the enzyme results from the enhanced stability of the enzyme–substrate bond, which, in turn, is due to the negligible loss of entropy accompanying bond formation.

The idea that the reduction in the entropy requirement for the rate-determining step of reaction is an important feature of enzyme catalysis has been developed recently by Page and Jencks (1971). These workers have argued (Jencks and Page, 1972) that the entropy of activation of bimolecular reactions is frequently underestimated by equating it with the approximately 9 eu requirement for formation of an "encounter complex." When the residual freedom of motion within the encounter complex is lost on covalent bond formation, the true entropy loss is significantly greater and may be as great as 30 eu. An enzyme which can utilize part of the free energy of substrate binding to reduce the entropy lost in the rate-determining step of a bimolecular reaction could, therefore, cause rate accelerations of 10^3–10^7-fold at the expense of enzyme–substrate binding energy.

The results presented here for elastase are entirely consistent with this view of enzymic catalysis, since the free energy of formation of an enzyme–substrate bond is such that

its entropy of formation may be interpreted to be nearly zero. It is tempting, therefore, to conclude that the basic idea behind the Page-Jencks model is applicable to elastase catalysis. A large part of the catalytic power of the enzyme may result from the rate-determining transacylation reactions occurring within a preexisting enzyme-substrate complex. The unusually favorable entropy of activation for these reactions could result in a greater than 10^4-fold rate acceleration over analogous bimolecular reactions.

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Reversible Lipid Titrations of the Activity of Pure Adenosine Triphosphatase-Lipid Complexes†

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ABSTRACT: Using pure complexes of synthetic phospholipids with ATPase derived from sarcoplasmic reticulum, a lipid titration technique has been developed which enables a rapid preliminary screening of the effect of added lipids on ATPase activity. The data from the titration experiments are compared with the properties of some pure ATPase-lecithin complexes in which the activity of the ATPase is highly sensitive to the structure of the lipid chains. The activity of ATPase complexes with saturated lecithins is completely inhibited below temperatures which probably correspond to a phase transition of the lipids. Preliminary experiments indicate that the lipid titration technique can be readily adapted to a wide range of membranes.

We have prepared complexes of pure ATPase from sarcoplasmic reticulum with three synthetic lecithins to examine the effect of the thermal phase transition on the activity of the enzyme. Complexes of the ATPase with dioleoyllecithin (DOL, 18:1; 18:1), dipalmitoyllecithin (DPL, 16:0; 16:0), and dimyristoyllecithin (DML, 14:0; 14:0) provide evidence for a critical effect of the chain conformation on the activity of the ATPase.[1] The use of such complexes, containing essentially pure lipid and almost free of detergent, is obligatory for definitive studies of the effect of the lipid phase transition on ATPase activity, especially where the transition temperature of the lipid may be influenced by the presence of the protein.

However, the preparation of these complexes by the lipid substitution technique which we have described elsewhere is lengthy, involving successive lipid equilibrations in cholate, alternating with long centrifugation steps (Warren *et al.*,

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[1] Abbreviations used are: DOL, dioleoyllecithin; DPL, dipalmitoyllecithin; DML, dimyristoyllecithin; DSL, distearoyllecithin; DOPE, dioleoylphosphatidylethanolamine; SR, sarcoplasmic reticulum; glc, gas-liquid chromatography.