Elastase-Catalyzed Amide Hydrolysis of Tri- and Tetrapeptide Amides†

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ABSTRACT: The kinetic constants for elastase-catalyzed amide hydrolysis of a series of tri- and tetrapeptide amides have been determined. These constants vary markedly with the nature of the group (P_4) three amino acid residues separated from, and N terminal to, the scissile bond. The interactions between P_4 and the enzyme are of two types: (a) those which strengthen enzyme–substrate binding, and (b) those which increase the rate of substrate hydrolysis but decrease enzyme–substrate binding. It is shown that the stronger binding arises from substrate occupancy of the region of the enzyme complementary to a P_4 amino acid's side chain. The rate enhancement, however, results primarily from substrate occupancy of the region complementary to a P_4 amino acid's amino group.

Recent studies of the substrate specificity of elastase (EC 3.4.4.7), a pancreatic serine proteinase, have indicated that the efficiency with which the enzyme hydrolyses peptide, amide, and ester bonds depends markedly on the chain length of the substrate (Atlas *et al.*, 1970; Thompson and Blout, 1970). The fourth amino acid residue of the substrate N terminal to the scissile bond, P_4,[1] was found to be particularly important in determining the rate of digestion of the substrate. More recently it has been shown that the major effect of a residue in this position is to accelerate the rate of the acylation reaction in the enzyme–substrate complex (Thompson and Blout, 1973b).

We report here that small changes in the P_4 residue of a series of tri- and tetrapeptide amides can cause large changes in the kinetic constants for amide hydrolysis. The kinetic data lead to a model in which specific regions of the S_4 subsite interact with the substrate to either decrease K_m or increase both k_{cat} and K_m for substrate hydrolysis.

The substrates utilized here are derivatives of the tripeptide amide H-Ala-Pro-Ala-NH_2. The choice of a C-terminal alanyl residue (P_1) was dictated by the fact that analogous peptides with amino acids other than alanine in this position, *e.g.*, glycine, leucine, and valine, interact less well with the S_1 subsite and have higher K_m's for the hydrolysis reaction (Thompson and Blout, 1973b). The specificity of the S_3 subsite has not been fully explored, but, as alanine was found to interact more favorably with S_3 than glycine (Thompson and Blout, 1973b), it was chosen as the P_3 residue of our model substrates.

Proline was chosen as the P_2 amino acid since the active center of elastase appears to bind this residue only at certain subsites; the choice of this amino acid therefore reduces the possibilities of nonproductive binding of substrates to the active center of the enzyme (Thompson and Blout, 1973a). Also, since the problems of nonproductive binding will increase with an increasing number of amino acid residues in the substrate, we chose to study the hydrolysis of amide, rather than peptide, bonds.

Since in this communication we probe the role of the S_4 subsite of the active center, it is convenient to define certain parts of this subsite. We assume the disposition of substrate groups around the P_4 α-carbon atom to be tetrahedral. Each group is then postulated to interact with a specific region of the S_4 subsite. The regions are defined as follows. The side chain of residue P_4 occupies the R region, the α-hydrogen atom the H region, and the nitrogen atom the A region of the S_4 subsite (Figure 1) when P_4 is an L-amino acid residue and residue P_5 interacts with subsite S_5. In this case the C^α–C′ bond angle (ψ) is defined as ψ_4. Since interaction with the S_5 subsite may be required to define the angle $\psi = \psi_4$, we assume a possible threefold degeneracy of binding in this subsite ($\psi = \psi_4$, $\psi_4 + 120°$, and $\psi_4 + 240°$) for substrates with no P_5 residue, until the situation can be proven otherwise.

It is, of course, not possible to state *a priori* than the properties of any region or subsite are independent of the substrate bound. The nomenclature proposed above is employed solely to facilitate discussion; it is not meant to imply that the active center of the enzyme is rigid.

Materials and Methods

Elastase-catalyzed hydrolysis of peptide amides was followed in a pH-Stat as described previously (Thompson and Blout, 1973a). No ninhydrin-positive products were observed on thin-layer chromatography (tlc) of the reaction products. This is consistent with exclusive hydrolysis of the amide bond.

Tlc of peptides was carried out as described previously (Thompson and Blout, 1973a).

Porcine pancreatic elastase (>99.8% pure) was purchased from Whatman Biochemicals, England.

The preparations of HCl·H-Ala-Ala·Pro-Ala-NH_2 (VI) HCl·H-D-Ala-Ala-Pro-Ala-NH_2 (VII), Ac-Ala-Ala-Pro-Ala

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[1] In this, and subsequent papers, 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_3, S_2, S_1, and S_1' subsites of the enzyme will be denoted by the abbreviation $S_{1\bar{2}\bar{3}1}$.

TABLE I: Effect of P_4 Acyl Groups on the Kinetics of Elastase-Catalyzed Hydrolysis of the Amide Group of P_4-Ala-Pro-Ala-NH_2.

P_4	k_{cat}/K_m (M^{-1} sec^{-1})	k_{cat} (sec^{-1})	K_m (mM)	[S][a] (mM)
CH_3CO (I)	21	0.09	4.2	0.5–5
CH_3CH_2CO (II)	240	0.15	0.65	0.2–5
$(CH_3)_2CH$ CO (III)	325	2.7	8.3	1–9
$(CH_3)_3C$ CO (IV)	170	3.7	22	0.1–10

[a] Range of substrate concentrations.

NH_2 (IX), Ac-D-Ala-Ala-Pro-Ala-NH_2 (X) (Thompson and Blout, 1973a), Ac-Ala-Pro-Ala-NH_2 (I), HCl·H-Gly-Ala-Pro-Ala-NH_2 (V), and Ac-Gly-Ala-Pro-Ala-NH_2 (VIII) (Thompson and Blout, 1973b) have been described previously.

Propionylalanylprolylalaninamide (II). A suspension of 100 mg (0.32 mmol) of alanylprolylalaninamide hydrochloride (Thompson and Blout, 1973b) in 10 ml of *N,N*-dimethylformamide and 0.08 ml (0.72 mg, 0.72 mmol) of *N*-methylmorpholine was stirred at 0°, while 0.04 ml (42 mg, 0.46 mmol) propionyl chloride was added. The mixture was allowed to warm to room temperature, evaporated, and dissolved in water. Excess Rexyn I-300 mixed-bed ion-exchange resin (Fisher) was added and the suspension was stirred 15 min. After filtration, the water was evaporated, leaving a hygroscopic white foam: 46 mg (43%), homogeneous on tlc $R_{F_{VIII}}$ 0.4, mp 90–93°. *Anal.* Calcd for $C_{14}H_{24}N_4O_4 \cdot 0.5H_2O$: C, 52.31; H, 7.84; N, 17.43. Found: C, 52.5; H, 7.7; N, 17.6.

Isobutyrylalanylprolylalaninamide (III) was prepared similarly to propionyl-Ala-Pro-Ala-NH_2 using isobutyryl chloride: yield, 64 mg (58%); homogeneous on tlc $R_{F_{VIII}}$ 0.4; mp 188–190°, $[\alpha]_D^{25}$ −191° (*c* 0.3, 10^{-2} M aqueous $CaCl_2$). *Anal.* Calcd for $C_{15}H_{26}N_4O_4$: C, 55.19; H, 8.03; N, 17.17. Found: C, 54.9; H, 7.9; N, 17.2.

Pivaloylalanylprolylalaninamide (IV) was prepared similarly to propionyl-Ala-Pro-Ala-NH_2 using pivaloyl chloride: yield, 50 mg (43%); homogeneous on tlc $R_{F_{VIII}}$ 0.5; $[\alpha]_D^{25}$ −168° (*c* 0.7, 10^{-2} M aqueous $CaCl_2$). *Anal.* Calcd for $C_{16}H_{28}N_4O_4$: C, 56.45; H, 8.29; N, 16.46. Found: C, 56.4; H, 8.2; N, 16.4.

Acetyl-α-aminoisobutyrylalanylprolylalaninamide (XI). Acetyl-α-aminoisobutyric acid (prepared according to Bell, 1958) (39 mg; 0.27 mmol) and alanylprolylalaninamide hydrochloride (80 mg; 0.27 mmol) were dissolved in *N,N*-dimethylformamide (10 ml). *N*-Methylmorpholine (0.03 ml; 0.27 mmol) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (58 mg; 0.27 mmol) were added and the mixture was stirred overnight at room temperature. After evaporation of the solvent, the residue was dissolved in water, treated with Rexyn I-300 resin (Fisher), and filtered. The residue, after evaporation of water, was crystallized from acetone–ether to give 29 mg (26%) of product: mp 148–152°, homogeneous on tlc $R_{F_{II}}$ 0.3. *Anal.* Calcd for $C_{17}H_{29}N_5O_5$: C, 53.25; H, 7.62; N, 18.27. Found: C, 53.1; H, 7.1; N, 18.2.

Results

The kinetic constants for cleavage of P_4 acyl peptides are shown in Table I. They illustrate the effects of methyl substitution of the P_4 α carbon in this series of peptides. The first

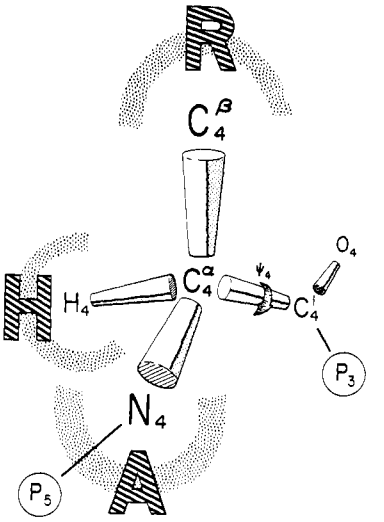

FIGURE 1: Diagrammatic representation of the S_4–P_4 enzyme–substrate contacts of a P_4 L-amino acid residue.

methyl group attached to the P_4 α-carbon atom results in an increased k_{cat}/K_m for hydrolysis. The increase results primarily from a decrease in the value of K_m since k_{cat} is not greatly affected by the presence of the α-methyl group (*cf.* propionyl- (II) and acetyl-Ala-Pro-Ala-NH (I)). The second α-methyl substitution, by way of contrast, hardly changes k_{cat}/K_m, but leads to large, compensating increases in k_{cat} and K_m (*cf.* isobutyryl- (III) and propionyl-Ala-Pro-Ala-NH_2 (II)). Further smaller increases in k_{cat} and K_m result from the third α-methyl substitution and lead to a small decrease in k_{cat}/K_m.

The effects of methyl substitution of the P_4 α carbon in the P_4 aminoacyl and P_5P_4 acetylaminoacyl series can be seen, from Table II, to depend on the configuration of the new asymmetric carbon atom. The introduction of an α-methyl group to give peptides with P_4 L residues results in changes similar to those observed for the first α-methyl for α-hydrogen substitution in the P_4 acyl series, *viz.*, k_{cat}/K_m values increased mainly through decreases in K_m (*cf.* P_4 alanyl (VI), and P_4 glycyl (V), P_5P_4 acetylalanyl (IX), and P_5P_4 acetylglycyl-Ala-Pro-Ala-NH_2 (VIII)). The introduction of an α-methyl group

TABLE II: Effect of P_4 Aminoacyl Residues on the Kinetics of Elastase-Catalyzed Hydrolysis of the Amide Group of P_4-Ala-Pro-Ala-NH_2.

P_4	k_{cat}/K_m (M^{-1} sec^{-1})	k_{cat} (sec^{-1})	K_m (mM)	[S][a] (mM)
Gly (V)	18	1.0	50	1–18
Ala (VI)	74	1.4	19	3–30
D-Ala (VII)	20	1.2	70	1–9
Ac-Gly (VIII)	118	1.75	15	1–7
Ac-Ala (IX)	2900	6.1	2.1	0.5–9
Ac-D-Ala (X)			K_i = 350	10–100
Ac-α-aminoisobutyryl (XI)	1750	9.0	5.1	1–7

[a] Range of substrate concentrations.

to give peptides with P_4 D residues leads to either unchanged or less favorable kinetic constants for hydrolysis.

Table II also shows the effect of P_4 α-amino groups on the kinetics of hydrolysis. The substitution of an amino group for a P_4 α-hydrogen atom of acetyl-Ala-Pro-Ala-NH_2 (I) to give glycyl-Ala-Pro-Ala-NH_2 (V) results in large increases in k_{cat} and K_m and only a small change in the ratio k_{cat}/K_m. Similar changes in these parameters are seen on comparing L-alanyl-Ala-Pro-Ala-NH_2 (VI) with the propionyl compound II. These changes result in all P_4 aminoacyl peptides tested having much higher k_{cat} and K_m values than the small P_4 acyl peptides I and II in Table I.

N-Acetylation of P_4 L-aminoacyl residues generally gives substrates with decreased K_m's and increased k_{cat}'s (*cf.* IX and VI, VIII, and V). In sharp contrast to these findings N-acetylation of the P_4 D-alanyl peptide leads to very unfavorable changes in both K_m and k_{cat} (*cf.* VII and X). Thus the acetylalanyl compound is hydrolyzed much more efficiently than the acetyl-D-alanyl analog, although the P_4 alanyl peptide is only a marginally better substrate than its P_4 D isomer (VI, VII). It therefore appears that the enzyme's ability to distinguish L and D residues binding at S_4 increases as the residue more closely mimics part of a long polypeptide chain.

Taking the results in Tables I and II together, it can be seen that P_4 α-carbon substituents fall into two main classes: (1) those that result in a decreased value of K_m, *i.e.*, the first α-methyl group and (2) those that result in increased values of both k_{cat} and K_m, *i.e.*, the first α-amino or α-acetamido (L) group and the second α-methyl group.

Interpretation of Results

By virtue of their -Ala-Pro-Ala- sequence, the peptides P_4-Ala-Pro-Ala-NH_2 will be bound strongly only to the S_{4321} subsites of elastase. For these peptides the observed K_m and k_{cat} may be equated, respectively, with K_s, the dissociation constant of the enzyme–substrate complex, and k_2, the rate constant for the acylation reaction (Thompson and Blout, 1973a). The results described above, therefore, imply that there are two types of enzyme–substrate contact occurring in the S_4 subsite, the first resulting in increased enzyme–substrate binding, the second resulting in a coordinate decrease in enzyme–substrate binding and increase in the rate of hydrolysis. Before these enzyme–substrate contacts can be identified, however, it is necessary to consider the possibility of a degenerate binding mode for the P_4 residue of the substrate. In the following, therefore, we consider the constraints which remove the possible degeneracy of the S_4–P_4 contact. The variation of K_m with the nature of P_4 is then used to determine how each S_4–P_4 contact affects the enzyme–substrate binding energy, ΔF_{ES}. The variation of k_{cat} with P_4 is similarly used to determine which S_4–P_4 contacts lead to increases in the rate of the acylation reaction.

Binding Mode of Residue P_4 of the Substrate. As shown previously, the topography of the active center of elastase ensures that residue P_4 of peptides with the formula P_4-Ala-Pro-Ala-NH_2 will be bound only to the S_4 subsite of the enzyme (Thompson and Blout, 1973a). However, in the absence of a significant contact between the S_5 subsite and P_5 residue, residue P_4 might be free to rotate about its C^α–C′ bond, introducing a threefold degeneracy into the S_4–P_4 contact (Figure 1, $\psi = \psi_4$, $\psi_4 + 120°$, $\psi_4 + 240°$).

In the work reported in this paper, degeneracy of the S_4–P_4 interaction is prevented by the strong binding of a P_4 α-methyl group to one region of the S_4 subsite. The existence of a favorable binding site for the α-methyl group follows from the K_m's of P_4 propionyl and alanyl peptides, which are low compared to those of the analogous P_4 acetyl and glycyl peptides, respectively. Since additional α-methyl substituents of the P_4 α-carbon atom after the first lead to weaker enzyme–substrate binding (*cf.* the K_m's of propionyl- (II), isobutyryl- (III), and pivaloyl-Ala-Pro-Ala-NH_2 (IV), Table I), there is only one favorable binding region for P_4 α-methyl groups among the A, R, and H regions of subsite S_4.

The ability of a P_4 α-methyl group to orient the P_4 residue can be illustrated by considering the energy required to remove the α-methyl group of propionyl-Ala-Pro-Ala-NH_2 from the most favorable to the second most favorable position in the S_4 subsite. The binding energy of a P_4 α-methyl group to its preferred position may be calculated from the K_m's of pairs of substrates with and without a single P_4 α-methyl substituent, *i.e.*, compounds IX and VIII, VI and V, II and I. The calculations give consistent values (−1.3, −0.8, and −1.1 kcal per mol, respectively). The P_4 α-methyl group will therefore bind to the single favorable binding region of S_4 with about −1 kcal/mol. We may estimate the energy of binding to the next available binding region for P_4 α-methyl groups by a comparison of the K_m's of propionyl- (II) and isobutyryl-Ala-Pro-Ala-NH_2 (III).[2] This energy is unfavorable to the extent of 1.6 kcal/mol. Thus, the best binding mode of propionyl-Ala-Pro-Ala-NH_2 is about $-(-1.0) + 1.6 = 2.6$ kcal/mol more favorable than any other.

With substrates which have more than one substituent attached to the P_4 α-carbon atom, other interactions may add to, or detract from, the orienting ability of the P_4 α-methyl group. Binding modes in which the P_4 α-methyl group does not occupy its preferred position could become significant if favorable interactions existed between the other P_4 α substituents and S_4. Examination of the K_m's of propionyl- (II), alanyl- (VI), and isobutyryl-Ala-Pro-Ala-NH_2 (III), however, shows that there are no favorable interactions between P_4 α substituents and S_4, other than the α-methyl interaction discussed above. Indeed, these data suggest that additional α substituents cannot be placed in the S_4 subsite without incurring an additional unfavorable energy of binding of at least 0.6 kcal/mol.[3] Thus, where there is more than one substituent on the P_4 α-carbon atom, and at least one of these substituents is an α-methyl group, the minimum energy difference between any binding mode in which this α-methyl group is not bound to the favored region of S_4 and the CH_3 (R), H (H), H (A) binding mode of propionyl-Ala-Pro-Ala-NH_2 can be calculated as follows. It is the sum of (a) the energy required to remove the P_4 α-methyl from its preferred region (1.0 kcal/mol), (b) the energy required to place the P_4 α-methyl group in the next available binding region (1.6 kcal/mol), and (c) the energy required to place the other P_4 α substituent in its binding region (>0.6 kcal/mol). In the following, therefore, we assume a unique S_4–P_4 interaction and the binding of a P_4 α-methyl group to the favored region unless the binding energy of the substrate is more than 3.2 kcal/mol ($= -(-1.0) + 1.6 + 0.6$) unfavorable with respect to that of propionyl-Ala-Pro-Ala-NH_2.

Effect of the S_4–P_4 Interaction on Enzyme–Substrate Binding. The preferred binding of P_4 α-methyl groups to one region of

[2] Since the binding energy to the favored region appears to be independent of the state of occupation of other regions of S_4, the same situation may be presumed to hold *vice versa*.

[3] This figure takes into account the cooperativity of binding to the A and H regions of S_4 discussed later in this paper.

TABLE III: A Summary of the Properties of Substrates Considered to Have Defined Binding Modes to the S_4 Subsite.

	Regions Occupied	ΔF_{ES} (kcal/mol)	k_{cat} (sec^{-1})
CH_3CO (I)		3.3 ± 0.1	0.09
CH_3CH_2CO (II)	R	4.5 ± 0.1	0.15
$CH_3CH(CH_3)CO$ (III)	R + A	2.9 ± 0.1	2.7
L-$CH_3CH(NH_2)CO$ (VI)	R + A	2.6 ± 0.1	1.4
L-$CH_3CH(CH_3CONH)CO$ (IX)	R + A	3.8 ± 0.1	6.1
D-$CH_3CH(NH_2)CO$ (VII)	R + H	1.6 ± 0.1	1.2
$(CH_3)_3CCO$ (IV)	R + A + H	2.3 ± 0.1	3.7
$(CH_3)_2CH(CH_3CONH)CO$ (XI)	R + A + H	3.2 ± 0.1	9.0

S_4 determines the relative binding mode of the P_4 groups of a series of substrates. We now show that the favorable P_4 α-methyl binding region is S_4R and thereby define the absolute binding modes of the other substrates listed in Table III. Assignment of the binding modes allows calculation of the free energy of interaction of α-amino, α-acetamido, and α-methyl groups with the A and H regions of S_4.

1. R REGION. P_4 α-Methyl Groups Bind Favorably to the S_4R Region.

Reference to Table II shows that acetylated tetrapeptide substrates VIII and IX bind more tightly and react more rapidly with the enzyme than the nonacetylated analogs, V and VI. From these data we infer the existence of an S_5 subsite, which the P_5 acetyl group may be presumed to occupy. A S_5–P_5 interaction fixes the angle ψ at ψ_4 and orients the P_4 α-methyl group of Ac-Ala-Ala-Pro-Ala-NH_2 (IX) toward the R region of S_4 (Figure 1). Since the binding energy of this α-methyl group is typical of the P_4 α-methyl group in other peptides (*vide supra*), we conclude that in each of the substrates II, VI, and IX, the strong α-methyl binding site of S_4 is the R region. P_4 α-methyl groups, therefore, bind to S_4R and contribute an approximate 1 kcal/mol to the free energy of enzyme–substrate binding.

2. A AND H REGIONS. a. α-Amino Groups: P_4 α-Amino Groups Bind Highly Unfavorably to both the A and H Regions of S_4.

Binding of the P_4 α-methyl group of H-Ala-Ala-Pro-Ala-NH_2 (VI) to the R region forces the α-amino group to occupy the A region of S_4. Similarly the α-amino group of H-D-Ala-Ala-Pro-Ala-NH_2 (VII) will be forced to occupy the H region of S_4. The binding energy of α-amino groups in the A and H regions may therefore be calculated from a comparison of the K_m's of the P_4 alanyl (VI) and P_4 D-alanyl (VII) substrates, with the P_4 propionyl compound (II). In both cases (where the remaining region (H or A) is to be unoccupied; see section 2d) the binding is highly unfavorable. An α-amino group requires 1.9 kcal/mol to occupy the A region, and 2.9 kcal/mol the H region, of the S_4 subsite.

b. α-Acetamido Groups: P_5P_4 α-Acetamido Groups Bind Unfavorably to the S_4A–S_5 Regions, and Highly Unfavorably to the S_4H Region.

In an analogous fashion to the above, the orienting influence of the α-methyl group can be used to determine the interactions of the A and H regions with α-acetamido groups. Compared to their rather similar properties with respect to α-amino groups, the A and H regions show very different characteristics on interacting with the α-acetamido groups of acetylalanyl- (IX) and acetyl-D-alanyl-Ala-Pro-Ala-NH_2 (X). The unfavorable binding energies of these compounds with respect to propionyl-Ala-Pro-Ala-NH_2 (II) are 0.7 and 3.8 kcal per mol, respectively.

The highly unfavorable interactions which are present in the D isomer must arise from an incompatibility between the α-acetamido group and the H region. The interactions are so strong (>3.2 kcal/mol) that we must question whether the true binding mode is CH_3 (R), CH_3CONH (H), H (A). It is possible that the P_4 α-methyl group may be forced out of the R region in an attempt to relieve the unfavorable P_4 acetamido–H region contact by rotation about the C^α–C′ bond (ψ). Whatever the actual binding mode of this substrate, it appears clear that a minimum estimate of the energy that must be supplied to place an α-acetamido group in the S_4H region is 3.8 kcal/mol. P_4 α-acetamido groups will therefore be directed strongly away from the S_4H region and toward the S_4A region and S_5 subsite of the enzyme's active center.

c. α-Methyl Groups: P_4 α-Methyl Groups Bind Highly Unfavorably to both the S_4A and S_4H Regions.

The preferred orientation of α-acetamido groups into the S_4A region and S_5 subsite allows us to determine the binding mode of isobutyryl-Ala-Pro-Ala-NH_2 and to calculate the energies of interaction of α-methyl groups with the A and H regions of S_4.

Considering the possible binding modes of acetyl-α-amino-isobutyryl-Ala-Pro-Ala-NH_2 (XI), if the binding of an α-methyl group to the R region were the sole orienting factor in this substrate, there would be two possible binding modes, *viz.*, CH_3 (R), CH_3CONH (A), CH_3 (H), and CH_3 (R), CH_3 (A), CH_3CONH (H) (interconvertible by rotation around the C^α–C′ bond). However, since the α-acetamido group is strongly directed away from H site (section 2b), the only binding mode available to this substrate is CH_3 (R), CH_3CONH (A), CH_3 (H). The unfavorable effect of binding a P_4 α-methyl group to the H site, when the A site is occupied, may therefore be calculated to be 0.6 kcal/mol from a comparison of the K_m of this compound (XI) with that of acetyl-Ala-Ala-Pro-Ala-NH_2 (IX) (CH_3 (R), CH_3CONH (A), H (H)).

The same unfavorable binding energy, 0.6 kcal/mol, may be calculated for the third α-methyl group of pivaloyl-Ala-Pro-Ala-NH_2 (IV) by comparison of its K_m with that of the isobutyryl analog (III). It is likely, therefore, that this α-methyl group can also be considered to occupy the H region, and that the binding mode of isobutyryl-Ala-Pro-Ala-NH_2 (III) will be CH_3 (R), CH_3 (A), H (H), rather than CH_3 (R), H (A), CH_3 (H). The energy of binding of an α-methyl group to the A region, when the H region is unoccupied, may then be calculated by comparison of the K_m of isobutyryl-Ala-Pro-Ala-NH_2 (III) with that of the propionyl analog (II), (CH_3 (R), H

(A), H (H)). This interaction may be seen to be unfavorable to binding by 1.6 kcal/mol.

d. Cooperativity of Binding to the A and H Regions. It is clear from the K_m's of propionyl-, isobutyryl-, and pivaloyl-Ala-Pro-Ala-NH_2 that binding of the second P_4 α-methyl group to S_4 is more difficult than binding the third. This raises the question as to why the second P_4 α-methyl group binds to a region of S_4 with the loss of 1.6 kcal/mol when another region, which apparently requires only 0.6 kcal/mol for occupation, is open. This paradox would be resolved if the figure of 0.6 kcal/mol, calculated for binding of the third group to the H region, was valid only when the A region was already occupied. The data are consistent with prior occupation of the A region facilitating subsequent occupation of H. Accordingly, we have concluded that binding of P_4 α-methyl groups to the A and H regions of S_4 might be a cooperative process, with the A region the primary binding site.

Effect of S_4–P_4 Interactions on Reaction Rates. The enzyme–substrate complexes formed by the peptides studied here differ in their S_4–P_4 contact; the S_4–P_4 interactions occurring in each complex are listed in Table III. By correlating the type of S_4–P_4 contact with the observed rates of hydrolysis, one can determine which features of the P_4 residue stimulate the enzyme's ability to cleave the P_1–P_1' amide bond.

1. R REGION: ONLY MINOR RATE ENHANCEMENTS RESULTS FROM P_4 α-METHYL GROUPS OCCUPYING THE S_4R REGION.

The rate accelerations resulting from the binding of an α-methyl group to the R region can be calculated using pairs of substrates with and without single P_4 α-methyl substituents. In IX and VIII, VI and V, II and I, we observe rate increases of 3.5-, 1.4-, and 1.7-fold, respectively. Except perhaps for the first case, these accelerations, due to occupation of the R region, are minor when compared to the rate acceleration due to occupation of the A and H regions (*vide infra*).

2. A AND H REGIONS: LARGE RATE ENHANCEMENTS RESULT FROM P_4 α-METHYL, α-AMINO, OR α-ACETAMIDO GROUPS OCCUPYING THE S_4A REGION AND FROM P_4 α-AMINO GROUPS OCCUPYING THE S_4H REGION.

As can be seen from Table III, the distinguishing feature of the class of rapidly reacting substrates is the occupation, in the enzyme–substrate complex, of either or both of the A and H regions of S_4. Rate enhancements of 10- to 20-fold, relative to propionyl-Ala-Pro-Ala-NH_2 (II), are observed on occupation of the A region by α-amino (VI) or α-methyl groups (III). α-Acetamido groups, which occupy both the S_4A region and S_5 subsite, produce even larger, 15- to 40-fold rate accelerations. These rate enhancements do not appear to depend on the simultaneous occupation of either the R or H regions since the P_4 acetylglycyl compound (VIII) also reacts rapidly.

The high k_{cat} of D-alanyl-Ala-Pro-Ala-NH_2 (VII) shows that occupation of the H region by an α-amino group can also lead to an approximate 10-fold enhancement of k_{cat}.

The data presented here do not allow the determination of the effect on reaction rates of P_4 α-methyl groups occupying the S_4H region. A comparison of the k_{cat} of acetylalanyl (IX) with that of acetyl-α-aminoisobutyryl-Ala-Pro-Ala-NH_2 (XI) and of the k_{cat} of isobutyryl (III) with that of pivaloyl-Ala-Pro-Ala-NH_2 (IV) would seem to indicate that a P_4 α-methyl group occupying the S_4H region results in only small changes in the rate of hydrolysis. However, it has been noted that the A and H regions of S_4 appear to be cooperatively linked, in the sense that the occupation of A facilitates later occupation of H. This facilitation of binding may result from an alteration of the structure of the H region, or of the substrate, following the occupation of S_4A. In XI and IV both the A and H regions of S_4 are occupied. It follows therefore that the H region seen by these substrates cannot be considered the same as the H region existing in the native enzyme, or in enzyme–substrate complexes where the A region is unoccupied, *e.g.*, D-alanyl-Ala-Pro-Ala-NH_2 (VII). Thus our data do not bear directly on the capacity of the P_4 α-methyl group–S_4H contact to induce high rates of substrate hydrolysis. The results do suggest, however, that rate enhancements resulting from substrate occupancy of S_4A and S_4H might be mutually exclusive. Clearly the potential effects of the S_4A and S_4H regions on rates, like their effects on K_m, cannot be considered separately since these regions are interdependent.

Discussion

The substrates listed in Tables I and II fall into two classes according to the rate of the acylation reaction. The slowly reacting substrates ($k_{cat} \sim 0.1$ sec^{-1}) form strong complexes with the enzyme ($K_m \sim$ mM). In contrast, substrates which react more rapidly with the enzyme form relatively weak enzyme–substrate complexes ($K_m \sim 20$ mM), except in the event they can bind to the S_5 subsite of the enzyme (IX and XI).

The correlation between a poor rate of reaction and good enzyme–substrate bindig could arise from the existence of a strong, nonproductive binding mode. As has been shown earlier (Thompson and Blout, 1973a), this is unlikely to be the case since the binding modes of these substrates are restricted to the productive one by the Ala-Pro-Ala sequence.

Both the rapid reaction rate and the reduced enzyme–substrate binding energy appear to result from occupation by the substrate of the A or H regions of the S_4 subsite. In *all* the rapidly reacting complexes one of these regions is occupied, whereas in *none* of the slowly reacting complexes is this the case. The nature of the substrate group occupying the A or H region does not markedly affect the kinetic constants for hydrolysis. Hydrophobic (III) and hydrophilic, hydrogen-bonding groups (VI and VII), both increase the k_{cat} and K_m for reaction. It is probable, therefore, that the space filling properties of the group in question are responsible for the changed kinetic parameters.

It has been argued previously that the effect of the S_{54}–P_{54} contact on substrate reaction rates is mediated by the enzyme, since the rate increase appears to be independent of the amino acid residues separating the P_{54} residues from the scissile bond (Thompson and Blout, 1973b). Consistent with the present results is the hypothesis that the initial event leading to changes in reaction rate is a conformational change induced by the steric incompatibility between the P_4 group of the substrate and the A or H regions of the S_4 subsite. A mutual repositioning of the P_4 residue and S_4 subsite upon binding to the A region would also explain the facilitation of subsequent binding to the H region and the fact that such binding induces little further increase in the rate of the acylation reaction. It therefore appears that the binding energy of the favorable enzyme–substrate contacts in S_5, S_4, S_3, S_2, and S_1 may be used to build a point of enzyme–substrate strain in the S_4 subsite, which can only be relieved by rearranging the local conformation of the enzyme. There appears to be little resistance to this conformational change if the S_1 subsite of the enzyme is unoccupied, or is occupied by a group with a tetrahedral configuration around the C′ carbon atom of residue P_1 (R. C. Thompson, to be published). The resistance observed when the planar P_1 amide group of a substrate occupies the S_1 subsite is therefore relaxed as this group approaches the tetrahedral

transition state for the acylation reaction. In this way the conformational change induced by the S_{54}–P_{54} contact could play an important role in reducing the free energy of activation for the acylation reaction.

Summary

By studying the kinetics of hydrolysis of a variety of substrates which form only productive enzyme–substrate complexes, it has been possible to separate the roles of the various S_4–P_4 interactions. The region of S_4 (R) normally occupied by the α-methylene group of an amino acid side chain has been shown to bind α-methyl groups with about 1 kcal/mol. S_4R therefore contributes to substrate binding; it does not appear to significantly influence rates of reaction. In contrast, the region of S_4 (A) which would normally be occupied by the NH group of a P_4 amino acid residue binds α-amino, α-acetamido, and α-methyl groups reluctantly, but this occupation greatly increases the rate of bond cleavage. Higher rates of substrate hydrolysis are therefore accompanied by poorer enzyme–substrate binding. This finding is consistent with the postulated role of a conformational change in the catalytic process.

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Micellar Aggregation of Δ^5-3-Keto Steroids Lacking a Polar C-17 Group and Its Relation to the Activity and Specificity of the $\Delta^5 \rightarrow \Delta^4$-3-Ketosteroid Isomerase of *Pseudomonas testosteroni*†

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ABSTRACT: A systematic study has been carried out on the activity and apparent specificity of the $\Delta^5 \rightarrow \Delta^4$-3-ketosteroid isomerase of *Pseudomonas testosteroni* with respect to potential substrates possessing hydrophobic C-17β substituents. The series of Δ^5-3-ketones with the C-17 groups H, CH_3, CH_2CH_3, $CH(CH_3)_2$, and C_8H_{17}, of graded hydrocarbon character have been evaluated and the results obtained have confirmed that micellar aggregation of hydrophobic potential substrates in aqueous solutions is the main reason for the inability of the enzyme to catalyze their isomerization under the standard assay conditions. Under conditions which ensure significant disaggregation of the micelles, each compound studied became a good substrate of the isomerase. The specificity effects attributable to the C-17β groups were minor although the presence of the large C_8H_{17} group did result in significantly lower isomerization rates. An acid-catalyzed isomerization kinetic method was used to monitor the formation and disaggregation of the steriod micelles. This technique was found to be much more sensitive than the more traditional methods such as, for example, that based on light scattering. The effectiveness of various organic solvents, and of Tween-80 and Brij-35, in solubilizing the steroids was surveyed. Of the methods evaluated, the use of methanol as cosolvent provided the best compromise for achieving a maximum of steroid solvation with a minimum of enzyme inactivation. The data obtained indicate that a C-17–ring D region binding locus of hydrophobic character is present at the active site and that binding of C-17 hydrophobic Δ^5-3-ketones in the ES complex helps to maintain the conformation of the active-site region against the inactivating effects of up to 30% methanol. Furthermore, it has been found that only a small proportion of the hydrophobic substrates needs to be solubilized for complete and rapid enzymic isomerization to take place since the solvated molecules and those present in the substrate micelles undergo facile equilibration.

The Δ^5-3-ketosteroid isomerase of *Pseudomonas testosteroni* (EC 5.3.3.1) is one of the most active enzymes known and many aspects of its capacity to isomerize a broad spectrum of steroid-5-en-3-ones to the corresponding Δ^4-3-ketones have been studied (Talalay, 1965; Sih and Whitlock, 1968; Malhotra and Ringold, 1965; Falcoz-Kelly *et al.*, 1968; Jones

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