step of reaction is dependent on remote enzyme–substrate contacts (Thompson and Blout, 1970, 1973b). The data presented here would be consistent with these contacts similarly affecting the rate constant for irreversible inhibition (k_2).

The development of chloromethyl ketone inhibitors of elastase points up both the enzyme's similarities and differences to the other serine proteinases. Like the other enzymes, the target for these alkylating agents appears to be the catalytically important residue His-45. Unlike the related enzymes, elastase appears to be alkylated only by *peptide* chloromethyl ketones. The peptide groups of elastase substrates and inhibitors, therefore, appear to be necessary for enzyme recognition of such compounds.

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Use of Peptide Aldehydes to Generate Transition-State Analogs of Elastase†

Robert C. Thompson‡

ABSTRACT: Two peptide aldehydes analogous to amide substrates of elastase have been synthesized. The aldehydes bind to the enzyme up to 5000-fold more tightly than the substrates. The elastase–aldehyde complexes appear to be good analogs of the transition-state complex for elastase-catalyzed amide hydrolysis.

A great deal of information about the specificity and catalytic mechanism of enzymes has been obtained by the study of complexes formed between the enzyme and substrates, or substrate analogs. In most cases the complexes have been analogous to the low-energy Michaelis complexes formed by initial reversible absorption of substrate to the enzyme. In many ways a more interesting species is the transition-state complex, that complex of highest energy on the pathway from the Michaelis complex to the reaction products. Differences between the solution and enzyme-catalyzed reactions are likely to be most pronounced in the structure of the substrate in their respective transition states. The nature of that difference should therefore be extremely relevant to defining the catalytic power of the enzyme.

The lifetime of the transition-state complex will be of the order of the vibrational period of a covalent bond (about 10^{-13} sec), precluding its direct study. However, certain analogs of the substrate, which form stable complexes with the enzyme, might mimic the true transition-state complex sufficiently closely to reveal something of the structure of the transient species. Transition-state theory predicts the enzyme–substrate binding in the true transition-state complex will be considerably stronger than that in the Michaelis complex from which it is formed (for a review, see Lienhard *et al.*, 1972, and Wolfenden, 1972). It is therefore likely that any analog which mimics relevant features of the transition-state

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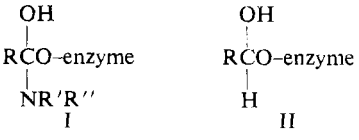
complex will also form a very strong complex with the enzyme.

The hydrolysis and alcoholysis of amides in aqueous solution is thought to proceed through a tetrahedral species which is either the transition state for reaction or a highly unstable intermediate. A similar species, I, has been proposed to be intermediate in the hydrolysis reactions catalyzed by several proteolytic enzymes, *e.g.*, the serine and cysteine proteinases (see Jencks, 1969). Breakdown of such a tetrahedral intermediate has been proposed to be the rate-determining step for α-chymotrypsin-catalyzed hydrolysis of anilides (Caplow, 1969). Alkylboronic acids, which readily form tetrahedral addition compounds, have recently been proposed as transition-state analogs for α-chymotrypsin (Koehler and Lienhard, 1971).

The tetrahedral intermediate I will be highly unstable by virtue of the good leaving groups on the "carbonyl" carbon atom. It will collapse easily to give either the acyl-enzyme or the substrate, both of which will be stabilized by delocalization of the π electrons of the new carbonyl group.



The hemiacetal II will be relatively stable since breakdown of this complex will give an aldehyde. Aldehydes are unique among carbonyl compounds in preferring to exist as tetrahedral addition complexes and are frequently unstable with respect to their hydrates and hemiacetals in aqueous or alcoholic solution.

There are clear analogies between the tetrahedral species, I, and hemiacetals of general structure II. Hemiacetals might therefore be expected to be good transition-state analogs for those hydrolases which react through tetrahedral species of general structure I. These hemiacetals should be easily generated from the corresponding aldehyde and the enzyme. I have, therefore, prepared two peptide aldehydes, analogous to peptide amide substrates of the proteolytic enzyme, pancreatic elastase, and have tested these for their ability to generate analogs of the transition-state complex for hydrolysis.

Independently of this work, Westerik and Wolfenden (1972) have shown that aldehyde analogs of certain substrates of papain have an abnormally high affinity for the enzyme. These investigators have interpreted this result in terms of the aldehyde generating an analog of a tetrahedral intermediate or intermediates in the hydrolysis reaction. Their results on a cysteine proteinase, and ours on a serine proteinase, confirm the basic similarity of mechanism of these two classes of enzymes.

Materials and Methods

Elastase-catalyzed hydrolysis of peptide amides and esters was followed in a pH-Stat as described previously (Thompson and Blout, 1970). Inhibition of the enzyme by the peptide aldehydes and alcohols was observed to be purely competitive.

Thin-layer chromatography (tlc) of peptides was carried out with silica gel plates (Q1) purchased from Quantum Industries, N. J. Plates were developed in 1-butanol–acetic acid–water (4:1:1, system II) and chloroform–methanol (9:1, system VIII). The plates were visualized by exposure to iodine vapor for several hours and sprayed with an aqueous solution of potassium iodide and tolidine (Reagent 32; Waldi, 1965).

Porcine pancreatic elastase was purchased from Whatman Biochemicals, England, and was assayed by the *p*-nitrophenyl-*tert*-butyloxycarbonyl alaninate assay of Visser and Blout (1972).

The preparation of acetylalanylprolylalaninamide (I) and acetylprolylalanylprolylalaninamide (IV) will be described elsewhere (Thompson and Blout, 1973b). L-Alaninol was purchased from the Fox Chemical Co., Los Angeles; it had an $[\alpha]_D$ of $+22.0°$ (*c* 2.0, ethanol) (lit. (Karrer *et al.*, 1948) $[\alpha]_D$ $+20.1°$).

Acetylprolylalanylprolylalaninol. Acetylprolylalanylproline (600 mg; 1.85 mmol) (Thompson and Blout, 1973b) and *N*-methylmorpholine (0.204 ml; 1.85 mmol) were dissolved in acetonitrile (20 ml). The mixture was stirred in a Dry-Ice–carbon tetrachloride bath and 0.24 ml of isobutyl chloroformate (1.85 mmol) was added. After 5 min, 270 mg of L-alaninol (3.7 mmol) was added and the mixture was allowed to warm to room temperature with stirring over a period of 1 hr. After a further 4 hr, the solvent was removed *in vacuo*.

The residue was dissolved in water and Rexyn I-300 resin (Fisher) was added and stirred 10 min. The resin was filtered and the aqueous solution was evaporated to give 580 mg (82%) of acetylprolylalanylprolylalaninol, pure by tlc $R_{F_{II}}$ 0.6, $[\alpha]_D^{25}$ $-220°$ (*c* 0.4, 10^{-2} M aqueous $CaCl_2$). *Anal.* Calcd for $C_{18}H_{30}N_4O_5$: C, 56.53; H, 7.91. Found: C, 56.3; H, 8.0.

Acetylalanylprolylalaninol was prepared from acetylalanylproline (Thompson and Blout, 1973b) and L-alaninol by a similar procedure to that used to prepare acetylprolylalanylprolylalaninol. The product was obtained in 87% yield from ethyl acetate: mp 173–175°, $[\alpha]_D^{25}$ $-171°$ (*c* 0.3, 10^{-2} M aqueous $CaCl_2$), single spot by tlc, $R_{F_{II}}$ 0.6, $R_{F_{VIII}}$ 0.5. *Anal.* Calcd for $C_{13}H_{23}N_3O_4$: C, 54.72; H, 8.13; N, 14.73. Found: C, 54.9; H, 8.0; N, 14.8.

Acetylprolylalanylprolylalaninal. Acetylprolylalanylprolylalaninol (550 mg; 1.38 mmol) was dissolved in ethanol-free chloroform (6 ml) and dimethyl sulfoxide (0.6 ml, 8.5 mmol, six-times excess). Dicyclohexylcarbodiimide (850 mg; 4.1 mmol, three-times excess) was added, followed by 7 × 50-μl aliquots of dichloroacetic acid over a period of 2 hr.

The chloroform and part of the dimethyl sulfoxide were removed at 30° and 0.1 Torr over a period of 6 hr. The residue was dissolved in chloroform and left at $-20°$ for 2 hr. The dicyclohexylurea was filtered off, and the filtrate was extracted twice with water. The combined aqueous phases were evaporated *in vacuo*, and the residue was triturated under ether to give 264 mg of a solid.

Then 150 mg dissolved in chloroform was absorbed onto a 20 × 2 cm column of silica gel and eluted with 200 ml of chloroform–methanol (90:10). Fractions containing the $R_{F_{VIII}}$ 0.6 spot were concentrated *in vacuo*, dissolved in chloroform (0.5 ml), and filtered. The filtrate was evaporated *in vacuo* to give 25 mg (5%) of a slightly hygroscopic white powder: $[\alpha]_D^{25}$ $-224°$ (*c* 0.06, 10^{-2} M aqueous $CaCl_2$), single spot by tlc $R_{F_{VIII}}$ 0.6. The nuclear magnetic resonance (nmr) spectrum of this material in $CDCl_3$ was consistent with the presence of an aldehyde group, having a single proton 9.75 ppm (s) from Me_4Si. Addition of 2% methanol completely removed this peak from the spectrum.

Acetylalanylprolylalaninal was prepared by a procedure similar to that used to prepare acetylprolylalanylprolylalaninal. The nmr spectrum of this peptide in $CDCl_3$ was consistent with the presence of one aldehyde group in the molecule, having a single proton 9.80 ppm (s) from Me_4Si, which was completely removed by the addition of 2% methanol.

TABLE I: Kinetic Constants for Elastase-Catalyzed Hydrolysis of Peptide Amides and Elastase Binding of Peptide Alcohols and Aldehydes.

Peptide	K_i (mM)	K_m (mM)	k_{cat} (sec^{-1})	k_{cat}/K_m (M^{-1} sec^{-1})
Ac-Ala-Pro-Ala-NH_2 (III)		4.2	0.09	21
Ac-Ala-Pro-alaninol (IV)	7.0			
Ac-Ala-Pro-alaninal (V)[a]	0.062			
Ac-Pro-Ala-Pro-Ala-NH_2 (VI)		3.9	8.5	2200
Ac-Pro-Ala-Pro-alaninol (VII)	0.6			
Ac-Pro-Ala-Pro-alaninal (VIII)[a]	0.0008			
Ac-Pro-Ala-Pro-alaninal (VIII)[b]	0.002			

[a] pH 7.00. [b] pH 4.00.

Results

The synthesis of peptides containing aldehyde groups represents a novel feature of synthetic peptide chemistry and is described briefly below. The tendency of this functional group to react with amino groups dictated that the aldehyde should either be synthesized after the rest of the peptide had been constructed, or should be protected in the form of an acetal during coupling reactions. The former approach was adopted since it promised to involve a shorter sequence of reactions.

The immediate precursors of the aldehydes, the peptide alcohols, were synthesized by a standard mixed-anhydride coupling procedure (Anderson *et al.*, 1967) from an optically active amino alcohol L-alaninol (Karrer *et al.*, 1948).

Oxidation of the alcohols was carried out by the dicyclohexylcarbodiimide–dimethyl sulfoxide reagent introduced by Pfiztner and Moffatt (1965). For this reaction, an excess of the dichloroacetic acid catalyst was added to the reaction in small aliquots over a period of several hours. This minimized losses of the carbodiimide by a side reaction leading to *N*-dichloroacetyldicyclohexylurea. This side reaction to an *N*-acylurea was found to be more troublesome if a pyridinium trifluoroacetate catalyst was used.

A crude reaction product obtained in good yield appeared to be largely the peptide aldehyde on the basis of its behavior on tlc. However, it required final purification by column chromatography, and a very poor yield was obtained after this step. No attempt has been made to elucidate the reason for the great loss of material at this stage.

The inhibition constants (K_i) of the peptide aldehydes were calculated from Dixon plots (Dixon, 1953), using as substrate acetyltrialanine methyl ester (Gertler and Hoffman, 1970). This allowed the use of very low concentrations of enzyme and the maintainence of a high inhibitor:enzyme ratio. The ester, which has a lower K_m than amide substrates, also competes more effectively with the aldehyde and allows the construction of an accurate Dixon plot. Use of the ester substrate allowed the determination of K_i's at low pH's, but necessitated working at pH 7.00, rather than at the standard pH 9.00, to reduce the background rate of base-catalyzed hydrolysis. This is not a serious objection to its use, since the K_i's of the aldehydes are not greatly affected by pH over the range pH 4.00–7.00, where k_{cat}/K_m for elastase-catalyzed ester hydrolysis is most clearly pH dependent (Hartley and Shotton, 1971) and where a variation of K_i might therefore be most reasonably expected. We, therefore, feel justified in comparing the K_i's of the aldehydes at pH 7.00 with the K_m's and K_i's of other peptides obtained at pH 9.00.

The K_i's of the peptide aldehydes and alcohols, and the kinetic parameters for hydrolysis of the corresponding amide substrates, are listed in the table. Immediately apparent are the extremely low values of K_i measured for the aldehyde. The enzyme–aldehyde complexes are the strongest yet observed between elastase and any peptide substrates or inhibitors. This is qualitatively consistent with the hypothesis that the aldehyde complexes are transition-state analogs. Below we consider the limitations placed on the structure of the enzyme–aldehyde complex by the known chemistry of aldehydes, and quantitative aspects of the analogy between these complexes and the true transition-state complexes.

Discussion

To define the species which accounts for the exceptionally strong binding of the aldehydes by elastase, three major possibilities are considered: noncovalent complexes of the enzyme with the aldehyde, or aldehyde hydrate, and the covalent complex, II. These three complexes will probably equilibrate rapidly with respect to the time scale of our experiments (2–5 min) since the formation of aldehyde hydrates and hemiacetals is subject to general-base catalysis. His-45 of the enzyme (homologous to His-57 of α-chymotrypsin) would be expected to facilitate formation of II by removal of a proton from Ser-188 (homologous to Ser-195 of α-chymotrypsin) in line with its role as a general-base catalyst of the hydrolysis reaction.

Formation of a stable noncovalent complex between the enzyme and the aldehyde is unlikely. The carbonyl group of the aldehyde will be approximately isosteric with that of analogous esters and amides and so must bind in close juxtaposition to the strongly nucleophilic hydroxyl group of Ser-188. Since the aldehydes considered here appear to form tetrahedral adducts with even weak nucleophiles,[1] the conjunction of the aldehyde carbonyl group with Ser-188 must be considered an unstable situation.

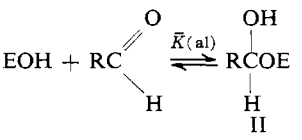
Noncovalent binding of the aldehyde hydrate to elastase would avoid the objections to placing a reactive carbonyl group in the active site. However, if this is the structure of the complex, it is difficult to see why aldehyde hydrates, which are *gem*-diols, should bind so much better than the analogous alcohols IV and VII. Hydrogen bonds between the enzyme and the second hydroxyl group are unlikely to favor the binding of a diol over the corresponding alcohol to the extent observed here. Since noncovalent binding of the aldehyde hydrate cannot satisfactorily account for the strong binding of aldehydes V and VIII to elastase, it is concluded that the hemiacetal II best represents the structure of the aldehyde–enzyme complex.

The dissociation constant of a transition-state analog is frequently used to estimate the degree to which this species resembles the true transition state (Wolfenden, 1972). A good transition-state analog, for example, might be expected to have a dissociation constant several orders of magnitude lower than that of a comparable Michaelis complex. By this criterion, the peptide aldehyde–enzyme complexes are good transition-state analogs, since they have dissociation constants up

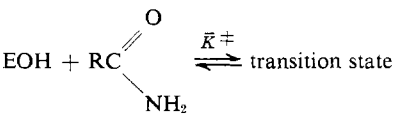
[1] The aldehyde proton cannot be observed in a deuteriochloroform–2% methanol solution, presumably due to the formation of a methyl hemiacetal.

to several thousandfold lower than Michaelis complexes.[2] However, the validity of such a comparison could be questioned since there is a covalent bond in the transition-state analog not present in the Michaelis complex. For this reason we prefer to justify the hypothesis that the enzyme–aldehyde complexes are good transition-state analogs by a somewhat different argument.

A good substrate will presumably react more rapidly than a poor one because of the greater stability of its transition-state complex relative to the ground state. This stability should be reflected in good analogs of the transition-state complex. Hence, those structural features which in substrates result in rapid reaction should, in transition-state analogs, result in enhanced stability and a lowered dissociation constant. Thus, if the equilibrium of peptide aldehydes V and VIII is a good analog of the pseudoequilibrium of peptide



amides III and VI, respectively, we might expect that the as-



sociation constant for the aldehydes, $\bar{K}$ (al), and the pseudo-equilibrium constant for formation of the transition state for amide hydrolysis, $K^{\ddagger}$, might be similarly sensitive to structural variation in the peptide. The value of β in eq 1 will therefore be close to unity.

$$\frac{\bar{K}\ (\mathrm{al})_{\mathrm{VIII}}}{\bar{K}\ (\mathrm{al})_{\mathrm{V}}} = \beta \frac{K^{\ddagger}_{\mathrm{VI}}}{K^{\ddagger}_{\mathrm{III}}} \qquad (1)$$

The quantity on the left-hand side of this equation can be derived from the observed K_i's of the peptide aldehydes as follows. Aldehydes V and VIII are in equilibrium with their hydrates in aqueous solution; hence

$$K_i(\mathrm{obsd}) = \frac{[\mathrm{E}][\mathrm{aldehyde}]}{[\mathrm{II}]} + \frac{[\mathrm{E}][\mathrm{aldehyde\ hydrate}]}{[\mathrm{II}][\mathrm{H_2O}]}$$

$$= \frac{[\mathrm{E}][\mathrm{aldehyde}](1 + K_{\mathrm{hy}})}{[\mathrm{II}]}$$

where K_{hy} is the formation constant for the aldehyde hydrate. Thus K_i (obsd) = $(1/\bar{K}\ (\mathrm{al}))(1 + K_{\mathrm{hy}})$ and $\bar{K}$ (al) = $(1 + K_{\mathrm{hy}})/K_i$ (obsd). Since to a first approximation, K_{hy} will be independent of remote structural features of the aldehyde

$$\frac{\bar{K}\ (\mathrm{al})_{\mathrm{VIII}}}{\bar{K}\ (\mathrm{al})_{\mathrm{V}}} = \frac{K_i\ (\mathrm{obsd})_{\mathrm{V}}}{K_i\ (\mathrm{obsd})_{\mathrm{VIII}}} = 78$$

The quantity, $K^{\ddagger}_{\mathrm{VI}}/K^{\ddagger}_{\mathrm{III}}$, on the right-hand side of eq 1 can be derived from the rate constants for hydrolysis of the peptide amides VI and III. According to transition-state theory, the rate constant for a chemical reaction is given by eq 2, where κ is a transmission coefficient, taken to be equal to

$$k = \kappa \frac{\mathbf{k}T}{h} K^{\ddagger} \qquad (2)$$

1 for most reactions. The relative rate constants for elastase-catalyzed hydrolysis of the amides III and VI will be entirely determined by the ratio of the pseudoequilibrium constants, $K^{\ddagger}_{\mathrm{VI}}$ and $K^{\ddagger}_{\mathrm{III}}$,[3] and

$$\frac{K^{\ddagger}_{\mathrm{VI}}}{K^{\ddagger}_{\mathrm{III}}} = \frac{(k_{\mathrm{cat}}/K_{\mathrm{m}})_{\mathrm{VI}}}{(k_{\mathrm{cat}}/K_{\mathrm{m}})_{\mathrm{III}}} = 105$$

The value of the proportionality constant β in eq 1 will therefore be 0.74. This is sufficiently close to unity to show that formation of the transition state for elastase-catalyzed amide hydrolysis is a very similar reaction to the binding of peptide aldehydes to the enzyme. The enzyme–aldehyde complexes must therefore be considered good transition-state analogs for the hydrolysis reaction. As shown above, the most reasonable structure for the enzyme–aldehyde complex is the hemiacetal II. The most plausible candidate for the transition state for elastase-catalyzed hydrolysis of amides will therefore be a tetrahedral adduct not very different from the proposed intermediate, I.

The use of aldehyde analogs of the substrate to probe enzymic reactions is not likely to be restricted to elastase, but might be of general use for a variety of proteases and esterases. It may be possible, for example, to distinguish acyl-enzyme and imino-enzyme mechanisms using these compounds. Where an enzyme mechanism is already fairly well understood, perhaps the most interesting results will come from X-ray crystallographic determination of the three-dimensional structure of hemiacetals like II. These structures might be expected to reveal features of the catalytic process which would otherwise prove elusive, *e.g.*, the possibility of general-acid catalysis of tetrahedral intermediate formation by the serine proteinases. In the case of elastase in particular, a comparison between the hemiacetals formed by aldehydes V and VIII may afford an interpretation at the molecular level of the rate enhancements resulting from enzyme–substrate contacts remote from the scissile bond (Thompson and Blout, 1970, 1973b).

Immediately prior to submission of this manuscript, I was informed of the work of Aoyagi *et al.* (1969) on the leupeptins, a novel class of naturally occurring protease inhibitors. The leupeptins have been shown to be peptide aldehydes and their structures have been confirmed by synthesis (Kawamura *et al.*, 1969). The results described above would suggest that the high affinity of leupeptins for certain serine proteinases, and their potent inhibitory properties, might arise from the enzyme–leupeptin complex being structurally similar to the transition state for the hydrolysis reaction.

[2] The observed K_m for the hydrolysis of peptide amides III and VI may be equated with the dissociation constant of the Michaelis complex since these peptides have a unique enzyme–substrate binding mode (Thompson and Blout, 1973a). and the acylation step of their hydrolysis appears to be rate determining.

[3] Since we wish to compare these values of $K^{\ddagger}$ with the association constants for the bimolecular reaction of the enzyme with aldehydes, the relevant rate constants will be the second-order constant, $k_{\mathrm{cat}}/K_{\mathrm{m}}$.

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Restrictions on the Binding of Proline-Containing Peptides to Elastase†

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ABSTRACT: In general, peptide substrates of pancreatic elastase have multiple binding modes to the enzyme and can give rise to heterogeneous reaction products. The presence of proline residues in the substrate is shown to restrict the activity of the enzyme and lead to fewer reaction products. Evidence is presented that the restriction of activity results from the inability of one subsite of the enzyme to bind proline residues of the substrate and the consequent reduction in the number of possible enzyme–substrate binding modes. These observations have led to the design of peptide substrates and inhibitors with a single enzyme binding mode.

The use of X-ray diffraction techniques makes it possible to study the detailed interactions of enzymes with substrate-like molecules. Frequently, however, the functional importance of the interactions observed cannot be deduced from static models of enzyme–substrate complexes. Because of their essentially dynamic nature, the function of enzyme–substrate interactions can often best be determined by studying their effect on the rate and equilibrium constants of the catalytic process. Unfortunately, the derivation of these constants from kinetic data is frequently complicated by the presence of secondary enzyme–substrate complexes.

The long substrate binding site of elastase (EC 3.4.4.7) (Atlas *et al.*, 1970; Thompson and Blout, 1970) and the polymeric nature of its substrates make secondary complex formation particularly likely for this enzyme. For hydrolyses occurring by an acyl-enzyme mechanism with the acylation reaction the rate-determining step, the observed value of the Michaelis constant, K_m, will be related to the dissociation constants for all possible enzyme–substrate complexes by eq 1, where K_p and K_n are the dissociation constants of "productive" and "nonproductive" enzyme–substrate complexes, respectively. Similarly, the observed value of an inhibition constant, K_i, will be related to the dissociation constants for all possible enzyme–inhibitor complexes by

$$\frac{1}{K_m} = \sum_p \frac{1}{K_p} + \sum_n \frac{1}{K_n} \quad (1)$$

$$\frac{1}{K_i} = \sum_n \frac{1}{K_n} \quad (2)$$

The measured value of K_m or K_i is simply related to the dissociation constant of a single enzyme–peptide complex

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