Reaction of Peptide Aldehydes with Serine Proteases. Implications for the Entropy Changes Associated with Enzymatic Catalysis†

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ABSTRACT: Peptide aldehydes and serine proteases combine to form hemiacetals which are analogues of the transition state for substrate hydrolysis. The free energies of hemiacetal bond formation have been determined for a number of peptide aldehydes and serine proteases by comparing the binding constants of the aldehydes with those of the analogous peptide amides. This free energy is found to be very dependent on the contact between the enzyme and the P_1 "side chain" of the peptide aldehyde. For chymotrypsin and two chymotrypsin-like enzymes, the free energy of the hemiacetal bond is almost 3 kcal mol^{-1} greater when this side chain is a benzyl group than when it is a hydrogen atom. For elastase the free energy of bond formation is also close to 3 kcal mol^{-1} greater for a methyl than a hydrogen "side chain". Earlier work has shown that the free energy of this "side-chain" contact in noncovalent enzyme–substrate complexes is not greatly affected by small displacements of the substrate. It is therefore unlikely that the enthalpy of the side-chain contact will differ in the amide and hemiacetal complexes. We may conclude that the contact between the benzyl group and the chymotrypsin-like proteases affects mainly the entropy of hemiacetal bond formation, making this quantity less negative by approximately 10 eu mol^{-1}. The contact between a methyl "side chain" and elastase makes the entropy of hemiacetal bond formation with this enzyme less negative by approximately 9 eu mol^{-1}. A corollary of these findings is that the side chain will increase the entropy loss associated with noncovalent complex formation between the aldehyde and enzyme by at least 10 eu mol^{-1}. Noncovalent complexes between serine proteases and specific ligands are therefore characterized by a low internal mobility, and covalent reactions within these complexes should occur with abnormally favorable entropies. The catalytic mechanism of the serine proteases involves as its rate-determining step a reaction very similar to hemiacetal bond formation. Application of the principles discussed above indicates that this reaction will occur with an abnormally low entropy of activation. Our results therefore support the hypothesis that a substantial part of the catalytic power of serine proteases is due to their reducing the entropy loss associated with the rate-determining step of reaction.

Theories seeking to explain the efficiency of enzymes as catalysts have mainly to account for the rapid rates of chemical reactions occurring within noncovalent enzyme–substrate complexes. Many such theories have emphasized the role of enzymes in facilitating these reactions by greatly limiting the relative mobility of the reacting groups within noncovalent complexes (for a review, see Bruice, 1970). In this respect enzyme-catalyzed reactions have something of the character of intramolecular reactions, which commonly proceed many orders of magnitude faster than analogous intermolecular reactions. Most recently the idea of the limited internal mobility of enzyme–substrate complexes has been presented in terms of the reduced loss of entropy which should accompany transition-state formation within such complexes (Jencks, 1975; Page, 1977). The entropy loss associated with transition-state formation in typical bimolecular reactions is now known to be large (Page & Jencks, 1971). A significant reduction in this loss could, therefore, account for a large part of the catalytic efficiency of enzymes. Unfortunately, the magnitude of the entropy loss for enzyme reactions has proved difficult to determine. Theoretical approaches to calculating the entropy loss founder on our lack of knowledge of low-frequency vibrations in noncovalent enzyme–substrate complexes. These vibrations could well retain large amounts of entropy that must be lost on subsequent covalent reaction. Experimental approaches based on measurements of the entropy changes associated with enzyme reactions founder on our inability to determine how much of the observed ΔS is due to the reduced mobility of substrate with respect to enzyme and how much is due to changes in solvation of both species. While relevant to an overall description of the reaction, the entropy change associated with changes in solvation is not pertinent to the question of how much of the increased rate of enzyme reaction is due to an abnormally small loss of independent mobility on the part of the reactants.

Determination of the entropy change associated with the reaction of enzyme–substrate complexes is sufficiently important that it is worth trying indirect approaches to this problem where more direct methods have failed. We have recently obtained data on the reaction of various peptide aldehydes with serine proteases. This reaction is an ideal one for the study of the characteristics of enzyme reactions from several points of view. It has a defined product, a hemiacetal of Ser^{195} and the aldehyde (Thompson, 1973, 1974; Lowe & Nurse, 1977; Kennedy & Schultz, 1979; Brayer et al., 1979). It is freely reversible, allowing the thermodynamic characteristics of the reaction to be determined from equilibrium constants, and it is closely analogous to the reaction for which the enzyme is the physiological catalyst. Accordingly, we have tried to estimate the entropy of this reaction. In this we have taken advantage of other work, discussed below, which suggested to us that the enthalpy of hemiacetal bond formation should be largely independent of the P_1 side chain of the peptide aldehyde. We show that the free energy of this reaction is strongly dependent on the P_1[1] side chain and conclude, therefore, that interaction of the P_1 side chain with the enzyme strongly influences, and can reduce by up to 10 eu, the entropy lost on hemiacetal bond formation. This result indicates that, for a serine protease·peptide aldehyde complex with a strong

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[1] Amino acid residue P_1 of an enzyme–peptide complex (Schechter & Berger, 1967) is that part of the ligand immediately N terminal to the scissile bond in a substrate, or the analogous bond in a substrate analogue.

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Table I: K_ms (mM) for the Peptide Amides Ac-Pro-Ala-Pro-NHC(R)HCONH$_2$, and K_is (mM) for the Peptide Alcohols Ac-Pro-Ala-Pro-NHC(R)HCH$_2$OH and Peptide Aldehydes[3] Ac-Pro-Ala-Pro-NHC(R)HCHO for Four Serine Proteases[a]

	SGP1		SGP3			α-chymotrypsin			elastase		
R	K_m am	K_i ald	K_m am	K_i alc	K_i ald	K_m am	K_i alc	K_i ald	K_m am	K_i alc	K_i ald
H	80[b]	1.2	17[b]	18	0.4 (15)	120[b]	110	10	22	1.0	0.12
CH_3	25	0.3	8	8	0.1	75[b]	80	3	3.9	0.6	0.0008 (0.002)
$PhCH_2$	2.4	0.0003 (0.003)	0.54 (1.2)	0.8	0.00005 (0.002)	3.4	3.5	0.003 (0.006)			

[a] At 37 °C, pH 9.00 (SGP1, SGP3, and elastase), pH 8.00 (α-chymotrypsin), and pH 4.00 (those values in parentheses). [b] K_i for a peptide amide.

P_1 side-chain interaction, the loss of internal mobility associated with the covalent reaction is small. Given the strong similarities between the enzyme's reaction with aldehydes and the formation of a transition-state complex with substrates (Thompson, 1973), it appears likely that the entropy loss for transition-state formation for specific substrates is also small. Our results, therefore, provide experimental support for theories which attribute the rapid rate of enzyme-catalyzed reactions in large part to the small entropy losses associated with reactions occurring within the enzyme–substrate complexes. They indicate that one of the major roles of the P_1 side-chain interaction with serine proteases is in holding such entropy losses to a minimum during the covalent step of substrate hydrolysis.

Materials and Methods

Bovine α-chymotrypsin was a 3× crystallized preparation from Worthington. Porcine elastase was an electrophoretically purified, lyophilized preparation from Sigma. SGP1[2] and SGP3[2] were homogeneous, lyophilized preparations, which have been described earlier (Bauer, 1977; Bauer & Löfqvist, 1973).

Substrate hydrolysis was followed in a pH-stat according to Bauer et al. (1976). K_i values were determined from Dixon plots (Dixon, 1953) and, in the case of tightly binding inhibitors, also from Henderson plots (Henderson, 1972). Inhibition was competitive.

Thin-layer chromatography (TLC) of peptides was carried out according to Thompson & Blout (1973).

L-Phenylalaninol was purchased from Fluka AG, Switzerland. All amino alcohols, aldehydes, and acids were of the L configuration. The preparations of Ac-Pro-Ala-Pro-OH, Ac-Pro-Ala-Pro-Gly-NH_2 and Ac-Pro-Ala-Pro-Ala-NH_2 (Thompson & Blout, 1973), Ac-Pro-Ala-Pro-Alaol (Thompson, 1973), Ac-Pro-Ala-Pro-Alaal and Ac-Pro-Ala-Pro-Pheal (Thompson, 1977), and Ac-Pro-Ala-Pro-Phe-NH_2 (Bauer et al., 1976) have been described previously.

Acetylprolylalanylprolylphenylalaninol. Acetylprolylalanylproline (65 mg, 0.2 mmol) was dissolved in warm acetonitrile (2 mL) and *N*-methylmorpholine (25 μL, 0.22 mmol) added. The mixture was cooled to −20 °C in dry ice–carbon tetrachloride, and isobutyl chloroformate (26 μL, 0.20 mmol) was added with stirring. After 5 min a solution of L-phenylalaninol (33 mg, 0.22 mmol) in acetonitrile (2 mL) was added. The mixture was stirred at −20 °C for 1 h and at room temperature for 3 h. The solvents were removed in vacuo; the residue was dissolved in water and treated with excess Rexyn I-300 resin. After the resin was removed by filtration, the solution was evaporated and triturated under ether to give a solid product, 57 mg (62%), homogeneous and clearly different from either starting material by TLC: R_f 0.9 in chloroform–methanol (9:1).

Acetylprolylalanylprolylglycinol. This peptide was prepared in 72% yield by a procedure identical with that used for the phenylalaninol analogue except that 100 mg of ethanolamine (eightfold excess) was used in place of phenylalaninol. This product was homogeneous and clearly different from either starting material by TLC: R_f 0.6 in chloroform–methanol (9:1).

Acetylprolylalanylprolylglycinal. Acetylprolylalanylprolylglycinal dimethyl acetal was prepared by a procedure identical with that used to prepare the phenylalaninol analogue except that 21 mg of aminoacetaldehyde dimethyl acetal (0.2 mmol, Aldrich) was used in place of phenylalaninol and the treatment with 400 mg or Rexyn I-300 resin was shortened to 5 min. The acetal in 2 mL of water was stirred with 0.5 g of Dowex AG50W-X8 resin for 4 h, filtered, and evaporated. It was dissolved in chloroform and left at −20 °C overnight, the faint precipitate was removed, and the filtrate was evaporated to give 56 mg (76%) of a solid homogeneous by TLC: R_f 0.5 in chloroform–methanol (9:1). The single spot was positive to the chlorine–KI–tolidine stain used for peptide bonds and the dinitrophenylhydrazine stain used for aldehydes (Thompson, 1977).

Results

Our experiments have been designed to measure, or estimate, the free-energy changes characteristic of the noncovalent and the covalent steps in the reversible reactions of several serine proteases with peptide aldehydes. The particular enzymes used were chosen on the basis of the extensive information available concerning their structures and mechanisms: the substrates and substrate analogues were chosen so we could study the effect of a range of side-chain interactions with the enzymes, interactions which permit rapid, slow, or no catalysis of hydrolysis. The free energies of formation of noncovalent and covalent complexes of these ligands with the enzymes were chiefly obtained from the dissociation constants of enzyme–ligand complexes measured by steady-state kinetic techniques, either as K_ms or as K_is. Arguments to the effect that each of these constants is the dissociation constant of a unique, productive complex have been presented previously (Bauer et al., 1976). A list of these constants is presented in Table I. Before considering the implications of these results for the magnitude of the entropy changes accompanying formation and reaction of the noncovalent complexes, there are a number of the conclusions that can be reached concerning the nature of the enzyme–aldehyde complex and the role of geometric destabilization in the catalytic mechanisms of these enzymes.

[2] Abbreviations used: SGP1 and SGP3, extracellular proteases of *S. griseus* identical respectively with PNPA-hydrolases II and I (Wählby, 1969), alkaline proteinases c and a (Narahashi, 1970), *S. griseus* enzymes III and II (Gertler & Trop, 1971), *S. griseus* proteases B and A (Johnson & Smillie, 1971), and guanidine-stable and lysine-free chymoelastase (Siegel & Awad, 1973).

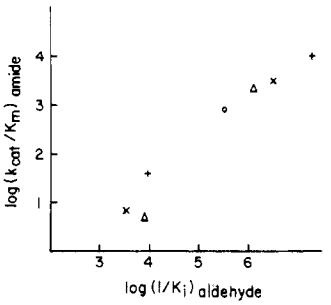

FIGURE 1: The similar linear relationship between $1/K_i$ of a peptide aldehyde and k_{cat}/K_m of the corresponding peptide amide for four serine proteases. (X) SGP1; (O) SGP3; (+) α-chymotrypsin; (Δ)elastase.

First and foremost, it is clear that peptide aldehydes show a much higher affinity for these enzymes than do either alcohols or amides. The enhancement in binding of the aldehyde with respect to the corresponding amide varies from 12-fold, in the case of α-chymotrypsin and Ac-Pro-Ala-Pro-Glyal, to 11 000-fold, in the case of SGP3 and Ac-Pro-Ala-Pro-Pheal.[3] For each enzyme the variation in the binding constants ($1/K_i$) of the aldehydes broadly parallels the variation in the second-order rate constant (k_{cat}/K_m) for hydrolysis of the corresponding amide. This is shown most clearly in Figure 1, where log ($1/K_i$) is plotted against those values of log (k_{cat}/K_m) that have been accurately determined. This is taken to support the hypothesis that the aldehydes bind as hemiacetals since the aldehyde–enzyme complexes clearly satisfy one of the criteria established for transition-state analogues (Wolfenden, 1976) and the transition-state complex is generally acknowledged to contain a substrate–enzyme covalent bond (Fersht & Requena, 1971; Fersht, 1972; Lucas et al., 1973). Figure 1 also shows that the transformation relating $1/K_i$ of an aldehyde to k_{cat}/K_m of the corresponding amide is similar for all four enzymes. This points to the strong similarity in the mechanisms of these enzymes.

The stability of the enzyme–aldehyde adducts studied here depends on pH, being lower at pH 4.0 than at pH 8.0 or 9.0 (Table I). The magnitude of the variation, however, is different for different enzymes, being greatest for SGP3 and least for α-chymotrypsin. The significance of these variations is unclear at present since they bear no obvious relationship to the maximum strength of the enzyme–aldehyde adduct or to the catalytic potential of the enzyme.

Table I shows that there is no intrinsic preference on the part of the active centers of α-chymotrypsin, SGP1, or SGP3 for tetrahedral as opposed to planar groups since these enzymes bind peptide amides and alcohols with equal affinity. This stands in marked contrast to elastase, which binds alcohols approximately tenfold more tightly than amides, a finding that has been taken to indicate an element of geometric destabilization in the catalytic mechanism of this enzyme (Thompson, 1974). Apparently, this cannot be a general aspect of the mechanism of serine protease catalyzed hydrolysis but may instead represent a special characteristic of an enzyme designed to hydrolyze substrates with small P_1 side chains.

Discussion

Nature of the Enzyme–Aldehyde Adduct. It has been proposed that the tight binding of peptide aldehydes to serine proteases is, in part, due to the formation of a hemiacetal bond between the aldehyde and the nucleophilic serine residue of the enzyme (Thompson, 1973). Several observations support the assignment of a hemiacetal structure to those serine protease–aldehyde adducts whose stability appears to be enhanced with respect to other enzyme–substrate analogue complexes. These include the observation that the serine residue is essential to the enhanced binding of aldehydes, since this phenomenon is not observed with anhydroelastase (M. L. Bender, personal communication) or with anhydrochymotrypsin (Kennedy & Schultz, 1979) and the observation that the species which binds to the enzyme is the unhydrated aldehyde (Thompson, unpublished; Kennedy & Schultz, 1979). Very recently a crystallographic study of the SGP3·Ac-Pro-Ala-Pro-Pheal complex at pH 4.1 has shown that there is a covalent bond between Ser^{195} of SGP3 and the aldehyde group of Ac-Pro-Ala-Pro-Pheal (Brayer et al., 1979) and hydrocinnamaldehyde has been shown to associate with α-chymotrypsin by a cross-saturation NMR technique (Lowe & Nurse, 1977).

The assignment of a hemiacetal structure to the enzyme–aldehyde adduct is also in good accord with our knowledge of organic chemistry. Amides are known to be able to adopt tetrahedral configurations in the active site of a serine protease (Huber et al., 1974; Sweet et al., 1974). Since the R—C=O groups of amides and aldehydes are approximately isosteric (Robin et al., 1970; Berthier & Serre, 1966) and the latter compounds show a much greater tendency to form tetrahedral adducts, it would perhaps be surprising if an aldehyde did not show a substantially greater tendency to form tetrahedral adducts with these enzymes than the corresponding amide.

There are, however, certain experimental results which may be interpreted as showing that serine protease–aldehyde adducts may not be hemiacetals. Thus, it has been shown by NMR (Gorenstein et al., 1976) and by UV spectroscopy (Breaux & Bender, 1975) that cinnamaldehyde and dimethylaminocinnamaldehyde bind to α-chymotrypsin in a simple, noncovalent manner. The failure of these aldehydes to form covalent complexes with the enzyme may reflect a number of factors including their general reluctance to form tetrahedral adducts (Rawn & Lienhard, 1974) and the unfavorable orientation of the carbonyl group and the serine hydroxyl, which is exemplified by the poor ability of α-chymotrypsin to catalyze the hydrolysis of esters of cinnamic acid (Bender & Zerner, 1962). The observation that a hemiacetal is not formed, however, is consistent with the observation that cinnamaldehyde–enzyme complexes do not show exceptional stability (Rawn & Lienhard, 1974) and the results therefore do not invalidate, but rather support, the proposed relationship between the enhanced stability of aldehyde–enzyme complexes and hemiacetal formation. All the aldehyde–enzyme complexes discussed here are considerably (12–11 000-fold) more stable[3] than the corresponding complexes of amides and alcohols (Table I). We have therefore interpreted our results as indicating the formation of tetrahedral adducts between the enzymes and all the aldehydes tested.

Another property of the enzyme–aldehyde complexes which is not clearly in accord with their being hemiacetals is the pH dependence of their dissociation constant (Thompson, 1973; Schultz & Cheerva, 1975; Breaux & Bender, 1975; Kennedy

[3] The K_is presented here are based on the total concentration of aldehyde present, i.e., the sum of the concentrations of aldehyde and aldehyde hydrate. Since the predominant inhibiting species is the free aldehyde (Thompson, unpublished; Kennedy & Schultz, 1979) and this is likely to be 10% or less of the total aldehyde present (Lewis & Wolfenden, 1977), the true K_i for the free aldehyde may be expected to be about tenfold lower than the figures quoted here. The relative values of K_i will be largely unaffected by this uncertainty as the hydration constant will not be highly sensitive to the amino aldehyde's side chain.

Table II: Free Energy of Formation (ΔG_{HA}, kcal mol^{-1}) and Effect of the Side Chain on the Entropy of Formation ($\Delta\Delta S_{HA}(R)$, eu mol^{-1}) of the Hemiacetal Bond in Enzyme Complexes with Aldehydes Ac-Pro-Ala-Pro-NHC(R)HCHO

	SGP1		SGP3		α-chymotrypsin		elastase	
R	ΔG_{HA}	$\Delta\Delta S_{HA}(R)$	ΔG_{HA}	$\Delta\Delta S_{HA}(R)$	ΔG_{HA}	$\Delta\Delta S_{HA}(R)$	ΔG_{HA}	$\Delta\Delta S_{HA}(R)$
H	−2.6		−2.3		−1.5		−1.3	
CH_3	−2.7	0.3	−2.7	1.3	−2.0	1.6	−4.1	8.9
$PhCH_2$	−5.5	9.4	−5.7	11.0	−4.3	9.0		

& Schultz, 1979). Although this differs from the pH dependence of binding of other uncharged substrate analogues (Johnson & Knowles, 1966; Schultz & Cheerva, 1975), the observed pH dependence of aldehyde binding is not that which might be expected of a good transition-state analogue. The catalytic activity of each of these enzymes depends on a group, histidine-57, with a pK_a between 6.7 and 7.0. The enzyme's activity at pH 4.0 should therefore be less than 0.2% of that at pH 8.0. In terms of transition-state theory, this translates to a more than 500-fold weaker binding of the transition state by the enzyme at the lower pH. The aldehydes are bound only 2- to 40-fold more weakly at pH 4.0 than at pH 8.0 or pH 9.0. This behavior, however, does not necessarily argue against a hemiacetal structure for the aldehyde adduct. It is to be expected that His^{57} will catalyze formation of the hemiacetal by acting as a general base, if this is its role in the catalysis of amide hydrolysis. Since the pK_a of a hemiacetal is about 13 (Bell & McTigue, 1960), the immediate product of the reaction will be rapidly protonated. Microscopic reversibility then requires that hemiacetal breakdown should be subject to specific base–general acid catalysis (Jencks, 1969) and so the stability of the complex will not necessarily be affected by pH. If, on the other hand, $His^{57}H^+$ catalyzes amide hydrolysis by acting as a general acid and donating a proton to the amide nitrogen in the transition state, it may be expected to have no effect on either the rate of formation or breakdown of the hemiacetal. Thus the pH dependence of the stability of serine protease aldehyde adducts, as opposed to the pH dependence of their rate of formation and breakdown, may not be of fundamental significance for the structure of the complex or for the mechanism of action of these enzymes. A similar analysis of the pH dependence of aldehyde inhibition of serine proteases has been proposed independently by Kennedy & Schultz (1979). In summary, the evidence adduced against hemiacetal formation is either restricted to certain special cases or can be as easily interpreted in terms of the hemiacetal structure as in its absence. On the other hand, the evidence in favor of hemiacetal formation between the enzymes and hemiacetals used here seems overwhelming.

Effect of the P_1 Side Chain on the Entropy of Formation of the Covalent Bond and the Noncovalent Complex. (a) A knowledge of the free-energy characteristic of covalent bond formation within enzyme–ligand complexes is of value in that it should, under special circumstances, allow us to estimate the loss of entropy to enzyme and ligand accompanying an enzyme reaction. One of the best defined reversible, covalent reactions of serine proteases is their reaction with aldehydes to give hemiacetals. This reaction has a defined starting material and product, and the atomic movements of the groups most closely involved may be inferred from a variety of crystallographic investigations. Unfortunately, it is not possible at present to determine the free energy of covalent bond formation directly, but a good approximation to this value may be obtained by subtracting the free energy of noncovalent binding of an amide from that for covalent binding of the corresponding aldehyde. Evidence supporting the implicit assumption that the free energy of noncovalent binding of aldehydes does not differ greatly from that of amides and esters comes from the data of Rawn & Lienhard (1974) showing that the dissociation constant of the chymotrypsin-*trans*-cinnamaldehyde complex is 2.5 mM. The dissociation constant reported for the chymotrypsin methyl-*trans*-cinnamate complex is 6.7 mM (Bender & Zerner, 1962). Additional evidence comes from the work of Kennedy & Schultz (1979) showing that the affinity of anhydrochymotrypsin for benzoylphenylalaninal is similar to that of the native enzyme for benzoylphenylalanineamide. It has also been shown (M. L. Bender, personal communication) that anhydroelastase has an affinity for Ac-Pro-Ala-Pro-Alaal which is similar to that of elastase for Ac-Pro-Ala-Pro-Ala-NH_2 (Thompson & Blout, 1973). All these findings support the hypothesis that these enzymes show no preference for aldehydes over amides in the absence of covalent bond formation. We are justified, therefore, in taking the enhanced affinity for aldehydes as a measure of covalent bond formation and have calculated the free energy of hemiacetal bond formation, ΔG_{HA}, according to the equation

$$\Delta G_{HA} = -RT(\ln 1/K_d(\text{aldehyde}) - \ln 1/K_d(\text{amide}))$$

where K_d(aldehyde) and K_d(amide) are the equilibrium constants for dissociation of the enzyme–aldehyde and enzyme–amide complexes.[4] Any systematic error introduced by this approximation will be removed when ΔG_{HA}s of the various aldehydes are compared.

Values of ΔG_{HA} have been listed in Table II. It is immediately apparent that, for each enzyme, the free energy of covalent bond formation is much greater for aldehydes with a "specific" P_1 side chain.[3] Thus for SGP1, SGP3, and chymotrypsin, ΔG_{HA} is about 3 kcal mol^{-1} greater for phenylalaninal than glycinal peptides, and for elastase ΔG_{HA} is 2.8 kcal mol^{-1} greater for alaninal than glycinal peptides.

The effect of the side chain on ΔG_{HA} may be due to its effect on either the enthalpy or entropy of the reactants and products. The side chain is sufficiently far removed from the carbonyl group to have little direct effect on its electrophilicity; therefore, any effect of the side chain on the enthalpy of the covalent reaction must result from an associated change in the interaction of the side chain with the enzyme. In the case of chymotrypsin, there is a variety of evidence indicating that any such changes will be small and that the potential energy of this interaction is, in any case, rather insensitive to the exact position of the side chain or of parts of the substrate outside the side-chain binding site. Thus, X-ray crystallographic studies, summarized by Blow (1976), indicate that the position of C^α and the P_1 side chain is very similar in covalent and noncovalent complexes analogous to those considered here. Solution studies of the affinity of chymotrypsin for substrates with similar sized but geometrically different side chains indicate that the precise position of the side chain does not

[4] In the case of elastase only, the K_d of the corresponding peptide alcohol has been used to calculate ΔG_{HA}. This is done because, in this case only, there is evidence that the amide is strained on binding to the active site of the protease.

Table III: Free Energy of Formation of a Tetrahedral Intermediate (ΔG_{T_1}, kcal mol^{-1}) and Effect of the Side Chain on Its Entropy of Formation ($\Delta\Delta S_{T_1}(R)$, eu mol^{-1}) from the Michaelis Complex of Enzyme and the Amides Ac-Pro-Ala-Pro-NHC(R)HCONH$_2$

	SGP1		SGP3		α-chymotrypsin		elastase	
R	ΔG_{T_1}	$\Delta\Delta S_{T_1}(R)$	ΔG_{T_1}	$\Delta\Delta S_{T_1}(R)$	ΔG_{T_1}	$\Delta\Delta S_{T_1}(R)$	ΔG_{T_1}	$\Delta\Delta S_{T_1}(R)$
H	>21.1		>21.1		>19.7		>17.9	
CH_3	18.6	>8.3	19	>7.0	19.7		16.6	4.3
$PhCH_2$	16.8	>14.3	16.8	>14.3	17.3	>8.0		

greatly affect the potential energy of the side-chain interaction. Thus acetylhexahydrophenylalanineamide and acetylphenylalanineamide bind equally well to chymotrypsin (Foster & Niemann, 1955) despite the fact that the chair conformation side chain of the former substrate must make different intermolecular contacts than the planar side chain of the latter. In addition, the study of substrates with a modified main chain indicates that distortion of substrate binding outside the side-chain binding site does not greatly affect the potential energy of side-chain binding. Substitution of methyl groups for hydrogen atoms on the P_1 amino group (Peterson et al., 1963) or C^α (Almond et al., 1962), for example, does not change K_m by more than a factor of 5. The potential energy of the side-chain contact is clearly not sensitive to small positional changes of the substrate, a finding that is clearly in accord with the predominantly hydrophobic character of the interaction.

X-ray crystallographic data on the other enzymes considered here show that their side-chain binding sites are less, not more, defined than that of chymotrypsin (Shotton & Watson, 1970; James et al., 1978; Brayer et al., 1978). We can safely conclude that, with these enzymes as well, transformation of a noncovalent complex to a hemiacetal is unlikely to seriously affect the potential energy of the side-chain interaction and that the side-chain interaction is correspondingly unlikely to affect ΔH_{HA}, the enthalpy of hemiacetal bond formation. We therefore ascribe the observed free-energy change primarily to an effect of the side chain on the entropy of reaction, ΔS_{HA}. Changes in the entropy of reaction, $\Delta\Delta S_{HA}$, due to the side-chain R, may then be calculated from the equation

$$\Delta\Delta S_{HA}(R) = S_{HA}(R = H) - \Delta S_{HA}(R) = (\Delta G_{HA}(R) - \Delta G_{HA}(R = H))/T$$

Values of $\Delta\Delta S_{HA}(R)$ calculated in this way are listed in Table II. For all four enzymes the optimal interaction between the side-chain binding site and a P_1 side chain appears to be capable of reducing the entropy of hemiacetal bond formation by about 7–11 eu mol^{-1}. This would correspond to a substantial reduction in the mobility of the noncovalent enzyme–aldehyde complex as a consequence of forming the enzyme–P_1 side-chain interaction, a point that will be discussed more fully below.

(b) Another reaction which involves reversible covalent bond formation within a noncovalent enzyme–ligand complex is the formation of a tetrahedral intermediate from a serine protease·amide complex. We have applied the reasoning described above to calculate the effect of the side-chain interaction on the entropy of formation of this covalent complex, although in view of the fact that the tetrahedral intermediate is not as well defined as the hemiacetal and that we cannot easily determine its free energy of formation we do not have the same degree of confidence in our figure for $\Delta\Delta S(R)$. The change in free energy of the enzyme and substrate on formation of a tetrahedral intermediate, ΔG_{T_1}, will be similar to that for formation of the transition state, which may occur shortly before or after the intermediate on the reaction pathway depending on the exact substrate used. We have, therefore, taken ΔG_{T_1} to be equal to the observed free energy of activation for amide hydrolysis $\Delta G^\ddagger$, calculated according to the equation

$$\Delta G_{T_1} = \Delta G^\ddagger = -RT \ln k_{cat}(h/kT)$$

where k_{cat} is the turnover number, which in this case will be equal to the rate constant for the acylation reaction (Bauer et al., 1976). Values of k_{cat} have been published previously by Thompson & Blout (1973), Bauer et al. (1976), and Bauer (1978). As discussed above for hemiacetal bond formation, it is unlikely that the changes observed in ΔG_{T_1} are due to differences in ΔH_{T_1}. Such changes could result only from changes in the enthalpy of the side-chain interaction, changes which are likely to be small in view of the minimal change required in the position of the side chain (Blow, 1976) and the insensitivity of the enthalpy of this interaction to small positional changes (see above). We have therefore assumed that the enthalpy of tetrahedral intermediate formation, ΔH_{T_1}, will be independent of the side chain. The effect of the side chain on ΔS_{T_1} may then be calculated from the changes in ΔG_{T_1} using the equation

$$\Delta\Delta S_{T_1}(R) = \Delta S_{T_1}(R = H) - \Delta S_{T_1}(R) = (\Delta G_{T_1}(R) - \Delta G_{T_1}(R = H))/T$$

Values of ΔG_{T_1} and $\Delta\Delta S_{T_1}(R)$ have been listed in Table III. With the exception of elastase, the rate constant k_{cat} for the hydrolysis of the glycinamide is too slow to be measured. Thus it is only possible to give a lower limit for $\Delta G^\ddagger$ and ΔG_{T_1} of this peptide and for $\Delta\Delta S_{T_1}(R)$ for all side chains with SGP3, SGP1, and α-chymotrypsin. Even the lowest estimates of $\Delta\Delta S_{T_1}(R)$ are greater than our estimate of the analogous quantity $\Delta\Delta S_{HA}(R)$. This may indicate that the effect of the P_1 side chain in reducing the entropy loss associated with tetrahedral intermediate formation is greater than its effect on the entropy loss associated with hemiacetal formation. This point will be discussed further below.

(c) The effect of the P_1 side-chain interaction in reducing the entropy loss associated with covalent bond formation within a noncovalent complex requires that it increase the entropy loss associated with formation of the noncovalent complex. This can be seen more clearly if we consider the overall covalent reaction between the enzyme and ligand, either an aldehyde or amide

$$E + L \rightleftharpoons EL \rightleftharpoons EL'$$

where EL is a noncovalent or Michaelis complex and EL′ a covalent complex. The most important feature reducing the entropy of the covalent complex below that of the separate reactants, E and L, will be the covalent bond. Noncovalent interactions between E and L, such as those between the enzyme and the P_1 side chain, will further reduce the entropy of the complex and will increase the entropy loss that will be associated with forming EL′, i.e.

$$\Delta\Delta S_{overall}(R) \leq 0$$

However, the overall effect of the side chain will be the sum of its effects on the noncovalent and covalent steps of reaction

$$\Delta\Delta S_{overall}(R) = \Delta\Delta S_{nc}(R) + \Delta\Delta S_{c}(R)$$

and therefore

$$\Delta\Delta S_{nc}(R) \geq -\Delta\Delta S_{c}(R)$$

Thus a P_1 side-chain interaction decreases the entropy lost in the covalent reaction should increase the entropy lost in the noncovalent reaction at least as much. We may conclude that the additional entropy lost to enzyme and ligand due to the P_1 side-chain interaction noncovalent complex formation is at least 9–11 eu mol^{-1} for a benzyl side chain and SGP1, SGP3, and α-chymotrypsin and at least 7 eu mol^{-1} for a methyl side chain and elastase.

Implications for the Mechanism of Serine Proteases. A good deal of information about the mechanisms of enzymes would be obtained if the precise nature of the noncovalent complex of an enzyme with its specific substrate was understood. These complexes may be analogous to rather loose, encounter complexes in which the reacting groups have a high relative mobility, or they may be much tighter complexes in which these groups have no greater relative mobility than similar groups which participate in many intramolecular reactions. The former type of complex will be formed with relatively little loss of entropy because the translational–rotational entropy of the substrate will be replaced in the complex by low frequency vibration entropy. In contrast, formation of the latter type of complex with fewer such vibrations will require a substantial loss of entropy. Subsequent covalent reactions within the former, but not the latter, complex will involve a large loss of entropy, because the force constant of the covalent bond will be too high to permit low-frequency vibrations near the reaction center. Because of this the enzyme's contribution to increasing reaction rates within a loose ES complex will primarily be one of reducing the enthalpic component of $\Delta G^{\ddagger}$, unless a second, tight ES complex is formed subsequently (Jencks, 1979). In contrast, for a reaction in which the covalent bond is virtually fully formed in the transition state, a large, and possibly dominant, role of the enzyme within a tight ES complex might be to reduce the entropic component of $\Delta G^{\ddagger}$. For a typical reaction this could lead to a 10^6- to 10^8-fold acceleration (Jencks, 1975).

The effect of additional enzyme–ligand contacts on the entropy of noncovalent complex formation and subsequent covalent reaction should be different for the two extreme models of an enzyme–substrate complex. If a specific substrate complex is of the "loose" type, like its nonspecific analogue, the additional contacts should not appreciably affect the entropy of either reaction. However, if a specific substrate complex is of the "rigid" type the new contacts should reduce the low frequency vibrational motion characteristic of the nonspecific complex, vibrational motion that would otherwise be lost only on covalent bond formation. The additional contact should then increase the entropy loss during noncovalent complex formation but reduce that loss during subsequent covalent reactions.

The results described above, together with crystallographic and other evidence indicating that the potential energy of the side-chain interaction will be similar in noncovalent and covalent complexes of α-chymotrypsin, argue strongly in favor of the P_1 side-chain interaction being one that reduces the entropy loss consequent to hemiacetal bond formation by up to 9–11 eu mol^{-1}. A strong side-chain interaction correspondingly increases the entropy lost due to noncovalent complex formation by at least this amount. The results, therefore, indicate that the noncovalent complex of the phenylalaninal peptide with SGP1, SGP3, or α-chymotrypsin and the complex of the alaninal peptide with elastase are tight complexes from which much of the vibrational entropy characteristic of nonspecific substrate complexes has been removed.

It is clearly of interest to inquire what proportion of the entropy loss normally associated with hemiacetal bond formation has been shifted to the noncovalent step of reaction. A simple calculation suggests that the residual entropy loss in the covalent reaction might be quite small. The enzyme complex with a glycinal peptide will probably not be less rigid than hydrogen-bonded or charge-transfer complexes which have entropies of formation of about −10 eu mol^{-1}. The additional entropy loss calculated for the P_1 side-chain interaction is at least 9–11 eu mol^{-1}, making the total entropy lost in forming the noncovalent complex between the enzyme and a specific aldehyde greater than 19–21 eu mol^{-1}. The entropy loss due to formation of a covalent "hemiacetal" bond between acetaldehyde and water is 24 eu mol^{-1} (standard state of water = 1 M, Bell & McDougall, 1960). The enzyme, through noncovalent complex formation, would appear to be capable of removing sufficient entropy from the aldehyde such that covalent bond formation would proceed with little or no further entropy loss.

The strong analogies between hemiacetal formation and tetrahedral intermediate formation, and the results of calculations based on the effect of the side-chain contact on k_{cat} (Table III), suggest that the enzyme's ability to catalyze amide hydrolysis is also rooted in its ability to reduce the entropy loss accompanying covalent bond formation. The P_1 side-chain contact can be expected to reduce the entropy of activation of the Michaelis complex for this reaction by at least 10 eu mol^{-1} and thereby increase k_{cat} more than 100-fold. The increase in k_{cat} may be even greater than this if, as suggested by the data in Table III, the entropic advantage due to the side-chain contact is greater for tetrahedral intermediate than for hemiacetal formation. As with hemiacetal formation the overall entropic advantage of preliminary noncovalent complex formation to the enzyme reaction will be substantially greater than that due to the side-chain contact alone. If the entropy of activation for amide hydrolysis is not less than the entropy loss due to hemiacetal formation, the $\Delta S^{\ddagger}$ for the enzyme reaction should be more favorable by not less than 20 eu mol^{-1}, corresponding to a rate advantage of more than 10^4. We may conclude that a substantial part of the rapid rate of reaction of specific amides with serine proteases is due to the low entropy of activation of these reactions and that the enzyme's contact with the P_1 side chain is one of the chief interactions determining this.

While this paper has been concerned mainly with the role of the side-chain contact in increasing k_{cat} through decreasing $\Delta S^{\ddagger}$, it is important to consider also the role of this contact in reducing K_m. The benzyl side chain, for example, reduces the K_m of the amides in Table I by more than 30-fold with SGP1, SGP3, and α-chymotrypsin. The two roles of the P_1 side chain may be unified by the concept of an intrinsic binding energy (Jencks, 1975). Only part of this binding energy will appear to stabilize the Michaelis complex, reducing K_m, because of the unfavorable entropy changes within the complex which accompany formation of the side-chain contact. The "missing" energy is applied to reducing the entropy of subsequent chemical reactions of the Michaelis complex, i.e., in increasing k_{cat}. The intrinsic binding energy of the P_1 side chain appears in full only when we consider its effect on the stability of the transition state with respect to the separate reactants. The ease of this conversion, which includes both the noncovalent and covalent steps of reaction, is reflected in

the quotient k_{cat}/K_m. This constant has units of M^{-1} s^{-1} and is equal to the second-order rate constant for substrate hydrolysis at substrate concentrations well below K_m. It is greater for Ac-Pro-Ala-Pro-Phe-NH_2 than for Ac-Pro-Ala-Pro-Gly-NH_2 by factor of more than 10 000, more than 17 000, and more than 800 for SGP1 (Bauer, 1978), SGP3, and α-chymotrypsin (Bauer et al., 1976), respectively.

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