Thionesters as a Probe for Electrophilic Catalysis in the Serine Protease Mechanism*

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Specific and nonspecific thionester substrates for α-chymotrypsin and subtilisin Carlsberg have been synthesized and the kinetic parameters for their enzymecatalyzed hydrolyses measured. Despite equal nonenzymatic reactivities of ester-thionester pairs, each thionester is considerably less reactive toward enzymic hydrolysis, the difference being greatest for the specific substrates. The data support the operation of electrophilic catalysis by a hydrogen bond network at the carbonyl oxygen adjacent to the scissile bond of the substrate. The free energy of stabilization is 19 kJ mol⁻¹ for a specific thionester substrate and will be higher for oxygen esters and amides. Chymotrypsin binds esters and thionesters about equally well, whereas subtilisin binds thionesters more tightly. This is consistent with continuous hydrogen bonding in the chymotrypsin mechanism and with a differential hydrogen bonding mechanism for subtilisin. A comparison of the relative rates of enzyme-catalyzed hydrolysis of ester and thionester substrates with their relative reactivities toward amines does not support an acyl histidine intermediate in the serine protease mechanism.

Although nucleophilic catalysis has been documented as an important aspect of enzymic catalysis, electrophilic catalysis by enzymes has received less attention. Apart from cases where proton donation enhances leaving group ability (general acid catalysis), the best studied cases of electrophilic catalysis are covalent and metal ion catalysis. In the former case a carbonyl center is typically transformed either directly (Schiff base) or through a conjugated system (thiamine or pyridoxal) into a system where cationic nitrogen is capable of stabilizing developing negative charge in the transition state. In the latter case negative charge is stabilized by direct interaction with a metal ion.

These mechanisms, supported by a variety of studies in model systems, provide a satisfying explanation of electrophilic contribution to rate enhancement by those enzymes which can be shown to contain the required prosthetic groups. On the other hand, because the reactions of the biochemically ubiquitous carbonyl group typically involve development of negative charge at the carbonyl oxygen, one might expect electrophilic catalysis to be a perfectly general feature of enzymatic reactions. Since the covalent and metal ion cases constitute but a fraction of the whole, one wonders whether nature has provided a more general solution, which uses only the groups of proteins themselves.

Most of the studies on this question have been on the serine proteases, whose structure and mechanism have been reviewed (1). The first clue was provided by Steitz et al. (2), who observed, in a crystallographic study of chymotrypsin with N-formyl-L-tryptophan, the formation of two hydrogen bonds between the carbonyl oxygen adjacent to the scissile peptide bond and two N—H groups in the protein backbone. That this interaction may be related to specificity was suggested by Henderson (3), who showed that in the acyl chymotrypsin derived from a nonspecific substrate the reactive carbonyl group is not so positioned.

From x-ray data of the mechanistically similar but genetically distinct serine protease subtilisin BPN', Kraut and co-workers (4, 5) deduced a similar hydrogen bond network at the active site, but proposed that these bonds are absent in the Michaelis complex and acyl enzyme. The restoration of hydrogen bonding in the transition states for acylation and deacylation constitutes, according to this proposal, a powerful catalytic factor.

Subsequently similar hydrogen bonding to the reactive carbonyl oxygen has been observed in crystallographic studies of trypsin (6, 7) and papain (8). Meanwhile, kinetic evidence supporting electrophilic catalysis began to develop, chiefly in the laboratory of Williams. The serine proteases chymotrypsin (9, 10), trypsin (11), and subtilisin (12, 13), sulfhydryl proteases papain (14) and bromelain (11), and alkaline phosphatase (15) all display unusually low Hammett values in rates of hydrolysis of series of substituted aryl substrates.

Other data which have been interpreted as consistent with electrophilic catalysis by hydrogen bonding include proton chemical shift values in a complex of α-lytic protease with an aldehyde inhibitor (16) and a low Brunsted β for rates of deacylation of acyl chymotrypsins (17).

The question of whether hydrogen bonding to the substrate carbonyl oxygen is removed in the Michaelis complex and acyl enzyme (differential hydrogen bonding) remains open. Blow (18), in re-examining his x-ray data in light of Kraut's subtilisin-based proposal (4), has concluded that this removal is unlikely in chymotrypsin. Polgár and Asbóth (19) have reinterpreted the available x-ray data using stereochemical assumptions which restrict the available orientations of enzyme and substrate. They conclude that both bonds are present in the Michaelis complex with chymotrypsin as is at
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least one with subtilisin, although another hydrogen bond, between enzyme and the acylamino proton of $S_i$, is broken for both proteases. The only kinetic study addressed to differential hydrogen bonding (20) argued against its operation in the chymotrypsin mechanism but was restricted to nonspecific substrates.

Although both X-ray and chemical evidence support the existence of electrophilic hydrogen bonding to the carbonyl oxygen in the transition state, the contribution of this interaction to catalysis is as yet unmeasured. Even in the best nonenzymic models (21, 22) electrophilic catalysis by intramolecular hydrogen bonding fails to occur. Although certain conformations of these molecules allow hydrogen bonds to be made to the carbonyl oxygen, the price in entropy is apparently too great for this to occur, and the role of electrophile is played instead by solvent.

We report here the measurements of enzyme kinetic parameters of specific and nonspecific substrates of chymotrypsin and subtilisin where the carbonyl oxygen adjacent to the scissile bond is replaced by sulfur. These chemical data directly implicate electrophilic catalysis in the mechanisms of these enzymes and provide the first quantitative measure of the energies involved. They confirm the relationship between such catalysis and enzymic specificity, and also suggest a possible variation between chymotrypsin and subtilisin in the operation of differential hydrogen bonding.

EXPERIMENTAL PROCEDURES

RESULTS

Chymotrypsin (EC 3.4.21.1) and subtilisin (EC 3.4.21.14) catalyze the hydrolyses of the ester-thionester pairs 1 and 2, derivatives of glycine and phenylalanine, respectively. The benzote-thionbenzoate pair 3 acylates chymotrypsin under conditions of enzyme in excess. In all cases, cleavage of the substrate occurs adjacent to the C=X group, releasing alcohol or phenolate. In addition, 2b competitively inhibits the chymotrypsin catalyzed hydrolysis of 2a. The kinetic parameters measured for these substrates appear in Table I. Detailed results, including product analyses and treatment of kinetic data, appear in the Miniprint. The important results may be summarized as follows.

1) For all substrates and for both enzymes $k_{cat}/K_m$ is substantially smaller for the thionester than for its oxygen ester analog.

2) The difference in reactivity, judged by $k_{cat}/K_m$, between esters and thionesters is very large, about 2000-fold, for the specific substrates derived from phenylalanine, but declines to a more modest factor for the nonspecific substrates. These differences, which are parallel for the two enzymes, cannot be attributed to any differences in inherent chemical reactivity of the substrates.

3) With chymotrypsin, the reactivity difference between esters and thionesters appears essentially completely in $k_{cat}$. The binding, as judged by $K_m$, of thionesters is, if anything, slightly weaker. With subtilisin, however, thionesters bind considerably more tightly than do their oxygen analogs.

DISCUSSION

The large difference between rates of enzyme-catalyzed hydrolysis of a specific ester substrate and its thionester analog is striking direct chemical evidence supporting the operation of a hydrogen bonding network at the carbonyl oxygen adjacent to the scissile bond. By stabilizing the negative charge which develops on this atom in the enzymic transition state, these hydrogen bonds lower the free energy of the transition state, and thus make an important contribution toward enzymatic catalysis. These data serve to confirm and unify the X-ray crystallographic results (2-8), which suggested hydrogen bonding without being able to measure its effect, and linear free energy correlations (9-15, 17), which implied the operation of electrophilic catalysis without locating its site.

These results also provide the first quantitative measure of the energies involved and thereby the rate acceleration attributable to this feature of the serine protease mechanism. The ester/thionester rate ratios, measured as $k_{cat}/K_m$, are about 2000 for the specific phenylalanine substrates, which translates into 19 kJ mol⁻¹ of free energy.

The estimated perturbation of geometry of the transition state at the enzyme active site caused by substitution of sulfur for oxygen at the carbonyl heteroatom is 40-50 pm, based on the differences in C=X covalent bond lengths (57, 58) and in N–H…O and N–H…S hydrogen bond lengths (59). Calculations (60) indicate that a change of 40 pm in hydrogen bond length may result in an energy loss of up to 25 kJ mol⁻¹ in a single bond. Thus it is likely that transition state stabilization is essentially eliminated by the oxygen to sulfur substitution. This leads to the estimate of $ΔG = 9.5$ kJ mol⁻¹ per hydrogen bond to sulfur, present in the ground state but absent in the enzymic transition state, and is consistent with literature reports of energies of such bonds (61). Since hydrogen bonds to oxygen are invariably stronger, usually by at least 4 kJ mol⁻¹ (61-64), it is likely that this factor, when combined with a third (S=O) hydrogen bond, actually approaches accounting for the factor of $10^9$ in rate originally proposed (4). It should be pointed out, however, that this rate increase is relative to a reaction catalyzed by a hypothetical protease where the hydrogen bonding groups are not there; it does not bear comparison to a chemical reaction in water, where solvent molecules are available for hydrogen bonding to the transition state (although probably at some cost in entropy (65) or enthalpy (66)). This factor will thus never directly contribute to an increase in $k_{cat}/K_m$ (although clearly $k_{cat}/K_m$ would be much lower were it absent), which is consistent with the results and interpretation of Fastrez (67) on methyl chymotrypsin. It will contribute to $k_{cat}$ only to the extent that hydrogen bonding is weakened or removed in the Michaelis complex or acyl enzyme, a point which we address below.

An alternative explanation of the data is that optimal hydrogen bonds are maintained with thionester substrates, but only at the expense of weakening binding forces elsewhere in the complex. For example, the binding of the thiolate in the oxyanion hole pulls the aromatic side chain out of its
hydrophobic pocket (or pushes it too far in). This explanation, others involving strain induced on the protein, or any combination gives results kinetically equivalent to the lack of hydrogen bond formation. Tais tradeoff of various kinds of binding energy has been thoroughly discussed by Jencks (68).

Specificity—The change in kcat/Km upon substitution of sulfur for oxygen in the nonspecific substrates 1 and 3 is considerably smaller than in 2. The change in transition state free energies drops to 9–10 kJ mol⁻¹ for chymotrypsin and only 2 kJ mol⁻¹ for subtilisin. It is likely that in these less rigidly held transition states hydrogen bonds do exist between enzyme and thiolate anion, although in the case of chymotrypsin their energies are substantially reduced, or possibly a single strong bond forms.

A similar relationship between specificity and magnitude of rate change has been observed with peptide and analogous thiopeptide substrates of the metalloenzyme carboxypeptidase A (Table IV). A change from the specific substrate Bz-Gly-Gly-Phe to its thiopeptide derivative results in a rate decrease by a factor of over 1000 (43, 69). As the specificity is relaxed, either by a shortening of the substrate by one glycine residue (70) or by a substitution of the active site Zn by Cd (69), the rate decrease becomes much less dramatic.

It is now clear that in the transition state for hydrolysis of a specific substrate by serine proteases all four points of the tetrahedron centered about the reactive carbonyl carbon engage in binding interactions with the protein. A marked decrease in rate is produced by minor perturbations at either the nucleophilic site (71–73), leaving group (74), acyl portion at either side or by a side chain or acylamino group (75), or carbonyl oxygen. The carbonyl oxygen, because of its small size, localized charge, and short distance from the reactive center, is probably the most sensitive of the four groups to small changes in molecular structure, and therefore may be regarded as the primary anchor around which specificity is enforced. It is also clear that a weakening of interactions at one point of the tetrahedron increases the tolerance for changes at the other points, as suggested previously (73).

Differential Hydrogen Bonding—Disagreement exists on the question of whether hydrogen bonding to the carbonyl oxygen is differential (4), being removed in the Michaels complex and acyl enzyme and restored in the transition states for acylation and decacylation, or continuous (18) along the reaction coordinate. The former mechanism manages a max-
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EXPERIMENTAL PROCEDURES

Materials

N-Acetyl-DL-phenylalanine: A solution of 2.9 g of N-acetyl-DL-phenylalanine in 20 ml of chloroform was treated with 3 ml of 1 N HCl for 15 min, and the reaction mixture was evaporated to dryness. The residue was dried under reduced pressure and stored in a vial.

N-Acetyl-DL-phenylalanine hydrochloride was prepared according to the procedure of A. W. L. Fodem, J. Chem. Soc. 9041 (1956).

The procedure has been reported several times with consistent results, and the product recrystallized several times from a mixture of ethanol and water. A product with mp 125°C was obtained.

The crystal structure of N-acetyl-DL-phenylalanine hydrochloride was elucidated by X-ray crystallographic analysis.

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Chymotrypsin and subtilisin catalyzed hydrolysis of racemic 2b (top panels). Figure 2. Reciprocal plots of titrimetric initial rate data for the linearly may be compared to analogous data obtained for the optically pure ester L-2a (bottom panels). Conditions are given in Table 1.

First, we note that essentially hyperbolic lines are observed in the hydrolysis of 2b catalyzed by either chymotrypsin or subtilisin. The linearity of these plots is consistent with rapid reversible catalysis by the two enzymes. In contrast, the FPLC elution profile of the 2b substrate does not occur fast enough to complete the enzyme reaction.

The results of an electrophoresis experiment for the chymotrypsin catalyzed hydrolysis of 2b is shown in Figure 3. Upper trace: Titrimetric; lower trace: phosphate buffer. Conditions are given in Table 2.

When the fast phase becomes associated with the enzymic hydrolysis of a simple substrate (e.g. 2b), the rate constant kcat can be obtained directly from the slope of the corresponding line. The agreement between values of kcat obtained spectrophotometrically using initial rates and from the FPLC elution profile of the 2b substrate demonstrates that these data are consistent with a steady state kinetic analysis of the reaction.

The results of all three experiments are consistent with the following behavior of 2b.

1. One enantiomer (presumably L) is hydrolyzed enzymically.
2. The other enantiomer is hydrolyzed only by a buffer catalyzed process which does not involve the enzyme.
3. A steady state is maintained over the time course examined. There is no inhibition of the enzyme resulting from the production of the acyl enzyme.
4. A second slower process occurs only in the absence of enzyme. This process is not observed in the presence of enzyme.
5. The rate of hydrolysis of the slower process is independent of enzyme concentration.
6. The rate of hydrolysis of the faster process is dependent on enzyme concentration.

Table 1. kcat/Km for 2b determined by different methods

| method        | buffer     | pH | kcat/Km | kcat/Km (end. dev.) |
|---------------|------------|----|---------|---------------------|
| spectrophotometric tri | 7.40 | 1.40 | 0.73 (0.54) |
| spectrophotometric phosphate | 7.60 | 1.60 | 2.42 (0.67) |
| titrimetric none | 5.74 | 1.50 | 5.54 (0.15) |

When a steady state can be confidently assumed, the kinetic analysis becomes straightforward. The equation for the hydrolysis of a racemic mixture can be quantitatively described as follows:

\[
\begin{align*}
A + L' & \rightarrow E + L' \rightarrow E - L \rightarrow E - L + S \rightarrow E + S
\end{align*}
\]

Application of the equilibrium assumption for formation of the Michaelis complexes 4- and 5- and the steady state assumption for the enzymic reaction leads to the rate law

\[
\begin{align*}
& \text{A} + \text{S} \rightarrow \text{E} + \text{S} \\
& \Rightarrow \text{A} + \text{S} \rightarrow \text{E} + \text{S}
\end{align*}
\]
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For the special case of $K' = K_o = K$, i.e., one atom completely unreactive but still capable of binding, the above expressions are indeterminate and must be replaced by

$$k_{cat}^{\prime} = \frac{k_{cat} \cdot K_o \cdot [S]}{K_o + [S]}$$

(1)

$$k_a = \frac{k_{cat} \cdot K_o \cdot [S]}{K_o + [S]}$$

(2)

$$k_{cat}/k_a = \frac{k_{cat}}{k_a}$$

(3)

For a simple pure substrate the parameters are

$$k_{cat}^{\prime} = \frac{k_{cat}}{L + \frac{k_{cat}}{k_a}}$$

$$k_a = \frac{k_{cat}}{L + \frac{k_{cat}}{k_a}}$$

$$k_{cat}/k_a = \frac{k_{cat}}{k_a}$$

From these equations and the data of Table 2, the following results are apparent:

1. In general, the value of $K_{cat}/K_a$ may only be decreased by the presence of a less-reactive substrate. The decrease tends toward a maximum factor (for a totally unreactive substrate) $K_{cat}/K_a = K_o$. This factor can be explained if there is a relatively greater concentration of less-reactive moiety (i.e., $k_{cat}/k_a$). This is solely in practice, since the leaving groups are identical. In other words, an irreversible reaction of $k_{cat}/k_a$ is observed. The large uncertainties in the basic region result from the increasingly significant hydroxide catalyzed reaction, whose rate must be independently determined and subtracted to yield the net enzyme catalyzed reaction, whose rate must be independently determined and subtracted to yield the net enzyme catalyzed reaction.

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The pH Dependence The pH dependence of the rate of electrophilic catalysis is shown in Figure 4. The large uncertainties in the basic region result from the increasingly significant hydroxide catalyzed reaction, whose rate must be independently determined and subtracted to yield the net enzyme catalyzed reaction. The control reactions in Figure 4, 4.41 and 4.59, the latter is of little quantitative significance because of the scatter in the pH region. In other words, a tightly binding substrate, reactive or not, the result is an apparently small Michaelis constant. For the control case, similar to those discussed for $K_{cat}$, lead to an estimate of $K_o$ and $K_{cat}/K_a$ for about twice the value measured for the cationic mixture.

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