Mechanism of Action of Serine Proteases: Tetrahedral Intermediate and Concerted Proton Transfer†

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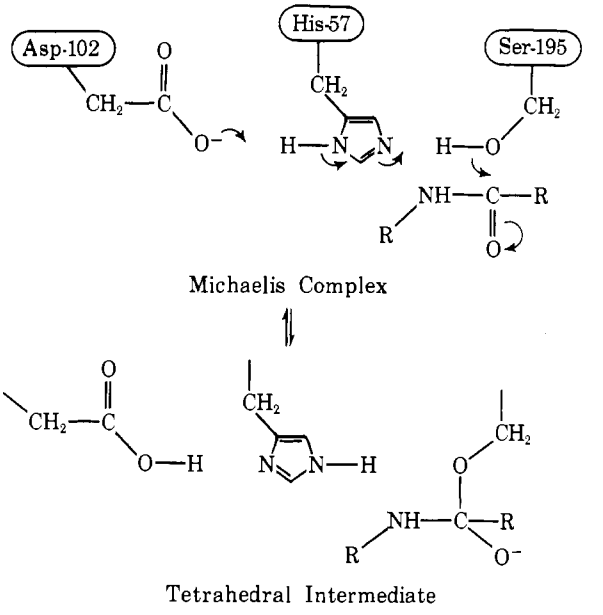
ABSTRACT: Stopped-flow spectrophotometry and proton inventory experiments have been used to define the reaction pathway for hydrolysis of a specific peptide substrate, Ac-L-Ala-L-Pro-L-Ala *p*-nitroanilide, by the serine proteases elastase and α-lytic protease. The stopped-flow studies reveal the existence and buildup of a tetrahedral adduct between the active site serine hydroxyl group and the sensitive carbonyl group of the substrate. The decomposition of this tetrahedral intermediate to the acyl enzyme and *p*-nitroaniline is the rate-limiting step for the hydrolytic reaction. The proton inventory data suggest the simultaneous transfer of two protons (presumably from the catalytic carboxyl of Asp-102 to N^{π} of the catalytic imidazole of His-57 and from N^{τ} of the imidazole to the anilide NH) in the transition state leading to breakdown of the tetrahedral complex. That these proton transfers occur in a concerted, rather than stepwise, process attests to the ability of enzymes to lower the enthalpy of activation most effectively when the precise alignment of a highly specific substrate and catalytic groups minimizes the entropy of activation.

The serine proteases are characterized by a precisely oriented catalytic triad of a carboxylate group of aspartic acid (Asp-102), an imidazole ring of histidine (His-57), and a hydroxyl group of serine (Ser-195) (Blow et al., 1969). These enzymes have maximal catalytic activity above pH 7 as protonation of a group in the catalytic triad with $pK_a \sim 6.7$ almost completely inactivates these enzymes. Carbon magnetic resonance studies (Hunkapiller et al., 1973, 1975) on the serine protease α-lytic protease, in which the C-2 carbon of the imidazole ring of the single histidine residue is enriched in ^{13}C, revealed that the C-2 carbon exhibits a significant change (2.4 ppm) in chemical shift around both pH 6.7 and 3.3, but exhibits a carbon–hydrogen spin–spin coupling constant characteristic of an imidazolium cation only below pH 3.8. This finding established the fact that this ring remains neutral on acquisition of a proton by the catalytic triad with pK_a 6.7 but that a nearby negative charge is neutralized. Accordingly, we concluded that the carboxylate anion of aspartic acid is the site of net acquisition of a proton. Based on this observation, we envisioned a catalytic mechanism that involves, during formation of the tetrahedral intermediate, the transfer of a proton from the serine hydroxyl group to the imidazole ring of histidine, concerted with the transfer of the proton from the other side of the imidazole ring to the carboxylate anion. Thus, the carboxylate anion of the aspartic acid residue in the catalytic triad is the ultimate base which accepts and stores the proton during nucleophilic addition of the serine hydroxyl group to the carbonyl group of the amide bond being hydrolyzed, the first species formed by this attack being a tetrahedral intermediate.

The existence of such a tetrahedral intermediate has been supported by x-ray diffraction studies of the binding of bovine pancreatic trypsin inhibitor (Ruhlman et al., 1973) and of soybean trypsin inhibitor (Blow et al., 1974) to trypsin, and, also, by kinetic studies on the hydrolysis of various synthetic substrates by chymotrypsin (Caplow, 1969; Fersht and Requena, 1971). The conclusion from these kinetic studies was that though some intermediate, presumably the tetrahedral

Scheme I



intermediate, does intervene between the ES complex and the acyl enzyme intermediate (to which the tetrahedral intermediate decomposes), the tetrahedral intermediate does not itself accumulate in observable quantities during the course of the enzymatic hydrolysis (Fastrez and Fersht, 1973).

In order to characterize more completely the sequence of dynamic events which occur during catalysis by serine proteases, we have sought (1) to establish the existence of the tetrahedral intermediate by direct observation and (2) to establish whether two protons are, in fact, transferred in the concerted fashion shown above. To this end, we have studied the hydrolysis of Ac-L-Ala-L-Pro-L-Ala *p*-nitroanilide to Ac-L-Ala-L-Pro-L-Ala and *p*-nitroaniline catalyzed both by α-lytic protease and by elastase. Three factors governed the choice of this particular substrate. (a) Hydrolysis can be fol-

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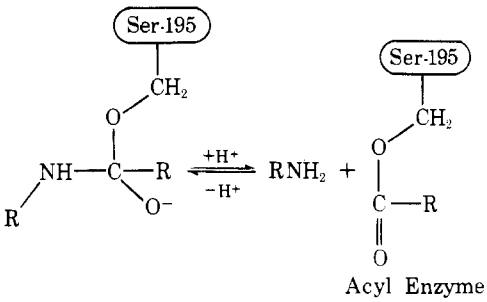
Scheme II



lowed conveniently and accurately by spectrophotometric observation of possible intermediates and released *p*-nitroaniline. (b) The strongly electron-withdrawing nature of the *p*-nitro group should stabilize any intermediate which still retains the *p*-nitroanilide residue and which bears a negative charge. (c) This particular tripeptide sequence, L-Ala-L-Pro-L-Ala, exhibits a strong, specific interaction with both elastase and α-lytic protease (Thompson, 1973; Hunkapiller et al., 1975). Extension of the peptide chain from the amino end of the hydrolytically sensitive alanyl group greatly enhances the interaction of the substrate with both enzymes, and placement of a prolyl residue adjacent to the sensitive residue virtually obviates vexatious nonproductive binding.

In order to search for the intervention of the tetrahedral intermediate prior to formation of the acyl enzyme, we used stopped-flow spectrophotometry. To determine the number of protons simultaneously in flight in the rate-determining transition state, we measured the catalytic turnover rate as a function of the mole fraction of deuterium in solvent water (Pollock et al., 1973).

The stopped-flow kinetic data establish both the existence and the buildup of a tetrahedral intermediate (to greater than 80% of the total enzyme with bound substrate) for the tripeptide anilide substrate and constitute the first direct spectrophotometric observation of such an intermediate during catalysis by serine proteases.

The kinetic isotope data reveal that $\sqrt{k_{cat}}$ (which for this substrate represents breakdown of the tetrahedral intermediate to the acyl enzyme and free *p*-nitroaniline) varies linearly with the mole fraction of deuterium in the solvent. This supports the view that two protons are being transferred simultaneously in the transition state for this reaction step, as outlined above. Such concerted transfers are, further, consistent with a tetrahedral intermediate in which the catalytic triad exists as a neutral carboxylic acid group and neutral imidazole ring (Hunkapiller et al., 1973, 1975).

Experimental Procedures

Deuterium oxide (99.8% ^2H) was obtained from Stohler Isotopes and distilled twice under argon in an all glass apparatus. NMR analysis showed no increase in ^1H content as a result of this purification which is absolutely essential if reliable experimental kinetic data are to be obtained. α-Lytic protease was prepared as described previously (Hunkapiller et al., 1973). Porcine elastase was prepared from Pancreatin (Sigma, grade III), as described by Shotton (1970).

Ac-L-Ala-L-Pro. Ac-L-Ala (18.6 g, 0.13 mol) and L-ProOMe (18.3 g, 0.13 mol) were dissolved in 300 ml of chloroform and cooled in an ice bath. Dicyclohexylcarbodiimide (32.1 g, 0.16 mol) was added, and the solution was stirred overnight at 4 °C. The solution was filtered, and the solvent was removed by rotary evaporation. The residue was extracted with 150 ml of warm water, and the extract was filtered and evaporated to dryness. The residue was dissolved in 300 ml of ethanol and 33 ml of 5 N NaOH, and the resulting solution was stirred for 2 h. The solution was adjusted to pH 1 by addition of 6 N HCl, and the solvent was removed by rotary evaporation. The residue was extracted with 500 ml of chloroform, and the solution was dried over Na_2SO_4, filtered, and evaporated to give a white solid, which was then crystallized from chloroform–*n*-hexane. Yield: 19.2 g (59%); mp 174–176 °C; lit. mp 171–175 °C (Thompson and Blout, 1973).

Ac-L-Ala-L-Pro-L-Ala p-Nitroanilide. Ac-L-Ala-L-Pro (4.6 g, 0.020 mol), L-Ala *p*-nitroanilide hydrochloride (Cyclo Chemicals, 5.0 g, 0.020 mol), and *N*-methylmorpholine (2.1 g, 0.020 mol) were dissolved in 50 ml of chloroform and cooled in an ice bath. Dicyclohexylcarbodiimide (4.6 g, 0.022 mol) was added, and the solution was stirred overnight at 4 °C. The solution was filtered, and the solvent was removed by rotary evaporation. The residue was extracted with 500 ml of 10% methanol, and the extract was stirred for 20 min with Rexyn I-300 (Fisher, 10–50 mesh). The ion-exchange resin was removed by filtration and the solvent was evaporated at 30 °C. The residue was dissolved in a minimum volume of chloroform and chromatographed in three portions on a 3 × 150 cm column of silica gel in chloroform–methanol (9:1). The fractions containing product were combined and the solvent was evaporated. The residue was dissolved in 40 ml of chloroform, treated with charcoal, and filtered. Ethyl ether (200 ml) was added and the product was crystallized overnight at room temperature. It was then recrystallized from methanol–water. Yield: 5.3 g (62%); mp 198–199 °C. Anal. Calcd: C, 54.41; H, 6.01; N, 16.70. Found: C, 54.42; H, 5.88; N, 16.72.

The product showed λ_{max} 315 nm, $\epsilon = 1.305 \times 10^4$ M^{-1} cm^{-1} in 4% methanol, 0.10 M Tris-HCl buffer (pH 8.75). Complete hydrolysis with α-lytic protease gave *p*-nitroaniline showing at pH 8.75 λ_{max} 381 nm, $\epsilon = 1.336 \times 10^4$ M^{-1} cm^{-1} based upon starting anilide. This indicates >99.7% optical purity based upon λ_{max} 381, $\epsilon = 1.340 \times 10^4$ M^{-1} for an authentic sample of *p*-nitroaniline.

Ac-L-Ala-L-Pro-L-AlaOMe. Ac-L-Ala-L-Pro (2.3 g, 0.010 mol) and L-AlaOMe (1.0 g, 0.010 mol) were dissolved in 30 ml of chloroform and cooled in an ice bath. Dicyclohexylcarbodiimide (2.3 g, 0.011 mol) was added, and the solution was stirred overnight at 4 °C. The solution was filtered, the filtrate was extracted four times with 100-ml portions of water, and the combined aqueous extracts were evaporated. The residue was crystallized from chloroform–ethyl ether. Yield: 2.8 g (89%); mp 156–157 °C. Anal. Calcd: C, 53.66; H, 7.40; N, 13.41. Found: C, 53.56; H, 7.29; N, 13.27.

Ac-L-Ala-L-Pro-L-Ala p-Nitrophenyl Ester. Ac-L-Ala-L-Pro-L-AlaOMe (0.94 g, 0.0030 mol) and α-lytic protease (2 mg) were dissolved in 20 ml of water. The solution was stirred and maintained at pH 8 by addition of 1 N NaOH until the pH held constant (about 30 min). The solution was titrated to pH 1 by addition of 1 N HCl and was extracted with four 100-ml portions of chloroform. The combined chloroform extracts were dried over Na_2SO_4, filtered, and concentrated to about 15 ml by rotary evaporation. *p*-Nitrophenol (0.54 g, 0.0030 mol) was added and the solution was cooled in an ice bath. Dicyclohexylcarbodiimide (0.68 g, 0.0033 mol) was added and the solution was stirred overnight at 4 °C. The solution was filtered and concentrated to about 5 ml by rotary evaporation. The product was chromatographed on a 3 × 100 cm column of silica gel with chloroform–methanol (9:1). The fractions containing product were combined, treated with charcoal, filtered, and concentrated to about 5 ml by rotary evaporation. Ethyl ether (50 ml) was added and the product

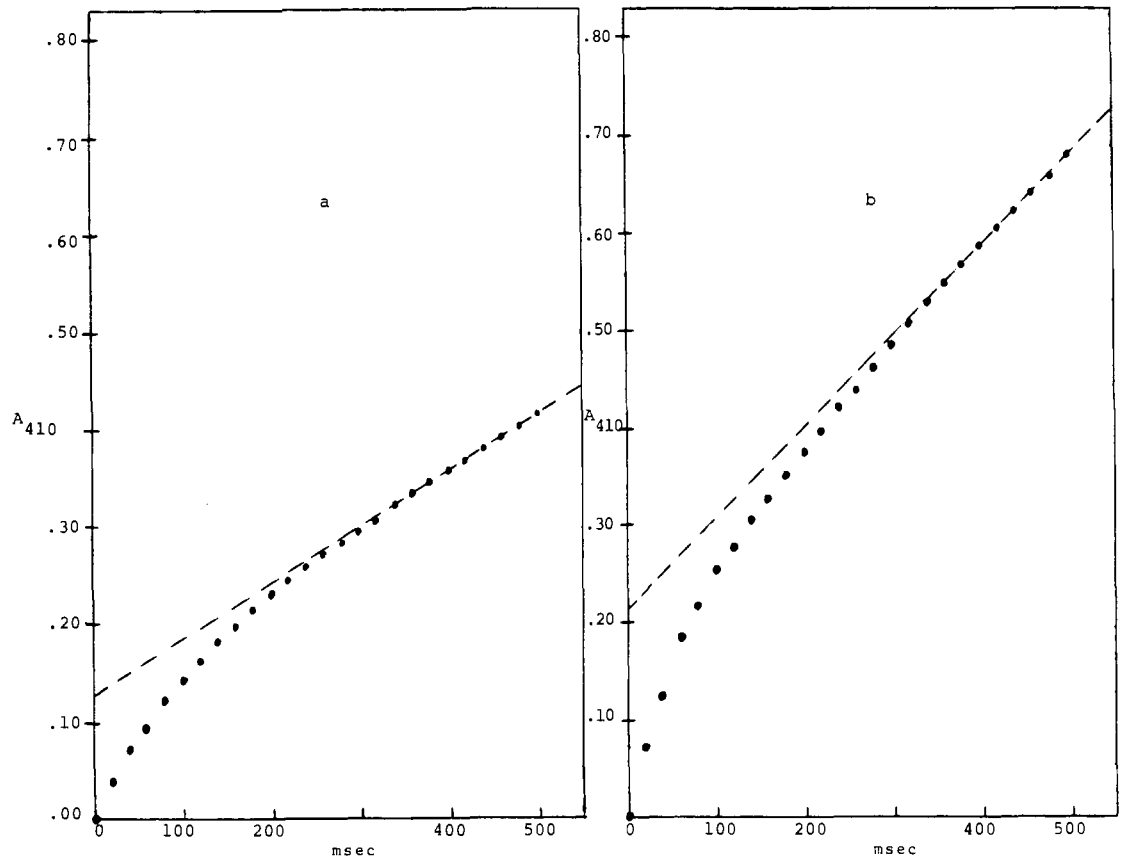


FIGURE 1: Increase in absorbance at 410 nm upon mixing elastase and Ac-L-Ala-L-Pro-L-Ala *p*-nitroanilide at 25 °C. $[S_0] = 1.50 \times 10^{-3}$ M. $[E_0] = 2.13 \times 10^{-5}$ M (a); $[E_0] = 3.49 \times 10^{-5}$ M (b).

crystallized overnight at room temperature. Yield: 0.84 g (67%); mp 175–178 °C.

Stopped-Flow Kinetics. Stopped-flow experiments were performed on a Durrum Model 110 stopped-flow spectrophotometer with a dead time of 5 ms. The syringe chamber and cuvette were thermostated at 25 °C. One syringe contained enzyme in 0.20 M Tris-HCl buffer (pH 8.75) and the other contained Ac-L-Ala-L-Pro-L-Ala *p*-nitroanilide in 8% methanol. Reaction progress was monitored at 410 nm ($\Delta\epsilon = 8.86 \times 10^3$ M^{-1} cm^{-1}).

Measurement of k_{cat} in D_2O–H_2O Mixtures. Samples of Ac-L-Ala-L-Pro-L-Ala *p*-nitroanilide were weighed into 5.00-ml graduated test tubes. Methanol or methanol-d_4 (0.20 ml) and 0.50 ml of 1.0 M Tris-HCl buffer (pH 8.75) in H_2O or D_2O were added to dissolve the substrate. The desired volume of D_2O was added and the sample was made up to 5.00 ml with H_2O. The substrate solution (2.00 ml) was transferred to a cuvette in a thermostated (25.00 ± 0.05 °C) cell compartment of a Beckman Acta C III spectrophotometer. The sample was stirred for 5 min to allow it to reach temperature equilibrium and 10.0 μl of enzyme solution was added. Stirring was continued for 30 s and then hydrolysis was monitored at 410 nm until about 2% of the substrate was hydrolyzed. Duplicate samples of each substrate solution were run and four to five substrate concentrations over a tenfold range around K_M were used for determination of k_{cat} at each D_2O concentration examined. k_{cat} was determined from standard Eadie plots of $[S_0]/v_0$ vs. $[S_0]$.

A single elastase solution and a single α-lytic protease solution were used for each set of experiments. The enzymes were dissolved in distilled water, filtered, and frozen in several aliquots. A fresh aliquot was thawed and used for each day's experiment.

Results

Stopped-Flow Kinetics. Figure 1 shows a typical result for the uv absorption at 410 nm vs. time for the hydrolysis at pH 8.75 of Ac-L-Ala-L-Pro-L-Ala *p*-nitroanilide catalyzed by porcine elastase. The biphasic nature of the curve is apparent. From a series of such observations one can extract two rate constants: ~17 s^{-1} for the initial rapid phase and 3 s^{-1} for the subsequent slower reaction. By extrapolation back to $t = 0$, one can calculate that the species formed in the initial rapid burst accounts for approximately 80% of the substrate bound to enzyme.

Stopped-flow data for the same reaction (hydrolysis at pH 8.75 of Ac-L-Ala-L-Pro-L-Ala *p*-nitroanilide) catalyzed by α-lytic protease are qualitatively similar to that for the elastase-catalyzed process in Figure 1. However, for the anilide hydrolysis catalyzed by α-lytic protease, the k_{cat} of 22 s^{-1} and K_M of 15 mM are sufficiently higher that stopped-flow kinetic studies are more difficult.

Kinetics of Ester and Anilide Hydrolysis. Table I collects k_{cat} and K_M values observed at pH 8.75 for hydrolysis catalyzed by elastase and α-lytic protease for the indicated substrates. For convenience, the k values for the initial burst are also included.

Solvent Kinetic Isotope Effects. Figure 2 plots the values of k_{cat} and $\sqrt{k_{cat}}$ for the α-lytic protease-catalyzed hydrolysis of Ac-L-Ala-L-Pro-L-Ala *p*-nitroanilide at pH meter reading of 8.75 in solutions of H_2O–D_2O with a mole fraction of deuterium (n) ranging from 0 to 1. The observed values of k_{cat} vary

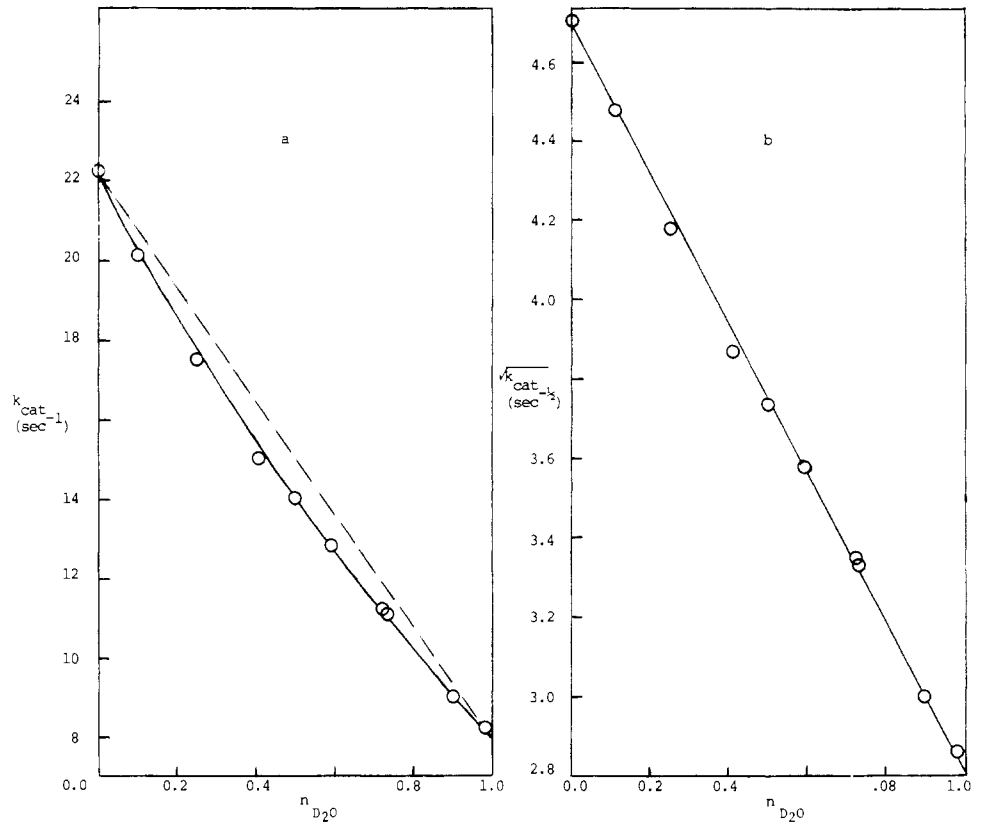


FIGURE 2: Dependence of k_{cat} and $\sqrt{k_{cat}}$ on mole fraction of solvent deuterium for α-lytic protease-catalyzed hydrolysis of Ac-L-Ala-L-Pro-L-Ala *p*-nitroanilide. (a) Curved line is theoretical line for $\phi_1^T = \phi_2^T = 0.60$, $k_H = 22.0\ s^{-1}$. (b) Solid line is linear, least-squares fit.

TABLE I.

Enzyme	Substrate	k_{cat} (s^{-1})	K_M	Initial Velocity (s^{-1})
α-Lytic protease	Ac-Ala-Pro-Ala *p*-nitroanilide	22	1.5×10^{-2} M	95
	Ac-Ala-Pro-Ala OMe	600	1×10^{-3} M	
	Ac-Ala-Pro-Ala *p*-nitrophenyl ester	600	1×10^{-3} M	
Porcine Elastase	Ac-Ala-Pro-Ala *p*-nitroanilide	2.8	1.6×10^{-3} M	17
	Ac-Ala-Pro-Ala OMe	300	1×10^{-4} M	
	Ac-Ala-Pro-Ala *p*-nitrophenyl ester	300	1×10^{-4} M	

nonlinearly as a function of n, but $\sqrt{k_{cat}}$ varies linearly with n. The average deviation per data point for a linear least-squares fit for k_{cat} vs. n is 6.8%, but for $\sqrt{k_{cat}}$ vs. n the average deviation is 0.8%. Duplicate determinations of k_{cat} at $n = 0.0$, 0.5, and 1.0 showed a reproducibility of 1.4%. Similar experiments (Figure 3) for the hydrolysis reaction catalyzed by porcine elastase also gave a linear dependence of $\sqrt{k_{cat}}$ vs. n (the average deviation per point from a linear least-squares fit was 1.4% compared with a deviation of 5.4% for k_{cat} vs. n). As noted above, the experiments were all performed in solutions of 0.10 M Tris-HCl (DCl) buffer with a pH *meter reading* of 8.75. As the value of k_{cat} is independent of pH in the range pH 8–11, the slight differences with deuterium content of the solvent between meter observed pH values and real pH values should not affect the reported values of k_{cat}.

Discussion

Tetrahedral Intermediate. The biphasic form of the stopped-flow kinetic data reveals the occurrence of two distinct events for which four possible interpretations should be considered.

(1) The steady-state (linear) rate could represent breakdown of the acyl enzyme, in which case the initial rapid increase in absorbance at 410 nm would reflect the rapid formation of the acyl enzyme with release of an equivalent amount of *p*-nitroaniline. This explanation seems to be eliminated by the other kinetic data collected in Table I. The acyl enzyme formed from all three substrates (Ac-L-Ala-L-Pro-L-Ala methyl ester, *p*-nitrophenyl ester, and *p*-nitroanilide) should be identical. The rate of breakdown of the acyl enzyme formed from these three substrates should, therefore, be identical. Accordingly, if the breakdown of the acyl enzyme is rate determining in all three cases, they should all show the same value of k_{cat}. They do not; the k_{cat} of 300 s^{-1} for the hydrolysis of the two esters (in which cases breakdown of the acyl enzyme is likely to be rate determining) exceeds by a factor of 10^2 the k_{cat} of 3 s^{-1} for the steady-state rate for the hydrolysis of the *p*-nitroanilide. Therefore, since deacylation has a rate of $\geq 300\ s^{-1}$, the observed value of k_{cat} for the hydrolysis of the *p*-nitroanilide must reflect an earlier, slower step.

(2) Just conceivably, the steady-state linear rate could represent a rate-limiting dissociation of *p*-nitroanilide from the enzyme following rapid formation of the acyl enzyme (in contrast to the two esters, in which cases the alcohols might

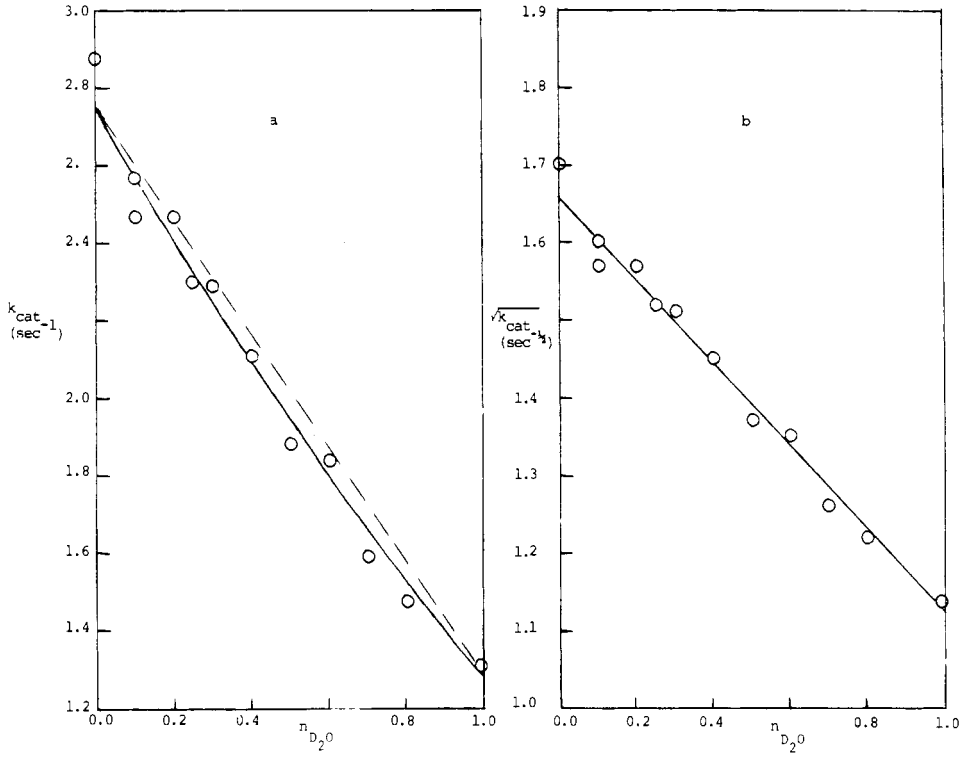


FIGURE 3: Dependence of k_{cat} and $\sqrt{k_{cat}}$ on mole fraction of solvent deuterium for elastase-catalyzed hydrolysis at Ac-L-Ala-L-Pro-L-Ala *p*-nitroanilide. (a) Curved line is theoretical line for $\phi_1^T = \phi_2^T = 0.68$, $k_H = 2.75$ s^{-1}. (b) Solid line is linear, least-squares fit.

dissociate much more rapidly from the enzyme). Two arguments can eliminate this possibility. First, the enzyme has little affinity for the nitrophenyl group; it does not measurably increase binding for the tripeptide derivatives (as compared to the methyl ester) and neither *p*-nitroaniline nor *p*-nitrophenol inhibit substrate hydrolysis. Secondly, no apparent reason exists for the release of *p*-nitrophenol to be faster by 10^2 than that for *p*-nitroaniline. Therefore, even if the observed k_{cat} for ester hydrolysis should represent rate-limiting dissociation of *p*-nitrophenol from the acyl enzyme–*p*-nitrophenol complex (a most unlikely situation in itself and one that has no precedence in the known dissociation behavior of serine protease substrate or inhibitor complexes (Smallcombe et al., 1972)), the value of k_{cat} observed for the hydrolysis of the *p*-nitroanilide derivative must reflect a slower and earlier step along the catalytic pathway.

(3) The initial rapid increase in absorbance could represent noncovalent binding of substrate to elastase, which causes a shift of more than 60 nm in the ultraviolet absorption spectrum of the anilide (possibly due to a conformational change induced in the substrate as a consequence of binding to the enzyme). Though inherently unlikely, this possibility can also be ruled out by consideration of the kinetic data for ester hydrolysis. The rate for anilide binding of the enzyme should approach the value (10^8 M^{-1} s^{-1}) for a diffusion controlled process (Smallcombe et al., 1972) and must, in any case, be at least as fast as 300 s^{-1} (the slowest rate observed in the hydrolysis of the *p*-nitrophenyl or methyl ester). Any process this fast would reach a steady-state condition in the dead time of the stopped-flow apparatus and would, therefore, appear as an instantaneous jump in absorbance at the initial time of absorbance monitoring. Thus, in the exceedingly improbable event that the value of k_{cat} of 300 s^{-1} reflects the binding of substrate by enzyme (the formation of the Michaelis complex), the initial rate ($k \sim 17$ s^{-1}) observed for anilide hydrolysis would have to represent a process that is slower, and later, than formation of the Michaelis complex. If substrate binding caused a conversion of a catalytically inactive conformation of the enzyme to an active form (Fersht and Requena, 1971), this should produce an exponential increase in the absorbance change, not the decrease observed in this work.

(4) The initial rapid increase in absorbance represents formation of a covalent, tetrahedral intermediate and the slower linear absorbance increase represents breakdown of this tetrahedral intermediate to acyl enzyme and free *p*-nitroaniline. The absorption spectrum of the tetrahedral intermediate should closely resemble that of unprotonated *p*-nitroaniline (λ_{max} 381 nm), while both are quite distinct from that of the substrate anilide (λ_{max} 315 nm) (Kallen and Jencks, 1966; Robinson, 1970). Therefore, any buildup of tetrahedral intermediate would appear as increased absorbance very near 381 nm. The breakdown of tetrahedral intermediate ($k \sim 3$ s^{-1}) to acyl enzyme and free *p*-nitroaniline is, thus, the slowest step in this sequence and is followed by the hydrolysis of the acyl enzyme at a rate which is 100 times faster ($k \sim 300$ s^{-1}).

Thus, we conclude that the hydrolysis of Ac-L-Ala-L-Pro-L-Ala *p*-nitroanilide by elastase proceeds through a tetrahedral intermediate whose rate of formation from the Michaelis complex is 17 s^{-1} and whose rate of decomposition to acyl enzyme and *p*-nitroaniline is 3 s^{-1}. Subsequent hydrolysis of acyl enzyme to enzyme and free acid has a first-order constant of 300 s^{-1}.

To our knowledge, this is the first direct observation during substrate hydrolysis of the tetrahedral intermediate whose intervention has previously been inferred from anomalous behavior of the pK_a's governing k_{cat} and K_M for several synthetic substrates of chymotrypsin (Caplow, 1969; Fersht and Requena, 1971). Extensive discussion of this evidence ensued (Renard and Fersht, 1973; Lucas et al., 1973; Fastrez and Fersht, 1973) with the conclusion that an intermediate between

the Michaelis complex and the acyl enzyme, presumably the tetrahedral intermediate, does exist but does not accumulate to an observable degree (less than 4%) during catalysis. Additional evidence from $^{14}N/^{15}N$ kinetic isotope effect observations of O'Leary and Kluetz (1970, 1972) in which the isotopic substitution was made in the nitrogen of the amide bond being hydrolyzed suggested that, if a tetrahedral intermediate does exist, its breakdown is rate limiting at alkaline pH (down to pH 7), while, in contrast, its formation is rate limiting at acidic pH. The clearest previous evidence for such a tetrahedral species has come from x-ray diffraction studies of the crystalline structure of enzyme–inhibitor complexes between trypsin and pancreatic trypsin inhibitor (Ruhlman et al., 1973) and between trypsin and soybean trypsin inhibitor (Blow et al., 1974). In those complexes, the covalent addition of the active site serine hydroxyl group to specific peptide carbonyl bonds of the inhibitors leads to a tetrahedral species (presumably an oxyanion) which is trapped, in these cases, by noncovalent interactions with the protein and steric restrictions on the movements necessary for its breakdown.

The experimental techniques of this work were essentially those of Fastrez and Fersht (1973) with Ac-L-Tyr *p*-acetylanilide and chymotrypsin, a system in which buildup of the intermediate was not detectable (less than 4% of the amount of substrate bound to enzyme). In the present case, the *p*-nitroanilide derivative should stabilize the oxyanion of the tetrahedral intermediate and, thereby, encourage its accumulation. (A change in the thermodynamic stability of the tetrahedral oxyanion relative to that of other forms of substrate bound to the enzyme by 3–4 kcal/mol would account for a change in concentration of the tetrahedral intermediate from <4 to 80% of the enzyme–substrate species present.) Further, we used a tripeptide derivative, which, by more closely resembling the true substrates of serine proteases (which are endopeptidases), may give results more closely reflecting the behavior of the enzymes in vivo. In this regard, further studies with leaving groups more natural than *p*-nitroaniline should be instructive.

Solvent Isotope Effect. Figures 2 and 3 show the dependence of k_{cat} for the hydrolysis of Ac-L-Ala-L-Pro-L-Ala *p*-nitroanilide catalyzed by α-lytic protease and by porcine elastase in solution with a mole fraction (n) of deuterium ranging from $n = 0$ to 1. The observed value of k_{cat} varies in a nonlinear fashion with n, but $\sqrt{k_{cat}}$ varies linearly with n.

The relationship between reaction velocity and deuterium content of mixed isotopic solvents has been described by Kresge (1964) and Gold (1969). In solvent containing a mole fraction of deuterium (n), the rate constant (k_n) is related to the rate (K_H) in pure water ($n = 0$) by isotopic fractionation factors of the reactant state (ϕ_j^R) and of the transition state (ϕ_i^T) (eq 1).

$$k_n = k_H [\Pi_i^{\nu}(1 - n + n\phi_i^T)]/[\Pi_j^{\nu}(1 - n + n\phi_j^R)] \quad (1)$$

Since exchangeable protons of proteins (except those of sulfhydryl groups) should have $\phi_j^R \sim 1$ (Showen, 1972), one can set the factors for the reactant state equal to 1 so that eq 1 reduces to eq 2.

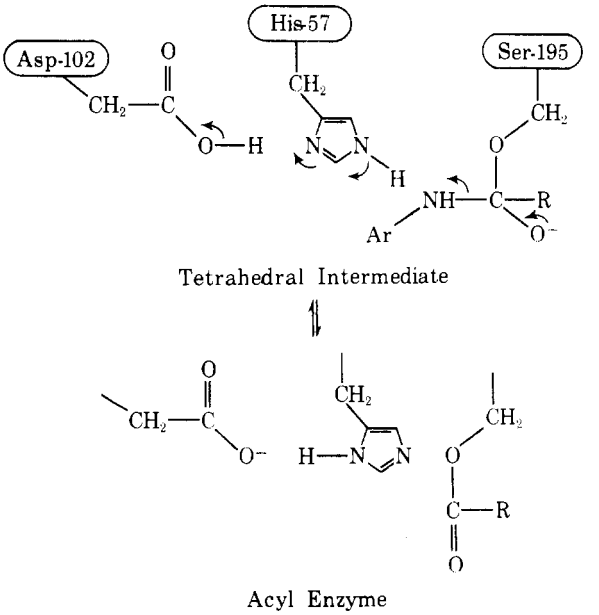
$$[k_n = k_H \Pi_i^{\nu}(1 - n + n\phi_i^T)] \quad (2)$$

The ratio k_n/k_H thus becomes a polynomial in n whose order, ν, specifies the number of protons moving in the transition state. The coefficients (ϕ_i^T) for the terms in the polynomial represent the kinetic isotope effect for each proton. Slight isotope effects for protons in the reactant state (i.e., $\phi_j^R < 1$) reduce the apparent order of the transition state contributions. Kresge (1974) has, in fact, postulated several instances in which reactions actually involving multiple proton transfers in the transition state could, nevertheless, produce a linear relationship between rate and mole fraction deuterium in the solvent. These situations could occur, because of the possibility that many sites might undergo a change in fractionation factor between initial state and transition state and the combination of these fractionation factors could obscure the curvature expected for a simple system with two protons moving in the transition state. As Kresge points out, the reverse situation could also presumably arise, in which one observed a nonlinear dependence with only a single proton actually moving in the transition state. However, this potential problem seems not to cause ambiguity in the case of the serine proteases because then one would not have expected to observe a linear dependence of reaction velocity on solvent deuterium content, which has characterized the results for most of the substrates examined to date (Pollock et al., 1973; Elrod et al., 1975). If only one proton is moving in the transition state ($\nu = 1$ in eq 2), then k_n should be a linear function of n; if two protons are simultaneously in flight in the transition state ($\nu = 2$), then $\sqrt{k_n}$ should be a linear function of n.

In the experiments reported above, $\sqrt{k_n}$ is shown to be linear in n, and this second-order dependence on n indicates the simultaneous movement of two protons in the transition state. Assuming perfect synchrony of movement and equal kinetic isotope effects for both protons ($\phi_i^T = \phi_2^T$), one calculates an isotope effect, $k_D/k_H = \phi^T = 0.60$ for each proton ($k_H/k_D = 1.67$) in the reaction catalyzed by α-lytic protease. Our data do not, of course, unambiguously require such perfect coupling, and dissimilar values of ϕ_1^T and ϕ_2^T are not ruled out. Nor is the possibility completely eliminated that *more* than two protons are simultaneously in movement, because the distinction between 2 and higher values for ν is less clear than the difference between $\nu = 1$ and 2. For example, the agreement between the calculated curve and the experimental points when $\nu = 3$ is 3.0%, compared with that of 6.8% for $\nu = 1$ and 0.8% for $\nu = 2$ in the reaction catalyzed by α-lytic protease.

This demonstration, that two protons move simultaneously in the breakdown of the tetrahedral intermediate to the acyl enzyme and free *p*-nitroaniline (a reaction step which is pre-

Scheme III



sumably microscopically analogous to the formation of the tetrahedral intermediate), confirms our earlier speculations about the dynamics of these processes, which were based on magnetic resonance studies of the ionization behavior of α-lytic protease as the free enzyme (Hunkapiller et al., 1973) and in the form of an enzyme–inhibitor complex that presumably mimics the tetrahedral intermediate (Hunkapiller et al., 1975). In these intermediates, both Asp-102 and His-57 are neutral, which leads to the suggestion that proton movement from N^{τ} of His-57 to NH of the anilide (concomitant with breaking of the C–N bond) is concerted with proton movement from the carboxyl group of Asp-102 to N^{π} of His-57. Such simultaneous movement of protons obviates the necessity for significant charge separation in the transition state whose formation, therefore, involves the transfer of the negative charge from the oxygen of the oxyanion to the carboxyl group of Asp-102.

Our experimental results contrast with those of Pollock et al. (1973) and Elrod et al. (1975) on hydrolysis catalyzed by serine proteases of less specific substrates, such as *p*-nitrophenyl acetate. They found that, in these cases, k_{cat} decreases linearly with the first power of the mole fraction of deuterium in the solvent, which indicates transfer of only a single proton in the rate-determining transition state. However, in the case of a tripeptide substrate (Bz-L-Phe-L-Val-L-Arg *p*-nitroanilide) specific for trypsin, they have recently observed linear dependence of $\sqrt{k_{cat}}$ on the mole fraction of deuterium (R. L. Showen, personal communication).

From these divergent observations, we conclude that the coupling of proton transfers (i.e., a concerted rather than a stepwise process) depends upon the nature of the fit between substrate and enzyme. Thus, extended peptide substrates, which have stereochemically precise interactions with these enzymes, exhibit concerted proton transfers. Less extended, less specific substrates have stereochemically less precise interactions with these enzymes and their hydrolysis is characterized by stepwise proton movements.

This difference in behavior may, with great sensitivity, reflect the stereochemistry of substrate–enzyme interaction. In fact, theoretical calculations suggest that shortening of hydrogen bond distances by as little as 0.25 Å can shift a reaction pathway involving two, stepwise proton transfers to a pathway involving a single, concerted transfer of two protons (Gandour et al., 1974; Elrod et al., 1975). The natural role for the serine proteases, such as trypsin, elastase, chymotrypsin, and α-lytic protease, is to act as endopeptidases catalyzing the hydrolysis of polypeptides. Accordingly, the alignment of substrate and catalytic groups is likely to be more nearly optimal for peptide derivatives than for derivatives of single amino acids. The very precise fit between trypsin and its polypeptide inhibitors convincingly illustrates this point.

Additional evidence that precise enzyme–substrate interactions favor concerted, as distinct from stepwise, proton transfers is provided by studies on the hydrolysis of asparagine or glutamine by asparaginase or glutaminase. In the cases of glutamine with glutaminase and asparagine with asparaginase, concerted transfers of two protons are observed. For glutamine with asparaginase and asparagine with glutaminase, stepwise proton transfers are observed (Elrod et al., 1975; R. L. Showen, personal communication).

Catalytic Mechanism. The finding that addition of a proton with a pk_a of 6.7 to the catalytic triad of the serine protease, α-lytic protease, generates a neutral Asp-102 and a neutral His-57 (Hunkapiller et al., 1973) led to a mechanistic proposal that the carboxylate anion of Asp-102 is actually the basic residue which stores the proton released when the serine hydroxyl group forms the tetrahedral oxyanion. The role of His-57 was suggested to be twofold: (1) to prevent the approach of water to the carboxylate anion of Asp-102, thereby localizing this group within an only slightly hydrophilic environment so that its pk_a is raised to the unusually high value of 6.7, which thereby causes it to be a more effective base than a normal aspartate side chain carboxyl group with a pk_a of 4–5, and (2) to act as a shuttle for net transfer of a proton from the serine hydroxyl group to the carboxylate anion of aspartate (by accepting a proton at N^{τ} while releasing a proton from N^{π}). If these proton transfer processes are concerted, the necessity for unfavorable charge separation in the hydrophobic interior of the enzyme–substrate complex is obviated and an enthalpically more favorable transfer of charge can occur. Such concertedness involves unfavorable entropic factors (Jencks, 1972) and is, therefore, rarely, if ever, encountered in reactions in solution or in model systems (Rogers and Bruice, 1974). In the enzymatic situation, in contrast, the prearranged precise alignments of catalytic residues and substrate can minimize these unfavorable entropic contributions to the activation energy for catalysis. Restricted mobility of the catalytic residue is apparent, for example, from the NMR relaxation parameters of the C-2 carbon of the imidazole ring of the histidine residue in the catalytic triad of α-lytic protease, both in the free enzyme (Hunkapiller et al., 1973) and an enzyme–inhibitor complex (Hunkapiller et al., 1975). If a specific substrate is anchored precisely with respect to the catalytic residues, which are themselves also precisely organized, concerted transfer of two protons with the attendant savings in enthalpy of activation can well provide the catalytic pathway of minimal overall activation energy.

Summary

The stopped-flow kinetic studies reported in this paper show that the rate-limiting step in the hydrolysis of a highly specific substrate (Ac-L-Ala-L-Pro-L-Ala *p*-nitroanilide) catalyzed by elastase or α-lytic protease is the decomposition of the tetrahedral intermediate (which has, furthermore, been shown to accumulate) to acyl enzyme and *p*-nitroaniline. The proton inventory experiments support the view that the two proton transfers which occur during this step take place in a concerted, not stepwise, manner. In contrast, when less specific substrates are cleaved (Elrod et al., 1975) the proton transfers may be uncoupled with a resultant loss in catalytic efficiency. Thus, only when both substrate and catalytic groups have been precisely aligned (as for enzymes in vitro catalyzing those reactions for which they are specific) does one observe the true catalytic mechanism with all of its potential features operational.

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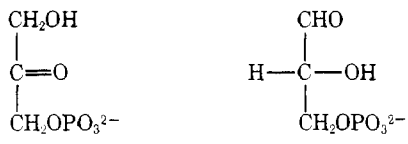
Deuterium and Tritium Exchange in Enzyme Kinetics†

W. John Albery and Jeremy R. Knowles*

ABSTRACT: The theory of the isotopic exchange of deuterium and tritium between an enzyme–substrate complex and the solvent is derived for 16 different types of experiment involving measurements of initial velocities and of the isotopic content of the reactants and products as a function of the extent of reaction. It is shown how the data from these experiments can be analyzed to obtain the rate constants for the individual steps and thereby the Gibbs free energies of the intermediates and transition states in the reaction. The effects of isotopic substitution on each intermediate and transition state are also found and this allows conclusions to be drawn as to the extent to which a proton is in flight in a particular transition state. Neither substrate handling (that is, on-off steps), nor the isotopic exchange with the solvent, is assumed to be rapid.

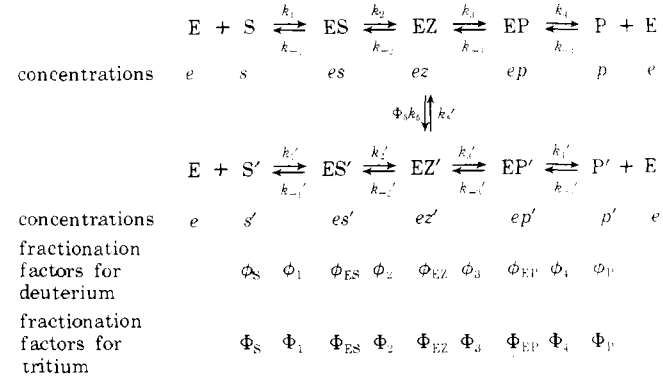
A very large number of enzyme-catalyzed reactions rely on acid–base catalysis for the movement of protons to and from the substrate(s) in the course of catalysis. In many of these, the substrate protons become partially or completely equilibrated with the solvent protons at an intermediate stage of the reaction. This paper presents the kinetic treatment for such reactions, in which the fate of a hydrogen isotope is followed when either the substrate or the solvent is isotopically labeled.

The present work arose from the investigation of the mechanistic details of the reaction catalyzed by the glycolytic enzyme triose phosphate isomerase. This reaction involves the interconversion of dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate:



The elegant work of Rose (1962) demonstrated that the triose phosphate isomerase reaction is a particular example of a class of enzyme reactions whose pathway is shown in Scheme I. This pathway is evidently followed by a large number of aldose-ketose isomerases and, with relatively minor modification,

SCHEME I: Pathway for a Reaction That Interconverts S and P, Catalyzed by an Enzyme E, in Which a Reaction Intermediate EZ Can Exchange Isotope with the Solvent.[a]



[a] The primes indicate isotopic substitution by either D or T; unprimed rate constants refer to the all-hydrogen system.

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