Rate-Controlling Step of Oxazolinone Formation. Secondary and Solvent Kinetic Isotope Effects

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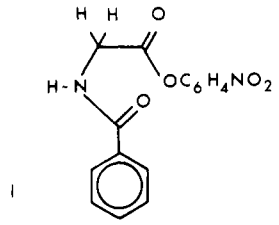
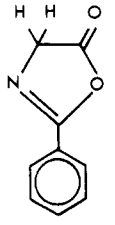
Abstract: The β-deuterium secondary kinetic isotope effect for the formation of 2-phenyloxazolin-5-one in the alkaline hydrolysis of *p*-nitrophenyl *N*-benzoylglycinate (p-$NO_2C_6H_4O_2CCL_2NHOCC_6H_5$, L = H or D) was determined to be k_H/k_D = 1.03 ± 0.02 at temperatures between 10.5 and 40.0 °C; activation parameters for the protium ester are ΔH^* = 21.7 ± 0.4 kcal mol^{-1} and ΔS^*_{298} = 31.9 ● 0.6 cal deg^{-1} mol^{-1}. These results, combined with those of previous studies, suggest that leaving-group expulsion is the only step contributing to rate limitation for conversion of the glycinate to the oxazolinone. The small, normal secondary isotope effect probably has complex origins, which may include relief of steric strain upon cyclization and hyperconjugative stabilization of the rate-controlling transition state. Apparent second-order rate constants for the formation of 2-phenyloxazolin-5-one in the alkaline hydrolysis of the isotopically unsubstituted ester were also obtained in aqueous solvent containing various mole fractions n of D_2O (k_n). The kinetic solvent isotope effects (KSIE's) are inverse at all values of n with $k_{1.0}/k_0$ = 1.75. Analysis of the bowl-shaped plot of $k_{1.0}/k_0$ vs. n indicates that fractionation of the reactant lyoxide ion in the mixed isotopic waters predominates the KSIE. A small, normal transition-state effect of about 1.3 makes the KSIE less inverse than would be expected if the entire effect originated in lyoxide fractionation.

Work by Williams[1] establishes three discrete steps in the formation of an oxazolinone intermediate during alkaline hydrolysis of *N*-benzoylglycine *p*-nitrophenyl ester (I): (a) abstraction of the α-amido N–H proton in a rapid preequilibrium step; (b) a second rapid preequilibrium involving addition of the oxygen of the α-amide anion to the ester carbonyl group, presumably to form a quasi-tetrahedral intermediate; (c) completely or partially rate-controlling breakdown of the intermediate to produce *p*-nitrophenolate anion and 2-phenyloxazolin-5-one (II).



I



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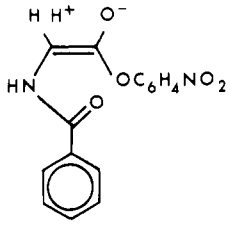

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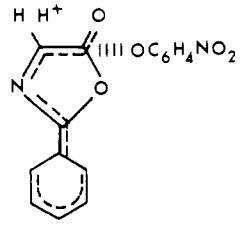

IV

In this paper, we report the β-deuterium secondary kinetic isotope effect (β-D KIE) and its temperature dependence for the formation of II from I. The β-D KIE is normal ($k_H/k_D > 1$), a rare occurrence in acyl-transfer reactions of activated esters. Our results further indicate that the transition-state (TS) properties revealed by kinetics-based probes are those for the TS of a single rate-controlling step and not of a weight-average, or "virtual" TS,[2,3] as would be the case if multiple parallel or serial steps contributed to rate limitation. Since the rate-controlling step for formation of II from I must involve solvent reorganization (SR), we also determined kinetic solvent isotope effects (KSIE's) in mixtures of H_2O and D_2O in hope of gaining additional insights about the structure of the TS.

Table I. β-D KIE on the Alkaline Hydrolysis of p-$NO_2C_6H_4O_2CCl_2NHCOC_6H_5$[a]

T,[b] °C	L	$10^{-3}k_L$,[c] M^{-1} s^{-1}	k_H/k_D
40.0	H	41.9 ± 0.4	1.03 ± 0.02
	D	40.9 ± 0.4	
25.0	H	7.00 ± 0.07	1.02 ± 0.02
	D	6.92 ± 0.07	
18.5	H	2.66 ± 0.08	1.03 ± 0.02
	D	2.58 ± 0.08	
10.5	H	0.855 ± 0.010	1.03 ± 0.01
	D	0.838 ± 0.010	

[a] In aqueous 0.025 M phosphate (pH 7.56, 25 and 40 °C) or borate (pH 8.90, 10.5 and 18.5 °C) buffers containing 3.2% acetonitrile, ionic strength 1.0 M (KCl); reaction progress was monitored by following the appearance of *p*-nitrophenolate anion at 400 nm. [b] Precision ±0.1 °C. [c] Apparent second-order rate constants k_L are the pseudo-first-order rate constants k_{obsd} divided by a_{OH}. The H and D esters were run in alternation: k_L is the mean and one standard deviation for six to eight determinations at the specified temperature; k_H/k_D is the mean and one standard deviation of the isotope effect for the six to eight H/D *pairs*.

Results and Discussion

Table I reports the apparent second-order rate constants at four temperatures for the alkaline hydrolysis of the isotopic versions of I. The rate constants at 25 °C are in good agreement with those previously determined at similar conditions.[1] The mean β-D KIE for the entire temperature range is 1.03 ± 0.02.

In a reaction consisting of one elementary step, perturbations of the experimental rate constant by changes in environmental factors such as temperature, or by isotopic substitution, can yield information about the structure of the TS for that step.[2] The experimental second-order rate constant for formation of II from I is a complex mixture of rate and equilibrium constants.[1] This is not necessarily an insurmountable impediment to obtaining TS information. The information may be obtained as properties of a "virtual" TS, whose structure is the weight average of multiple, partially rate-controlling TS's.[2,3] The extraction and interpretation of TS information from the experimental rate constant is usually simpler if a single step is completely rate-controlling, however. The temperature independence of the β-D KIE for the alkaline hydrolysis of I, and the fact that the hydrolysis rate is proportional to hydroxide ion activity over a 1 000 000-fold range,[1] indicates that a single elementary step is rate-controlling at all experimental temperatures.[3-5] This step must be leaving-group expulsion, since

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substituted aryl esters of *N*-benzoylglycine exhibit a Brønsted slope of −0.8 in the hydrolysis reaction.[1] The rate-controlling attack of the oxygen of an α-amide anion on ester carbonyl would be expected to give a smaller Brønsted slope, similar to the −0.23 obtained in the intermolecular attack of hydroxide ion on substituted phenyl acetates.[6] Although β-D KIE's are expected to increase with increasing temperature,[5] the effect of temperature is small, and the data of Table I are too imprecise to detect any trend.

The special case of a multistep reaction in which a single elementary step is rate-controlling may be treated as a single-step reaction; the observed rate constant is informative of the differences between the reactant state (RS) and the TS for the rate-controlling step.[2] The foregoing considerations justify the determination of activation parameters from the rate coefficients at the various temperatures by means of Eyring plots. For the protium ester, the linear plot (correlation coefficient 0.9995, *F* ratio 16043) gives $\Delta H^* = 21.7 \pm 0.4$ kcal mol^{-1}, $\Delta S^*_{298} = 31.9 \pm 0.6$ cal deg^{-1} mol^{-1}, and $\Delta G^*_{298} = 12.2 \pm 0.1$ kcal mol^{-1}.

β-D KIE's on acyl-transfer reactions are thought to originate from differences in delocalization into the ester carbonyl group of the electron density of the β-C–L (L = H or D) σ bonds in the RS and TS.[4,5,7] In valence-bond language, this delocalization corresponds to hyperconjugation.[8] When the rate-limiting step in the alkaline hydrolysis of an activated ester is the addition of hydroxide ion to the ester carbonyl group, a small, inverse ($k_H/k_D < 1$) β-D KIE is predicted; the hyperconjugative stability of the RS declines at the TS because some rehybridization of the carbonyl group from sp^2 to sp^3 has occurred.[4,5,7] These predictions are usually realized;[4,7,9] the maximum β-D KIE, for a TS closely resembling a tetrahedral intermediate, is estimated to be 0.91/2D.[4,9] Similarly, the rate-controlling collapse of a quasi-tetrahedral intermediate should produce an inverse β-D KIE.

Although unusual, the normal β-D KIE described here is not unique. Gandour et al.[10] have reported $k_H/k_D = 1.035$ for the intramolecular nucleophilic carboxylate catalysis of *p*-bromophenyl succinate and *p*-bromophenyl succinate-d_4, another reaction in which leaving-group expulsion is rate-controlling.[10,11] They attributed the normal isotope effect primarily to the relief of ground-state steric interactions in driving ring closure to a late TS resembling a *p*-bromophenoxide ion and succinic anhydride. The β-D KIE for the formation of oxazolinone II could reflect normal contributions of this kind. In addition, the hypothetical delocalized TS IV presents more opportunity for hyperconjugative stabilization than the RS (I and III). Our results are consistent with a maximum normal contribution of 1.03/0.91 = 1.13 to the β-D KIE for a TS resembling a tetrahedral-like intermediate precursor of the oxazolinone; the minimum contribution, for a TS resembling the oxazolinone and *p*-nitrophenoxide, is 1.03/1.00 = 1.03.

Information about SR processes is available in principle from KSIE's obtained in mixtures of H_2O and D_2O ("proton inventories").[12] The Gross–Butler equation describes the perturbation of an elementary rate coefficient measured in light water, k_0, by inclusion of a mole fraction *n* of D_2O in the solvent (eq 1).

$$k_n = k_0 \prod(1 - n + \phi_j^T n) / \prod(1 - n + \phi_i^R n) \qquad (1)$$

The parameters ϕ_i^R and ϕ_j^T are isotopic fractionation factors for the *i*th RS and *j*th TS hydrogenic sites, respectively. The magnitudes of KSIE's at various values of *n*, hence the shapes of proton inventory curves, depend on the number and relative importance of hydrogenic interactions at RS and TS sites.[12] Small, nonspecific interactions ("generalized SR") at a large number of RS or TS sites ("infinite sites"), or both, may be expressed as a parameter Z^n, where *Z* is the aggregate of fractionation factors for the individual sites.[12c] The *Z*-sites contribution to the KSIE reflects the net change in generalized SR in going from the RS to the rate-controlling TS and is appropriately entered in the numerator of the Gross–Butler equattion.[12c]

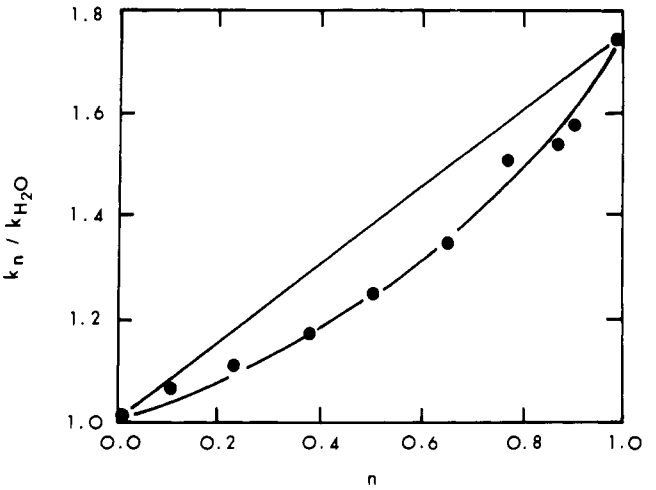

Figure 1. Proton inventory for the alkaline hydrolysis of *p*-nitrophenyl *N*-benzoylglycinate at 25 °C. Reaction mixtures contained 0.10 M phosphate buffer in L_2O of total ionic strength 1.0 M (KCl), 50 μM ester, and 3.2% acetonitrile. Apparent second-order rate constants k_n and k_0 are the pseudo-first-order constants divided by OL^- activity. The pH of the protium oxide data point was 7.56. The curve is for the relationship $k_n/k_0 = (0.75)^n/(1 - n + 1.25n)(1 - n + 0.70n)^3$ (see text).

Figure 1 shows the proton inventory for the alkaline hydrolysis of I. One standard deviation of the KSIE at each value of *n* is within ±2% of the average value represented in the figure. The overall KSIE is inverse, and the data points bow downward from a straight line drawn between the points for nearly pure H_2O and D_2O.

Because the formation of oxazolinone in the hydrolysis of I appears to involve a single rate-controlling kinetic step,[1] potential complications[12] of the incursion of parallel or serial TS's may be ignored in seeking the origins of the KSIE. We first considered that the KSIE might result entirely from fractionation of the RS lyoxide ion in L_2O. Published data[13] indicate that in aqueous solution, lyoxide exists as the trisolvated ion $[L_bOL_a]_3OL_c^-$ ($\phi_a = 0.70$, $\phi_b = 1.00$, and $\phi_c = 1.25$, overall $\phi_{OL} = 0.43$). The experimental $k_{1.0}/k_0 = 1.75$ would require an overall ϕ_{OL} of 1/1.75 = 0.57 if all the KSIE originated in lyoxide ion fractionation. Although the error in ϕ_{OL} is uncertain, it is unlikely of sufficient magnitude to justify the contention that lyoxide fractionation is the only source of the KSIE. The ratio 0.43/0.57 = 0.75 provides an estimate of the aggregate TS contribution to $k_{1.0}/k_0$. Thus, the experimental $k_{1.0}/k_0$ corresponds to an inverse RS effect of 1/.43 = 2.33, partially compensated by a small, normal TS effect of 0.75 (1/1.33).

Consideration of the effect of simple TS solvation models on the midpoint KSIE ($k_{0.5}/k_0$) indicates that a more detailed picture of TS solvation cannot be obtained from the proton inventory curve. The midpoint KSIE was selected since it usually exhibits the largest departure from linearity in a proton inventory curve.[12a]

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For the simple case of a TS involving reorganization of one or more equivalent protons, the TS contribution to the midpoint KSIE may be represented by two extremes: the linear single-site ($1 - n + 0.75n$) and exponential infinite-site (0.75^n) models. With these TS models and the RS lyoxide fractionation factors formulated as previously mentioned, the midpoint KSIE calculated from eq 1 declines only from 1.27 to 1.25 as the number of hypothetical TS hydrogenic sites increases from one to infinity (experimental $k_{0.5}/k_0 = 1.25$). Even without taking into account experimental errors or uncertainty in the fractionation factors of the lyoxide ion, the single-site and infinite-site models are essentially indistinguishable. The same conclusion is reached by utilizing all the k_n/k_0 vs. n pairs of data points. With the experimental values of k_n/k_0, n, and RS lyoxide ion fractionation factors specified, a curve-fitting procedure[14] was used to arrive at the TS fractionation factor giving the best least-squares fit of the data to the single-site and infinite-sites models. For both models, this value is 0.75 ± 0.01. Thus, the expanded data set is not helpful in clarifying the nature of the TS contribution to the KSIE. The reasonably good match between the theoretical curves and the experimental data, illustrated in Figure 1 for the infinite-sites model, does suggest that correct formulations for RS and TS contributions to the KSIE are not significantly more complex than those considered here.

Certainly most of the KSIE originates from changes in fractionation of the RS lyoxide ion and its specifically interacting L_2O during activation to the rate-controlling step. The large, positive ΔS^*, which undoubtedly is predominated by SR,[15] suggests a substantial decrease in solvation upon activation to the rate-controlling step. A large part of this entropy change probably results from SR associated with the initial proton abstraction; the equilibrium ΔS for autoprotolysis of water, an analogous process in reverse, is near −20 eu.[16] Whatever is the source of the small, normal TS contribution to the KSIE, we are confident that it does not represent residual fractionation of the RS lyoxide ion and its solvating L_2O. Because the proton abstraction that initiates the reaction precedes the rate-controlling step, the base and its specifically bound L_2O will have been transformed to bulk water ($\phi_w = 1.0$)[12] before this step occurs.

Conclusions. The normal β-D KIE associated with the formation of oxazolinone in the alkaline hydrolysis of I is consistent with a TS in which cyclization of the acyl moiety to a tetrahedral-like intermediate has occurred. In conjunction with the large leaving-group dependence of oxazolinone formation in activated esters,[1] the temperature independence of the β-D KIE indicates that expulsion of *p*-nitrophenoxide from a tetrahedral-like intermediate is completely rate-controlling at ordinary temperatures. The inverse effect generated by transformation of the solvated RS lyoxide ion to bulk L_2O in the course of activation to the rate-controlling TS predominates the KSIE but is partially offset by a small, normal TS effect of undetermined origin.

Experimental Section

Synthesis. To a solution of 1.79 g (1.0 mmol) of *N*-benzoylglycine and 1.39 g (1.0 mmol) of *p*-nitrophenol in 25 mL of $CHCl_3$ at 10 °C was added 2.06 g (1.0 mmol) of dicyclohexylcarbodiimde. The mixture was stirred 3 h, filtered to remove dicyclohexyl urea, and the solvent was evaporated at reduced pressure. Three recrystallization of the residue from ethyl acetate–hexane gave 2.03 g, 64%, of *p*-nitrophenyl *N*-benzoylglycinate, mp 169–170 °C [lit.[1] mp 170 °C].

The synthesis of the C_α-dideuterated ester was designed to circumvent intermediate formation of oxazolinone and to minimize exposure to protic solvents, thereby minimizing loss of deuterium. To 2.25 g (0.030 mol) of glycine-*2,2-d*$_2$ (98 atom % D, KOR Isotopes, Inc.) and 4.55 g (0.045 mol) of triethylamine (TEA) in a mixture of water (20 mL) and dioxane (20 mL) was added 7.39 g (0.030 mol) of 2-([*tert*-butoxycarbonyl)-oxy]imino)-2-phenylacetonitrile (Pierce Chemical Co.). The reaction mixture was stirred at room temperature for 2 h, and 50 mL of water was added. The aqueous layer was extracted with 60 mL of ethyl acetate, acidified with 5% citric acid solution, and extracted with ethyl acetate. The residue obtained upon evaporation of the dried ($MgSO_4$) organic layer was recrystallized from ethyl acetate–hexane to give 4.6 g, 87%, of *N*-*tert*-butyoxycarbonyl)glycine-*2,2-d*$_2$, mp 89.5–90.0 °C [protium lit.[17] mp 90 °C]. This product was converted to the *p*-nitrophenyl ester as described above for the *N*-benzoylglycinate; the *p*-nitrophenyl *N*-(*tert*-butoxycarbonyl)glycinate-*2,2-d*$_2$, recrystallized from ethyl acetate–pentane, had mp 69 °C [protium lit.[17] mp 70 °C]. The N-protecting group was removed by 15-min exposure to 4 M HCl in dioxane to give *p*-nitrophenyl glycinate-*2,2-d*$_2$ as the solid hydrochloride. To the hydrochloride, 2.32 g (1.0 mmol) suspended in $CHCl_3$ at 10 °C, was added 1.41 g of benzoyl chloride (1.0 mmol), followed by dropwise addition of 2.0 g (2.0 mmol) of TEA. The mixture was stirred for 2 h and filtered, and the solution was evaporated at reduced pressure. Recrystallization of the residue from ethyl acetate–hexane gave 2.11 g, 70%, of *p*-nitrophenyl *N*-benzoylglycinate-*2,2-d*$_2$, mp 169 °C; the protium content of the glycinate methylene was <3% by ^1H NMR. Each *p*-nitrophenyl ester released the theoretical amount of *p*-nitrophenoxide ion upon complete alkaline hydrolysis.

Solutions. Buffer ingredients were analytical grade materials from several sources. Deionized water was brought to a resistivity of 10 MΩ by passage through the activated carbon and deionizing cartridges of a Continental Water System. Deuterium oxide (Norrell, 99.8 atom % D) was distilled through a glass apparatus before use. For convenience, the mole fraction n of D_2O in solutions of this solvent is designated 1.0 in the text.

Mixtures of H_2O–D_2O (L_2O) containing the desired mole fraction n of D_2O were prepared volumetrically, taking into account the difference in density at 25 °C between H_2O (d = 0.997 g/cm^3) and D_2O (d = 1.1044 g/cm^3).[18] Accurately weighed amounts of a buffer pair in the ratio needed to give a pL near the desired value, plus the amount of KCl necessary to give a final ionic strength of 1.0 M, were diluted to volume with L_2O. Isotopic dilution of the L_2O solutions by protons released from the buffer salts was considered in calculation of the final value of n. The measured pH of the final solutions was determined to within ±0.01 unit on a Orion Model 701 A digital pH meter equipped with a Ross combination electrode. The difference between pL and pH (meter reading), determined from a series of meter readings for solutions of 1 mM LCl in 1 M KCl, was found to conform within 0.03 unit at all values of n to the equation

$$\text{pL} = \text{pH(meter reading)} + 0.076n^2 + 0.3314n$$

derived by Salomaa et al.[19] for H_2O–D_2O mixtures 1 mM and 0.1 M in LCl and 0.01 M in $LiClO_4$. The pH(meter reading) of the buffer solutions was converted to pL by use of this equation. The dependence of the ion-product constant of water on n was computed from the equation

$$K_n = K_0(1 - n + 0.69n)^3(1 - n + 1.25n)(1 - n + 0.70n)^3$$

This equation accounts for the effects on water autoprtolysis in H_2O–D_2O mixtures of the isotopic fractionation of the three protons of H_3O^+ (0.69/H),[12e] the proton of hydroxide ion (1.25), and one proton of each of three water molecules specifically solvating the hydroxide ion (0.70/H).[13] The pK_0 at 25 °C was taken as 13.753,[12c] and a_{OL} was calculated as K_n/a_{L_3O}.

In temperature dependence studies, the pH values of solutions were measured at the temperatures at which the kinetics experiments were conducted. The value of K_0 at each experimental temperature was determined from interpolation of a published compilation.[12c]

Kinetics. Kinetics experiments were carried out by addition of 0.1 mL of ester dissolved in acetonitrile to 3.0 mL of buffer in a cuvette in the thermostated cell compartment of a Varian-Cary Model 219 spectrophotometer. The absorbance increase owing to production of *p*-nitrophenolate ion was recorded at 400 nm. In preliminary studies, plots of log ($A_\infty - A_t$) vs, time[20] yielded straight lines through >90% reaction. For the experiments reported here, a Bascom-Turner Model 4120 Data Center was used to acquire and store evenly spaced voltages related to the absorbance increases through 5–7 half-lives. The stored data were treated according to the Guggenheim method[20] to obtain pseudo-first-

(14) Curve fitting of models to the KSIE data employed a computerized least-squares iterative fit of the data by the Marquadt–Levenberg method (BBN Research Systems, Cambridge, MA).

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order rate constants k_{obsd}, which were divided by a_{OL} to produce the apparent second-order rate constants. The Guggenheim treatment utilized data from at least three half-lives; the constant difference between readings taken at a series of times and a series of times later was selected to be approximately two half-lives.

Determinations of the β-D KIE's utilized the same stock buffer, and the labeled and unlabeled esters were run in alternation; 6–8 H/D pairs of rate constants were obtained at each temperature. For the proton inventory, each determination of k_n was followed by determination of a k_0. Experiments for a proton inventory curve were completed within a 10-h period. The results from three inventories were averaged to give the final isotope effects.

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Transition-State Properties for the Association of α-1-Protease Inhibitor with Porcine Pancreatic Elastase

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Abstract: The proton inventory (rate measurements in mixtures of H_2O and D_2O) was determined for the association of human α-1-protease inhibitor (αPI) with porcine pancreatic elastase (PPE). The overall solvent deuterium isotope effect [$^D(k_E)$] is 0.91 ± 0.06. As simpler models of this reaction, the proton inventories were also determined for the reaction of PPE with Suc-$(Ala)_3$-pNA [$^D(k_E)$ = 2.02 ± 0.09] and human leukocyte elastase (HLE) with MeOSuc-Ala-Ala-Pro-Val-pNA [$^D(k_E)$ = 1.51 ± 0.04]. For all three reactions, the general shape of the proton inventory was dome-shaped, and, in fact, for the association of αPI and PPE, the curve displayed a maxima. These curves could be described by the single expression

$$k_{E,n}/k_{E,0} = Z^n\left[\frac{C_1}{\Phi_1{}^n} + \frac{C_2}{(1 - n + n\phi_2)^2}\right]^{-1}$$

where n is the mole fraction solvent D_2O, Z is a composite fractionation factor corresponding to solvent reorganization and general "medium effects", C_1 and C_2 are the contributions made by each of the two partially rate-limiting transition states of k_E, a physical step (C_1), and the chemical steps of acylation (C_2), Φ_1 is a composite fractionation factor corresponding to the transition state for the physical step, and ϕ_2 is one of two identical fractionation factors corresponding to the two exchangeable protonic sites of the charge-relay system. These experiments suggest that the association of αPI and PPE proceeds through a transition state that derives its stability in part from some sort of protolytic catalysis and may therefore be at least partially rate-limited by acylation. In addition, these results point out the general importance of solvent reorganization in associative processes of enzymic reactions.

α-1-Protease inhibitor[1] is a glycoprotein of molecular weight near 53 000 that inhibits, with varying degrees of potency, all serine proteases. The minimal kinetic mechanism that can describe reactions of αPI[2] and proteases is in Scheme I. According to this view, the rapid association of protease and inhibitor (k_1 = 10^5–10^7 M^{-1} s^{-1}) produces a stable complex (E:I) that is resistant to both dissociation (k_{-1} = 10^{-4}–10^{-6} s^{-1}) and hydrolysis (k_2 = 10^{-5}–10^{-7} s^{-1}). Interaction of the two molecules involves the enzyme's active site and a portion of the inhibitor known as the "reactive center" and results in a single, specific cleavage at Met 358 of the inhibitor. The products of the hydrolytic decomposition of E:I are free, active enzyme, inactivated inhibitor (I'), and a small polypeptide.

The central feature of the mechanism depicted in Scheme I is E:I.[3] Considering its great stability, this complex is of considerable interest. Likely candidates for E:I are the various intermediates that normally occur during protease-catalyzed acyl-transfer reactions.[1b] These species include tetrahedral intermediates (TI),

Scheme I

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E{:}I \xrightarrow{k_2} E + I' + \text{peptide}$$

resulting from attack of the active site serine on the carbonyl carbon of the targeted Met residue, acyl-enzymes, formed upon collapse of tetrahedral addition adducts, and possibly even simple noncovalent complexes, if they can be sufficiently stabilized by van der Waals interactions, hydrogen bonds, and salt bridges. A goal of protease chemistry is a structural elucidation of E:I and an understanding of the origin of this intermediate's stability. An experimental strategy for addressing the latter problem emerges if we make the assumption that interactions stabilizing E:I are first brought to bear in the transition state for its formation. Thus, studying the transition state of k_1 could lead to an understanding of how E:I is stabilized and possibly the structure of this intermediate. To this end, kinetic studies were conducted for the association[4] of αPI and the protease, porcine pancreatic elastase. Also, to serve as models of this process, kinetics were determined

(1) (a) Travis, J.; Salvesen, G. S. *Annu. Rev. Biochem.* **1983**, *52*, 655-709. (b) Laskowski, M.; Ikunoshin, K. *Annu. Rev. Biochem.* **1980**, *49*, 593-626.

(2) Abbreviations: αPI, human plasma α-1-protease inhibitor; PPE, porcine pancreatic elastase; HLE, human leukocyte elastase; Suc, *N*-succinyl; MeOSuc, *N*-methoxysuccinyl, pNA, *p*-nitroanilide.

(3) It is likely that the interaction of α-1-protease inhibitor with serine proteases proceeds through the intermediacy of complexes other than just E:I. These would include loose Michaelis-type complexes and possibly a post-hydrolysis complex of protease and inactive inhibitor. At the present time, however, there is little evidence to support the existence of such intermediates.

(4) Throughout this paper, terms such as "association reactions" or "associative processes" refer to second-order reactions of enzyme with substrate or αPI. These processes are governed by the rate constants k_c/K_m or k_1 (see Scheme I), respectively, and reflect the energy difference between the reactants free in solution and the transition state of highest energy preceding the first irreversibly formed intermediate. Binding of substrate or inhibitor to the protease, conformational changes of initially formed encounter complexes, and enzyme acylation will all make contributions, with varying degrees of importance, to the rate determination of these associative reactions.

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