Correlative Variations in Enzyme-Derived and Substrate-Derived Structures of Catalytic Transition States. Implications for the Catalytic Strategy of Acyl-Transfer Enzymes[1]

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Abstract: Acetylchymotrypsin, acetyl elastase and (carbobenzyloxy)glycyl elastase all undergo hydrolysis with the same overall solvent isotope effect, which arises from a single protonic site [$k_n/k_1 = 2.45(1 - n + n/2.45)$]. [(Carbobenzyloxy)glycyl]-chymotrypsin, however, shows a larger effect arising from at least two sites [$k_n/k_1 = 3.34(1 - n + n/1.85)^2$]. Formylchymotrypsin and acetylchymotrypsin undergo deacylation with α-deuterium and β-deuterium secondary isotope effects, respectively, that suggest fractional tetrahedral character at the transition state of about 0.44 (vs. 0.58–0.66 for similar nonenzymic reactions) when compared to equilibrium isotope effects for complete addition. The effect for acetyl elastase suggests much less tetrahedral character (0.27). Addition of an *N*-acyl function leads to a more inverse isotope effect, per deuterium, and thus to an apparent increase in tetrahedral character: to 0.84 for [(carbobenzyloxy)glycyl]chymotrypsin; to 0.43 for (carbobenzyloxy)glycyl elastase. It is concluded that enzyme–substrate interactions at the transition state can alter both enzyme structure, as shown by the solvent isotope effects, and substrate structure, as shown by the substrate isotope effects. Such alterations, in the combination of enzyme with natural substrate, probably adjust both structures for optimal catalytic interaction.

The catalytic power of enzymes derives from the free energy released upon binding of the transition state for the uncatalyzed reaction to the enzyme.[2] Commonly, complexes of enzyme and substrate are also formed, an inherently inhibitory phenomenon.[3] Therefore, it is important for enzymes to evolve a strategy for transition-state stabilization that allows for a minimal wastage of catalytic power in the binding of substrates, products, or intermediate compounds.[2-4] Alternatively stated, the enzyme must develop a distribution of its catalytic power between V/K terms (transition-state stabilization) and V terms (transition-state stabilization diminished by stable-state stabilization). This distribution should ideally satisfy both the chemical constraints imposed by the reaction that the enzyme catalyzes and the biological constraints imposed by its organismic context.[4,5]

The serine proteases[4,6] constitute a particularly interesting object for study in this regard. In their natural task of polypeptide hydrolysis, these enzymes initially form a covalently derivatized enzyme molecule ("acyl enzyme") in which the carboxyl fragment of the substrate esterifies the hydroxyl function of the eponymous serine. This is an extreme form of stable-state binding, characteristic of "ping-pong" enzymes, and leads to the requirement for fission of a covalent bond to the enzyme during release of at least one of the products.

In fact, the spontaneous hydrolysis of an ester similar in structure to an acylated serine protease is expected[7] to have a rate constant of only about 10^{-7} s^{-1} at 298 K, while the rate constant[8] for hydrolysis of (*N*-acetylphenylalanyl)chymotrypsin is actually about 10^2 s^{-1}. Thus, a catalytic acceleration factor of around 10^9 is generated in release of the second-product fragment by serine proteases. In this paper, we will discuss some features of the catalytic process for this partial reaction.

We will use solvent isotope effects to obtain information about the transition-state status of the exchangeable hydrogenic sites of the enzyme. We assume that the "active" hydrogenic sites, those which produce a solvent isotope effect, are among the sites that make up the acid–base catalytic entity (ABCE) of the enzyme. Presumably this entity consists of the chain of hydrogen bonds connecting the active-site serine through His-57 to Asp-102 and perhaps beyond,[9] although the solvent isotope effects themselves do not contain any information about the structural location of the "active protons". The number of protonic sites contributing to the overall solvent isotope effect (and presumably to catalysis) can be estimated by the proton-inventory method,[10] in which the rate constant k_n is determined in mixed isotopic waters, HOH and DOD, as a function of n, the atom fraction of deuterium in the solvent. If only transition-state sites generate the isotope effect, $k_n(n)$ reflects their number and the magnitude ot the effect at each site. A single contributing site produces linear behavior (eq 1),

$$k_n(n) = k_0(n)(1 - n + n\phi_T) \quad (1)$$

$$k_n(n) = k_0(n)(1 - n + n\phi_{T1})(1 - n + n\phi_{T2}) \quad (2)$$

where ϕ_T, the transition-state isotopic fractionation factor, gives the inverse kinetic isotope effect k_D/k_H for the single site. Two contributing sites generate the quadratic function of eq 2, where ϕ_{T1} and ϕ_{T2} measure isotope effects at the two sites. More complex situations produce more complex dependences.[10] A very large number of contributing sites, each producing a very small isotope effect, will generate an exponential form (ln k_n linear in n). This is expected if generalized solvation changes or subtle enzyme-conformational changes give rise to the solvent isotope effect.

We recently[11] examined serine proteases acting upon various substrates, including *minimal* substrates, i.e., substrates that lack most of the structural features of the natural substrate. Then the

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enzyme is deprived of subsite interactions with substrate-derived structural elements in the catalytic transition state. In this situation, the systems exhibit *one-proton catalysis*. The enzymes presumably rely only on the proximal hydrogen bridge of the ABCE, in the manner of nonenzymic protolytic catalysts. When some features of the evolutionarily anticipated substrate structure were added back, *two-proton catalysis* (better, multiproton catalysis, since distinction of, say, quadratic and cubic or quartic proton-inventory functions is not always easy) comes into the picture. It was suggested that, possibly, the added, attractive subsite interactions lead to a compression of enzyme structure. This compression, acting across the hydrogen-bond chain of the ABCE, could couple the protonic sites through their increased proximity and induce stronger, multiproton, catalytic hydrogen bridging.[11]

In the present report, we examine the degree to which the transition-state status of the ABCE, as reflected in the solvent isotope effect, is coordinated with the status of heavy-atom reorganization in the substrate-derived acyl group, at the catalytic transition state. Our tool for probing this latter feature of transition-state structure is the β-deuterium secondary isotope effect.[12,13] Strictly speaking, this isotope effect reflects only changes in the substrate *force field* on conversion of reactants to transition state. However, most changes in force field are correlated with changes in structure. Thus we attempt, through the use of both empirical[13,15] and theoretical[14] arguments, to relate the isotope effects to structural changes. For reactions in which acyl transfer is accomplished by a two-step mechanism through a tetrahedral intermediate or by a one-step mechanism with a quasi-tetrahedral transition state, deuterium substitution on the carbon adjacent to carbonyl ("β-D" substitution) is expected to increase the rate. This is because in the partially tetrahedral transition state (whether before, after, or instead of the tetrahedral intermediate), hyperconjugation from the β-CH(D) bonds is reduced, increasing the electron density in the bonds and strengthening them (thus giving an inverse isotope effect).[12] The limiting isotope effect for conversion of a trigonal reactant to a hypothetical fully tetrahedral transition state should be $K_H/K_D = 0.955$ per D, as estimated from the equilibrium isotope effect for ketone hydration.[13] For transition states of intermediate structure, the isotopic difference in free energies of activation is expected to change in rough proportion to the nucleophile–carbon (or carbon-leaving group) bond order, if this bond order affects the β-CH force constants in a proportional manner.[14] Thus, an approximate measure $\hat{I}$ of the "tetrahedrality" developed at the transition state can be defined by eq 3. This quantity has been

$$k_H/k_D = (K_H/K_D)^{\hat{I}} \quad (3)$$

determined for various cases of nonenzymic nucleophilic reactions of acetate ester substrates.[15] Included were not only $\hat{I}$ values derived from β-D isotope effects but also the corresponding values from α-D isotope effects with formate substrates, as measured by Cordes and his group.[16] The two measures generally agreed.[17]

Table I. Zero-Order Rates and Kinetic Isotope Effects for Hydrolysis of p-$NO_2C_6H_4O_2CCH_2NHCbz$ and p-$NO_2C_6H_4O_2CCD_2NHCbz$ Catalyzed by α-Chymotrypsin and Elastase

enzyme	$10^{11} V_0^{2H}$ ($M\ s^{-1}$)	$10^{11} V_0^{2D}$ ($M\ s^{-1}$)	k_{2H}/k_{2D}
α-chymotrypsin[a]	5103		
		5444	0.937
	5089		0.935
		5553	0.917
	5283		0.951
		5741	0.920
	5277		0.919
		5730	0.921
			0.925 ± 0.009[c]
elastase[b]	3798		(1.052)[d]
		3610	0.967
	3490		0.948
		3683	0.963
	3547		0.955
		3715	0.974
	3620		0.961 ± 0.010[c]

[a] [α-Chymotrypsin] = 7.3×10^{-6} M; [substrate-h_2] = 2.026×10^{-4} M; [substrate-d_2] = 1.980×10^{-4} M; pH 4.83, 0.10 M acetate buffer; 1.66% acetonitrile; 25.15 ± 0.01 °C. [b] [Elastase] = 5.9×10^{-6} M; [substrate-h_2] = 2.05×10^{-4} M; [substrate-d_2] = 2.06×10^{-4} M; pH 4.83, 0.10 M acetate buffer; 1.66% acetonitrile; 25.01 ± 0.01 °C. [c] Mean ± SD. [d] Not included in the calculation of the mean ± SD.

The examples most relevant for the present report were four cases of general base (protolytically) catalyzed attack of water on ester carbonyl; all gave $\hat{I}$ values between 0.58 and 0.66.

Therefore, we discuss, in the present paper, the transition-state carbonyl structure (various degrees of tetrahedral structure, as reflected in the secondary isotope effects) and the transition-state status of the ABCE (as reflected in the solvent isotope effects) for various enzyme–substrate combinations. Results are given for chymotrypsin and elastase, as representative serine proteases, and for acetyl and *N*-(carbobenzyloxy)glycyl substrates. Deacylation of the acyl enzyme is under observation in all cases.

Results

Table I shows zero-order rates of hydrolysis of p-$NO_2C_6H_4O_2CCH_2NHCbz$ and p-$NO_2C_6H_4O_2CCD_2NHCbz$, catalyzed by bovine α-chymotrypsin and porcine elastase, under conditions given in the footnotes, and the corresponding kinetic isotope effects. Under these circumstances, rapid acylation of both enzymes is followed by rate-determining deacylation of the acyl enzyme.[20] The reaction under study is thus that of eq 4. Table

$$RCO\text{–}OCH_2Enz + H_2O \rightarrow RCO_2(H) + EnzCH_2OH \quad (4)$$

II contains similar data for p-$NO_2C_6H_4O_2CCH_3$ and p-$NO_2C_6H_4O_2CCD_3$. Table III presents rates in mixtures of protium and deuterium oxides.

The secondary isotope effects in Table I and the solvent isotope effects in Table III were determined at pH 4.7–4.8 and at corresponding pL in solvents containing deuterium oxide (a corresponding pL places the measurement at an equivalent point on the pL/rate profile in HOH, DOD, and their mixtures). The secondary isotope effects in Table II and the corresponding solvent isotope effects (reported earlier[11]) were measured at pH 7.5–8. The lower pH in the case of the (carbobenzyloxy)glycyl substrates was necessary in order to prevent rapid, nonenzymic background reactions. No effect of the pH change on the isotope effects is expected, however (or course, the absolute enzymic rates for the

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Table II. Zero-Order Rates and Kinetic Isotope Effects for p-$NO_2C_6H_4O_2CCH_3$ and p-$NO_2C_6H_4O_2CCD_3$ Catalyzed by α-Chymotrypsin and Elastase

enzyme	$10^7 V_0^{3H}$ (A_{400} s^{-1})	$10^7 V_0^{3D}$ (A_{400} s^{-1})	k_{3H}/k_{3D}
α-chymotrypsin[a]	4602	4816	0.956
	4657	4932	0.958
	4657	4880	0.936
			0.944
			0.954
α-chymotrypsin[b]	4240	4502	0.942
	4195	4486	0.932
	4177	4530	0.935
			0.931
			0.922
α-chymotrypsin[c]	5220	5556	0.940
	5197	5525	0.935
	5209	5552	0.941
			0.943
			0.938
			0.940 ± 0.010[h]
elastase[d]	8558	8797	0.972
	8633	8814	0.979
	8508	8667	0.979
			0.965
			0.982
	8469	8905	0.977
	8604		(0.951)[i]
			0.967
elastase[e]	8140	8235	0.988
	8224	8364	(0.999)[i]
	8093	8300	0.983
			0.968
			0.975
elastase[f]	7496	7700	0.974
	7516	7583	0.976
	7636	7541	0.991
			(1.007)[i]
			(1.013)[i]
elastase[g]	7217	7425	0.972
	7121	7376	0.965
	7142	7223	0.965
			0.968
			0.989
			0.975 ± 0.007[h]

[a] [α-Chymotrypsin] = 0.18 mg mL^{-1}; [substrates] = 7.29×10^{-5} M; pH 7.50, 0.03 M Tris buffer; 1.61% acetonitrile; 25.00 ± 0.03 °C. [b] Same as footnote *a* except [substrates] = 6.13×10^{-5} M. [c] Same as footnote *b* except pH 8.00, 0.01 M Tris buffer. [d] [Elastase] = 0.304 mg mL^{-1}; [substrates] = 4.31×10^{-4} M; pH 7.50, 0.03 M Tris buffer; 3.13% acetonitrile; 25.00 ± 0.03 °C. [e] Same as footnote *d* except elastase solution had been frozen and thawed. [f] Same as footnote *d* except [elastase] = 0.313 mg mL^{-1} and 0.01 M Tris buffer. [g] Same as footnote *f* except [elastase] = 0.297 mg mL^{-1}. [h] Mean ± SD. [i] Not included in the average (excluded by the 2σ test).

individual isotopic species should be affected). In accord with this, both the overall solvent isotope effect and dependence on atom fraction of deuterium for p-$NO_2C_6H_4O_2CCH_2NHCbz$ with elastase (pH 4.8) are the same as for p-$NO_2C_6H_4O_2CCH_3$ with chymotrypsin and elastase, both at pH 7.4–7.5 (see below).

Discussion

Protonic Reorganization at the Transition State. The transition-state status of the ABCE can be deduced for the various enzyme–substrate combinations from the proton-inventory data (Table IV). Under the conditions employed here, the effective reactant state is acyl enzyme and the effective transition state that

Table III. Proton Inventories for the Action of α-Chymotrypsin and Elastase on p-$NO_2C_6H_4O_2CCH_2NHCO_2CH_2C_6H_5$

reaction and conditions	atom fraction of deuterium [$10^{10} V_n$ (M s^{-1})]
α-chymotrypsin, 0.43 mg mL^{-1}; pH 4.72 and equivalent,[10] 0.10 M acetate buffer; substrate, 2.03×10^{-4} M; 1.6% acetonitrile; 24.98 ± 0.03 °C	0.00 (915, 899); 0.20 (761, 762); 0.39 (593, 584); 0.58 (507, 481); 0.75 (418, 381); 0.94 (310, 303)
elastase, 0.37 mg mL^{-1}; pH 4.72 and equivalent,[10] 0.10 M acetate buffer; substrate, 1.01×10^{-4} M; 1.6% acetonitrile, 25.12 ± 0.03 °C	0.00 (599, 611); 0.20 (557, 520); 0.40 (448, 480); 0.60 (415, 379); 0.80 (314, 332); 1.00 (245, 246)

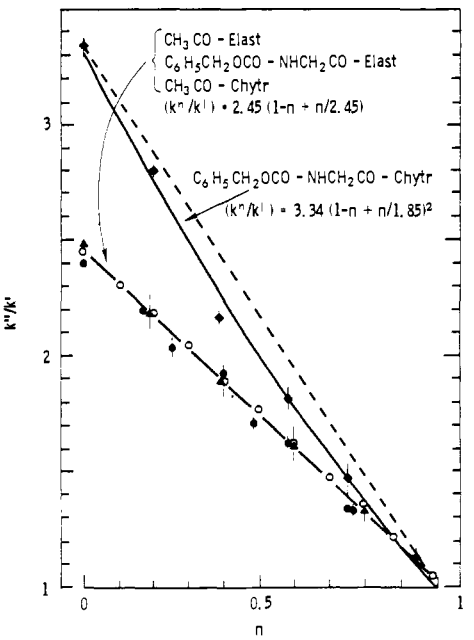


Figure 1. Partial solvent isotope effects, k_n/k_1, as a function of atom fraction n of deuterium in binary mixtures of protium and deuterium oxides for the hydrolyses of four acyl enzymes. The straight line represents the combined data for reactions of acetylchymotrypsin, acetyl elastase, and (carbobenzyloxy)glycyl elastase. All are described by the common line, $(k_n/k_1) = 2.45(1 - n + n/2.45)$. The curved line represents the data for [(carbobenzyloxy)glycyl]chymotrypsin and is described by the line $k_n/k_1 = 3.34(1 - n + n/1.85)^2$. The dashed line is straight and connects the point for $n = 0$ with that for $n = 1$.

for deacylation.[20] Relevant data are plotted in Figure 1, where the ordinate is the partial solvent isotope effect k_n/k_l (k_n is the rate constant in a mixed isotopic solvent with atom fraction n of deuterium; k_l is the rate constant in pure deuterium oxide) and the abscissa is n. In such a plot the intercept at $n = 0$ is k_0/k_1, the overall solvent isotope effect.

The lower, straight line in Figure 1 describes, by a single relationship, the results for *three* different enzymic deacylation reactions: (1) the deacetylation of acetylchymotrypsin;[11] (2) the deacetylation of acetyl elastase;[11] (3) the deacylation of CbzGlyOelastase. The overall solvent isotope effects and the partial solvent isotope effects in all isotopic solvent mixtures for all three of these reactions are equal. The data are described by the linear equation shown in Figure 1, suggesting that the solvent isotope effect arises in all cases from a single protonic site and that the magnitude of the effect is $k_H/k_D = 2.45$, regardless of enzyme or substrate structure.

For deacylation of CbzGlyOchymotrypsin (upper curve), the situation is different. The overall solvent isotope effect is about 3.34 and the nonlinear, roughly quadratic dependence of k_n upon n indicates multiproton catalysis. The simplest model, corresponding to the curve shown, is for two-proton catalysis with isotope effects $k_H/k_D \sim 1.85$ at each of the two protonic sites.

Table IV. Secondary Substrate Isotope Effects and Mixed Isotopic Solvent Effects for Nonenzymic and Enzymic Acyl-Transfer Reactions

system	secondary substrate isotope effect, k_H/k_D	$\hat{I}^a$	proton inventory results[b] ϕ_{T_1}	ϕ_{T_2}
nonenzymic protolytic catalysis[c]				
(1) four examples of protolytically catalyzed hydrolysis of aryl formates and acetates	0.813–0.833 (α-D)	0.58–0.66	(0.40–0.60)[d]	
	0.914 (β-D_3)	0.65		
deacylation of acylchymotrypsins				
(2) HCO-Enz + H_2O[e,f]	0.870 ± 0.023 (α-D)	0.44		
(3) CH_3CO-Enz + H_2O[g]	0.940 ± 0.010 (β-D_3)	0.44	0.42	1.00
(4) $C_6H_5CH_2OCONHCH_2CO$-Enz + H_2O[h]	0.925 ± 0.009 (β-D_2)	0.84	0.55	0.55
deacylation of acyl elastases				
(5) CH_3CO-Enz + H_2O[g]	0.975 ± 0.007 (β-D_3)	0.27	0.41	1.00
(6) $C_6H_7CH_2OCONHCH_2CO$-Enz + H_2O[h]	0.961 ± 0.010 (β-D_2)	0.43	0.41	1.00

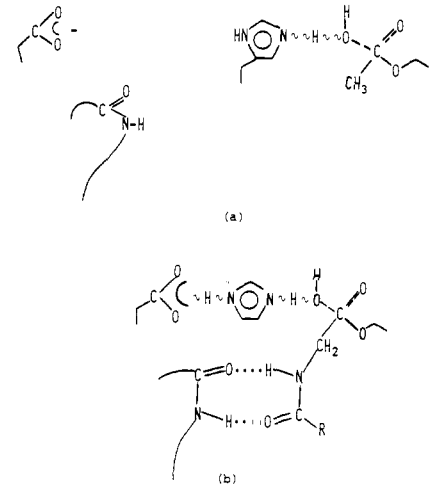
[a] For α-D effects, $I = \log(k_H/k_D)/\log 0.73$; for β-D_3 effects, $\hat{I} = \log(k_H/k_D)/\log 0.87$; for β-D_2 effects, $\hat{I} = \log(k_H/k_D)/\log 0.91$. Not more reliable[15] than ±0.1–0.3. [b] From nonlinear least-squares fit of k_n $(n) = k_0$ $(1 - n + n\phi_{T_1})(1 - n + n\phi_{T_2})$. [c] From ref 15 and 16. [d] Commonly observed range.[19] [e] From ref 18. [f] pH 5.8–6.8. [g] pH 7.5–8. [h] pH 4.7–4.8.

Hunkapiller, Forgac, and Richards[21] similarly observed two-proton catalysis in the acylation of elastase and α-lytic protease by AcAla-Pro-Ala *p*-nitroanilide, as we did[11] for trypsin with BzPhe-Val-Arg-*p*-nitroanilide (probably acylation but not demonstrated). Deacylation of $C_6H_5CH_2CH_2COO$chymotrypsin[11] gave two-proton catalysis with individual contributions of $k_H/k_D = 1.9$ and 1.5. Thus, as in the present study, substrates of truncated but not minimal structure sometimes but not always experienced one-proton catalysis (trypsin with BzArgOEt, α-chymotrypsin with $AcTrpNH_2$, thrombin with BzArgOEt).[11] Multiproton catalysis was observed with substrates having more extended "polypeptide tails", in the acylation of elastase, α-lytic protease, and trypsin. Multiproton catalysis is observed in two deacylation reactions [(3-phenylpropanoyl)chymotrypsin and [(carbobenzyloxy)glycyl]chymotrypsin] where only a single fragment of structure beyond the minimal acetyl group is present. We can therefore summarize the solvent isotope effect results now available for deacylation of the serine proteases studied, as follows: (1) As a rule, minimal substrate structures such as acetyl produce one-proton catalysis with $k_H/k_D = 2.5$, independent of enzyme structure. (2) Addition to the minimal acetyl group of one or two simple features of the structure of the evolutionarily anticipated, natural polypeptide substrate *may* lead to a retention of one-proton catalysis, commonly with k_H/k_D remaining about 2–3: addition of the first unit of the polypeptide tail, CbzGlyONp with elastase, $k_H/k_D = 2.4$; addition of the first unit of the polypeptide tail and the C_α side chain anticipated by the particular enzyme, BzArgOEt with trypsin, $k_H/k_D = 3.0$, and with thrombin, $k_H/k_d = 2.9$. (3) On the other hand, addition of one simple feature of the natural substrate *may* also lead to multiproton catalysis: addition of the first peptide unit, CbzGlyONp with chymotrypsin, $k_H/k_D = 1.8$[2]; addition of the C_α side chain, $C_6H_5CH_2CH_2COONp$ with chymotrypsin, $k_H/k_D = (1.9)(1.5)$.

Thus, when only the transition-state interactions between enzyme and substrate directly at the reacting center are possible (formation of the C–O bond from water to carbonyl or fission of the C–O bond from carbonyl to serine and "oxyanion-hole" interactions with carbonyl oxygen[4]), then the enzyme is unable to mobilize its multiproton-catalytic capability. The enzyme seems to function in the same way that protolytic general-base catalysts operate in nonenzymic systems, where isotope effects in the range $k_H/k_D = 2$–3 are common and arise from a single center.[19]

The addition of further interactions, either at the *N*-acyl binding site or at the C_α side chain "specificity pocket",[4] may or may not lead to mobilization of the multiproton catalytic apparatus. The resulting isotope effects at each site seem somewhat different from the 2.5 observed at the single site in the one-proton mechanism, for minimal substrates. When the more elaborate substrate continues to use a one-proton mechanism, the effects seem slightly increased (to 3.0 and 2.9 in the examples above). In the multiproton cases, the effects at each site seem to be reduced (1.5–1.9 in the examples above, when analyzed on a two-proton model).

Scheme I



As was noted in the introduction, we cannot use the kinetic isotope effects to localize the region of the enzyme structure that produces them. It is no great extension of our combined knowledge of the structures of the serine proteases and of the reaction mechanism of protolytically catalyzed ester hydrolysis, however, to suppose that the protonic site that generates an isotope effect around 2.5 with minimal substrates is that between the imidazole of the active-site histidine and the oxygen atom of the attacking water molecule or the departing serine, according to which step is rate determining. This hypothesis is portrayed in Scheme I (a). The other elements shown there are the active-site aspartate carboxylate group (of the charge-relay system) and a backbone amide group representing the *N*-acyl binding site.[4] Because the minimal substrate bears no *N*-acyl function, this site is empty. The distance from the imidazole to carboxylate is shown as rather large, corresponding to no extremely strong interaction. This is suggested for the transition state by the absence of any isotope effect that could be attributed to the proton between imidazole and carboxylate. That the situation is similar in the reactant state is suggested by various NMR and crystallographic studies, recently summarized by Kossiapov and Spencer.[22]

Now in some cases, the addition of the first interacting group to the minimal substrate produces multiproton catalysis. Scheme I (b) pictures a hypothesis about how an *N*-acyl function might give rise to this effect. The *N*-acyl function is shown as interacting with the appropriate binding site in the transition state. This attractive interaction, when combined with the anchoring of the substrate carbonyl to the active-site serine, exerts a compressive force across the nearby protein structure. The result is a shortening of the carboxylate–imidazole distance and the development of a second catalytic proton bridge. In addition, reorganization of structure at the first protonic site and within the reacting carbonyl function may also be induced. The formation of the new hydrogen

(21) Hunkapiller, M. W.; Forgac, M. D.; Richards, J. H. *Biochemistry* **1976**, *15*, 5581.

(22) Kossiapov, A. A.; Spencer, S. A *Biochemistry* **1981**, *20*, 6462–6474.

bridge, and possibly effects of reorganization within the carbonyl function (see below), releases sufficient energy to pay the energetic costs of the initial compression process ("utilization of intrinsic binding energy").[2]

Of course, the introduction of, say, an *N*-acyl group with a particular enzyme–substrate pair might or might not produce a sufficient energy release to allow the compression to be effected and multiproton catalysis to be activated. This would depend on such factors as the nature of other interacting groups present in the substrate and the resistance to compression offered by the detailed structure of the particular enzyme. In cases where, for example, a second interacting group presents a block to the compressive force of the first, or the enzyme structure is rather stiff, then compression and activation may not occur. Thus, we find that addition of the *N*-acyl function to the minimal acetyl *does* lead to multiproton catalysis with chymotrypsin but *does not* with elastase. Introduction of *both* *N*-acyl and an appropriate C_α side chain *does not* activate the multiproton capacities of trypsin or thrombin. Thus, one may be dealing, in the "natural" transition-state combination of the enzyme and evolutionarily anticipated substrate (with a polypeptide tail and the appropriate C_α side chain), with a delicate mechanical balance of forces. Omission of one or more structural elements may lead to a result difficult to predict at this stage of development in the mechanistic model.

Relationship to Diffraction and Spectroscopic Studies of Stable Enzyme States. The serine proteases, and their complexes and derivatives, have been the object of intensive direct examination by NMR and other kinds of spectroscopy, as well as by X-ray diffraction and by neutron diffraction techniques. Kossiapov and Spencer,[22] who characterized monoisopropylphosphoryltrypsin by neutron diffraction, have recently summarized those structural investigations which have concentrated on the acid–base catalytic entity (ABCE). Essentially all of these studies can now be interpreted as showing that, in the enzymes themselves or in complexes or derivatives sufficiently stable to accumulate to high concentrations in solution or in the solid state, the histidine and aspartate groups of the "Asp-His-Ser triad", here taken as at least a part of the ABCE, behave independently of each other and have the normal chemical characteristics (such as pK_a) of the carboxylate and imidazole functional groups.

The solvent isotope effect results just described suggest, on the other hand, that in the *transition state* for catalytic reaction of the enzymes with substrates having an approximation to the full, evolutionarily anticipated structure, and thus an approximation to the full, evolutionarily anticipated complement of transition-state enzyme–substrate interactions, something quite different is true. In such transition states, a number of sites along the Asp-His-Ser chain (and perhaps further into the enzyme structure) seem to cooperate in forming sites of substantial isotopic fractionation; we interpret these as representing a chain of strong hydrogen bonds that stabilize the transition state and contribute to catalysis. In this regard, it would be useful if some stable complex of a serine protease could be invented, which so simulated the transition-state energetics that this cooperative character of the ABCE could be elicited and directly examined, e.g., by spectroscopy or crystallography. Such a project should, however, be very difficult if not in fact impossible. It seems likely that transient features, such as the pK of the nucleophilic center, present only in a true transition state, may be critical for producing the cooperative effect that is seen kinetically.

Current theories of catalysis, most clearly expressed by Jencks[2] and Fersht,[4] are completely in accord with the combined structural–kinetic model just described. The ideal catalyst expresses its catalytic power only in the transition state but does not do so in reactant or other stable states. The transition state is then maximally stabilized, stable states are minimally stabilized, and energy barriers to the reaction are maximally reduced. A good ABCE functions fully only in the transition state, as observed.

Thus, kinetic studies and structural studies are both producing the expected, necessarily consistent results. Some wrong conclusions have nevertheless been drawn. Kossiapov and Spencer[22] write, "It can be concluded, therefore, that the catalytic effectiveness of the Asp-His-Ser triad is not due to some chemical effect perpetuated through the hydrogen bonding system as proposed in the charge-relay [Blow et al., 1969 (our ref 23)] and the double proton transfer [Hunkapiller et al., 1973 (our ref 24)] mechanisms but is rather a function of the standard chemical characteristics of these groups made maximally effective and efficient by their precise stereochemical positioning in the enzyme-substrate complex". This is erroneous. The error is to extend the lack of cooperative function of the ABCE, inferred from studies of stable materials, to the transition state (which is not accessible to direct structural study) and thus to the reaction mechanism. Theoretically,[2-4] function is *expected in the transition state*, where it is observed in kinetic studies, *but not in stable states*, where it is indeed not observed in the structural studies.

Substrate Structural Reorganization at the Transition State. From Table IV we may also deduce some things about the transition-state status of the carbonyl group and surrounding atoms from the α-D and β-D secondary isotope effects. In this connection, it is worth recalling an observation reported earlier[15] that emphasizes the structural plasticity of transition states for nucleophilic reactions at carbonyl. The β-D_3 secondary isotope effect for reaction of hydroxide ion with phenyl acetate is $k_{3H}/k_{3D} = 0.980 \pm 0.009$, corresponding to $\hat{I} = 0.15 \pm 0.09$. The same effect for the nucleophilic reaction of water with phenyl acetate, general base catalyzed by acetate ion, is 0.914 ± 0.023 ($\hat{I} = 0.65 \pm 0.29$). (Note the close similarity of the latter process to enzymic deacylation, which is a nucleophilic attack of water on the esterified enzyme, general base catalyzed by the enzymic ABCE.) The values of $\hat{I}$ show the carbonyl structure to be quite different in the two cases, with the structure more nearly resembling a tetrahedral adduct in the general catalysis reaction ($\Delta\hat{I} = 0.50 \pm 0.37$). On the other hand, the free energy difference between the two transition states[15] (calculable from the rate constants) is only 3.6 kcal/mol. The conclusion is that with the expenditure of a few kilocalories per mole, the structure of a transition state for nucleophilic displacement at carbonyl may be altered very significantly. Thus, if a chemical or biological opportunity presents itself to an acyl-transfer enzyme (in the course of biological evolution) that entrains an alteration in the carbonyl-group structure, the enzyme may be expected to be able to take advantage of the opportunity with a minimal expenditure of energy.

First, let us consider the results in Table IV for *minimal substrates* (acetyl or formyl) with both chymotrypsin and elastase, in comparison with the results for nonenzymic protolytic catalysis. We note the agreement of α-D and β-D effects with each other, in terms of $\hat{I}$ values, for both enzymic and nonenzymic cases. The results for formylchymotrypsin ($\hat{I} = 0.44$) and acetylchymotrypsin ($\hat{I} = 0.44$) may indicate very slightly less tetrahedral character than is present in nonenzymic reactions ($\hat{I} = 0.58$–0.66), but because of the probable errors, such a conclusion would be of little or no validity. For acetyl elastase, however, the situation is clearer ($\hat{I} = 0.27$) so that for this enzymic transition state, a less tetrahedral structure compared to that of the nonezymic transition state seems to exist. This suggests an "explosive" effect of the enzyme, lengthening the bond beween the carbonyl carbon and the atom which interacts with the hydrogen-bond chain of the ABCE. Elastase appears to achieve this effect even with the minimal structure acetyl. Whether chymotrypsin produces any such effect is debatable, and in any case the magnitude of the structural change—if present—is much attenuated. Thus, clearly, the *nature of the enzyme*, even within the serine protease family, is critical in this structural alteration of the transition state.

The other two enzymic results in Table IV are for (carbobenzyloxy)glycyl enzymes. Thus, the first step is taken in proceeding from the minimal acetyl structure toward the natural substrate: the first fragment of the polypeptide tail is added. The effect is the same for both enzymes: a strong *compression* of the structure toward the tetrahedral (for chymotrypsin, $\hat{I}$ is changed

(23) Blow, D. M.; Birktoft, J. J.; Hartley, B. S. *Nature (London)* **1969**, *221*, 337.
(24) Hunkapiller, M. W.; Smallcombe, S. H.; Whitaker, D. R.; Richards, J. H. *Biochemistry* **1973**, *12*, 4732.

from 0.44 to 0.84 $\Delta\hat{I} = 0.40$; for elastase, $\hat{I}$ is changed from 0.27 to 0.43, $\Delta\hat{I} = 0.16$). In spite of the probable errors in $\hat{I}$ values (partly from theoretical considerations),[15] the unambiguous character of this result can be appreciated by a direct of examination of the kinetic isotope effects: in both cases, addition of the *N*-acyl fragment produces a kinetic isotope effect for *two* deuteriums (0.925 for chymotrypsin, 0.961 for elastase) that is more inverse—or larger magnitude—than the previous isotope effect for *three* deuteriums (0.940 for chymotrypsin and 0.975 for elastase).

The compressed structure for the *N*-acylglycyl elastase transition state is similar to that for nonenzymic reactions, although conceivably still slightly exploded ($\hat{I} = 0.43$ vs. 0.58–0.66). The structure for the (*N*-acylglycyl)chymotrypsin transition state is, however, far *more* compressed than the nonenzymic transition states ($\hat{I} = 0.84$ vs. 0.58–0.66).

Eventually we would like to be able to interpret results like these, which bear on mechanical alterations of the transition state by the enzyme and the effect of these alterations on the function of catalytic entities such as the ABCE, in terms of a general theory of structure and function in catalysis. At this point we cannot do this because our data are too fragmentary and our understanding of them is too primitive. There are a few tentative observations possible, however. We recall that the chief structural difference[4] between chymotrypsin and elastase is in the "specificity pocket", just "south" of the nucleophilic serine in the usual presentation of the active site. Chymotrypsin has a large, open, hydrophobic cavity, while elastase has the same region occupied by molecular fragments, with only a small depression (capable of accommodating the C_α-methyl of alanyl derivatives) remaining open. With these points in mind, we note the following features of the present results: (a) Even when only minimal substrate structure is present, the enzyme seems to alter the transition-state carbonyl framework from its nonenzymic base-line structure, in an *explosive* direction, toward a less tetrahedral character, with the effect being pronounced in the case of elastase but doubtful or absent in the case of chymotrypsin. The only sites at which enzymic attractions or repulsions can act to produce such an effect are at the carbonyl oxygen and on the serine "arm" of the enzyme. (b) Addition of the first fragment of the polypeptide tail, a structural feature we earlier argued to be important in compressing the ABCE, seems to produce a compression also of the carbonyl structure, *at least when the proper C_α side chain is missing.* In fact, with chymotrypsin, where the missing C_α side chain is large and would occupy a significant region of the active site, a very strong compression results from the *N*-acyl interaction. In elastase, where only a C_α-methyl is evolutionarily expected, and where the active site is "stiffened" by the presence of structure in the specificity pocket, the compressive effect induced by the *N*-acyl group is less strong. The carbonyl structure seems to remain perhaps slightly exploded relative to the nonenzymic transition states.

One highly speculative model that accommodates both the isotope-effect findings and the enzyme structural data is this. The enzyme must lower the free energy of the transition state in such a way as not to "waste" catalytic power in stable states.[2-4] To do this, the enzyme in the transition state may (a) explode the carbonyl structure, (b) compress the ABCE, and (c) use the C_α side chain together with nearby protein structure as a pivot or block to separate the explosive motion (near the serine in the "northeast" active site) from the compressive motion (in the "northwest").

The explosive alteration of the carbonyl structure could adjust the effective p*K* of the nucleophilic oxygen to an optimum for interaction with the ABCE. The compressive alteration of the ABCE could couple the hydrogen bonds of this entity to allow a cooperative, transition-state-stabilizing interaction with the nucleophilic oxygen. Such interactions would be highly specific to the transition state and would not be expressed in preceding or succeeding states.

The difference in behavior seen for elastase and chymotrypsin may arise from the fact that chymotrypsin depends on a substrate C_α side chain for effective "blocking" between the compressive and explosive forces, while elastase depends only on protein structure for this purpose. Thus, introduction of the first *N*-acyl fragment (thought to exert a compressive force) produces little effect in elastase but an "overshoot compression" with chymotrypsin when the requisite C_α side chain is missing.

When the enzyme and the natural, evolutionarily anticipated substrate interact in the transition state, then an optimal explosive carbonyl p*K* adjustment and compressive coupling of the ABCE combine to produce strong stabilization. In the acyl enzyme, no such effect can result nor can it in the tetrahedral intermediate. Thus, the model describes a technique for reducing the free energy of the transition state while not reducing the free energies of stable states. The result is a lowered barrier to passage through the transition state without increased, inhibitory complexation of enzyme in stable states.

Sources of Error. An important source of error in comparing the isotope effects for the acetyl and *N*-acylglycyl derivatives with each other arises from the assumption that the intrinsic isotope effect for complete carbonyl addition is the same for both species. This could be incorrect, because β-D secondary isotope effects are known[25] to depend on the stereochemical relationship of the CH(D) bond involved and the empty or partially empty orbital into which hyperconjugative electron release is occurring. If the enzyme, by binding the *N*-acyl group, produces a significant change in the average relative orientation of the β-CH(D) bond and the carbonyl π orbitals, some change in the limiting isotope effect and thus the $\hat{I}$ values of Table IV would result. The magnitude of the experimental effects militates to some degree against a reversal of the conclusions on this basis: the two-dimensional isotope effects for the *N*-acyl compounds are actually more inverse than the three-dimensional isotope effects for the acetyl compounds. This argument is weak, however, and until further work elucidates the way in which the limiting effect is quantitatively altered by enzyme binding (if at all), the interpretations given above must be considered highly tentative.

A reservation about the solvent isotope effects, already expressed above, should be emphasized. Since the origin of these effects cannot be ascribed rigorously to the ABCE, other sites might give rise to them. For example, compression of enzyme structure by the *N*-acyl fragment of the polypeptide tail could conceivably generate very strong hydrogen bonds in the oxyanion hole. This seems less likely to us than an effect in the closer ABCE, which is almost surely the site of the one-proton isotope effects. At present, however, it cannot be excluded.

Conclusion. Solvent isotope effects and secondary substrate isotope effects for deacylation reactions of chymotrypsin and elastase, derivatized by minimal and near-minimal substrates, indicate that both the degree of multiproton function of the acid–base catalytic apparatus of the enzyme and the degree of tetrahedral structure at the substrate-derived carbonyl group depend on the structure of the substrate and the structure of the enzyme and thus presumably on enzyme–substrate interactions present in the catalytic transition state.

This view does not directly address one problem raised in the introduction: why do the serine proteases develop a covalent bond to the acyl group, an extrene form of intermediate-compound stabilization? A speculative suggestion, in the spirit of the conclusion above, is that only in this way can sufficient leverage be developed to permit mechanical deformations about the carbonyl group. Only by such deformations, in turn, can the requisite p*K* adjustments be effected to allow strong transition-state stabilization involving the ABCE: a form of stabilization unique to the transition state and thus satisfying the fundamental requirement for effective catalysis.

Experimental Section

Materials and Solutions. Buffers, salts, enzymes, and solvents were as previously described.[11] *p*-$NO_2C_6H_4O_2CCD_3$ was prepared from *p*-nitrophenol and CD_3COCl (from Diaprep CD_3CO_2D and thionyl chloride; esterification in benzene with pyridine) and contained >95% deu-

(25) Forsyth, D. A.; Lucas, P.; Burk, R. M. *J. Am. Chem. Soc.* **1982**, *104*, 240, and references cited therein.

terium in the acetyl group by NMR.

p-$NO_2C_6H_4O_2CCL_2NHCbz$ (L = H, D). $HO_2CCD_2NH_3Cl$ was obtained by refluxing diethyl acetamidomalonate (Aldrich) overnight in 20% DCl/D_2O (Sigma). Treatment in 1 M $NaHCO_3$ with benzyl chloroformate produced $CbzNHCD_2COOH$, which was coupled to p-nitrophenol with dicyclohexylcarbodiimide in ethyl acetate (30 m, 40 °C; 90 m, 25 °C). The labeled substrate (L = D) was recrystallized twice from absolute ethanol containing 0.1% acetic acid [white crystals, mp 124 °C; lit. mp (L = H) 128 °C (ref 26), 124-125 °C (ref 27)]. Incorporation of deuterium was >95% by NMR. The protiated substrate was prepared similarly except that H_2O was appropriately used in place of D_2O.

Kinetic Procedures. Rates were obtained by automated spectrophotometry as described before.[11] Runs with isotopic substrates were conducted in alternation and with use of the same stock solutions.

Registry No. p-$NO_2C_6H_4O_2CCH_2NHCbz$, 1738-86-9; p-$NO_2C_6H_4O_2CCD_2NHCbz$, 84712-45-8; p-$NO_2C_6H_4O_2CCH_3$, 830-03-5; p-$NO_2C_6H_4O_2CCD_3$, 81408-98-2.

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Monooxygen Donation Potential of 4a-Hydroperoxyflavins As Compared with Those of a Percarboxylic Acid and Other Hydroperoxides. Monooxygen Donation to Olefin, Tertiary Amine, Alkyl Sulfide, and Iodide Ion

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Abstract: The reaction of the hydroperoxides diphenylhydroperoxyacetonitrile (**4**), methyl diphenylhydroperoxyacetate (**5**), and 5′,6′,7′,8′-tetrahydro-4a′-hydroperoxy-3′-methylspiro[cyclohexane-1,2′-(4a′*H*)quinazoline]-4′(3′*H*)-one (**6**) with I^-, thioxane, and *N*,*N*-dimethylbenzylamine (DMBA) are first order in both hydroperoxide and substrate. For both **5** and **6**, I_3^- is produced in 100% yield. Product analysis for the reaction of **4**, **5**, and **6** with thioxane and DMBA established that the hydroperoxides are converted to the corresponding alcohols and that thioxane sulfoxide and *N*,*N*-dimethylbenzylamine *N*-oxide are formed. The reactions are quantitative. The reaction of **4** with I^- proved to be complicated. The alcohol generated from **4** is the cyanohydrin of benzophenone. The dissociation of the benzophenone cyanohydrin product is competitive with I_3^- formation so that CN^- produced in the dissociation reacts with I_3^- to yield ICN. Kinetic and thermodynamic analyses have provided the pertinent rate and equilibrium constants associated with the overall time course for reaction of **4** with I^-. The second-order rate constant for the reaction of *m*-chloroperbenzoic acid (**1**) with I^- has been determined and the second-order rate constant for reaction of **1** with thioxane was obtained from experiments in which thioxane and I^- were employed as competitive substrates. The second-order rate constants for reaction of **1**, **4**, **5**, and **6** with I^-, thioxane, and DMBA were compared with like constants for the reactions of 4a-hydroperoxy-5-ethyl-3-methyllumiflavin (**2**), 1-carba-1-deaza-4a-hydroperoxy-5-ethyl-3-methyllumiflavin (**3**), *t*-BuOOH (**7**), and H_2O_2 (**8**). A log-log plot of the rate constants for monooxygen transfer from hydroperoxides to thioxane (k_S) and to DMBA (k_N) was found to be linear and of slope 1.0. The best line for the plot of log k_S vs. the log of the rate constants for reactions with I^- (k_I) was of slope 1.1. The points for *m*-chloroperbenzoic acid were found to fit the log k_S vs. log k_I plot. These results show that the second-order rate constants for reactions of I^-, thioxane, and DMBA are of like dependence on the electronic and steric characteristics of the hydroperoxides and percarboxylic acid **1**. A linear free energy plot correlates the log of the second-order rate constants vs. pK_a of YOH for oxygen transfer from YOOH = **1**, **2**, **4**, **5**, **7** and **8** ($\beta_{lg} = -0.6$). In these reactions the 4a-hydroperoxyflavin **2** is the most efficient monooxygen donor of the hydroperoxides investigated, being 10^3-10^6 more reactive than *t*-BuOOH and $\sim 10^3$ less reactive than the peracid **1**. The kinetics of epoxidation of 2,3-dimethyl-2-butene by the hydroperoxides **2**-**6** were investigated by following both hydroperoxide disappearance and product formation. The results of these investigations, which include further reaction of epoxide with hydroperoxide to provide pinacol and 2,3-dimethyl-1-buten-3-ol, are discussed. Evidence for epoxidation of 2,3-dimethyl-2-butene by **2** or its 1-carba-1-deaza analogue **3** could not be obtained. The order of monooxygen donation to I^-, :S< and :N< by the hydroperoxides, for which **2** and **3** were most reactive, does not apply to the epoxidation reaction. The flavin hydroperoxides are decomposed in $CHCl_3$ by a free radical reaction. Hydroperoxide **2** exhibits a chemiluminescent oxidation of *p*-tolualdehyde, whereas hydroperoxides **4**-**6** do not.

4a-Hydroperoxyflavins are important intermediates in oxygen activation in biochemistry. Both enzyme bound[1] and free,[2] these substances oxidize I^-, sulfides, and secondary and tertiary amines at rates exceeding those for alkyl hydroperoxides by many orders of magnitude. Our present interest is in the comparative reactivities of 4a-hydroperoxyflavins and other electron-deficient hydroperoxides and percarboxylic acids.

Brill and Indicator[3] showed that low yields of stereospecifically derived epoxides are obtained when olefin and *t*-BuOOH are reacted neat and that the reaction is catalyzed by certain metal ions. In the hands of Sharpless and co-workers, the employment

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