BIOORGANIC CHEMISTRY 7, 69–76 (1978)

The Question of Differential Hydrogen Bonding in the Mechanism of Catalysis by Serine Proteases

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Received September 7, 1977

Two mechanisms recently proposed to account for the action of serine in proteases differ on the matter of hydrogen bonding between enzyme and substrate in the Michaelis complex and acyl enzyme. One mechanism involves the removal of hydrogen bonds as a means of raising the free energy of the Michaelis complex toward that of the transition state and thereby effecting catalysis. This report shows that the kinetic parameters k_2/K_s and k_3 for the α-chymotrypsin-catalyzed hydrolysis of a series of halogen-substituted phenyl acetates respond to electron withdrawal and concomitant changes in hydrogen bond energy in a nearly identical fashion. Reference to thermodynamic studies of hydrogen bonding in free solution shows that these data are not consistent with hydrogen bond removal in the Michaelis complex and acyl enzyme but do support the alternative mechanism, in which hydrogen bonding of substrate is maintained along the entire reaction coordinate.

INTRODUCTION

It is now accepted that enzymic catalysis derives from the binding of enzyme and transition state of the reaction catalyzed (*1–3*). Furthermore, for catalysis to occur, the enzyme must bind the transition state more strongly than the Michaelis complex (*2*). The factors which contribute to this differential binding constitute the central subject of the mechanism of enzyme action.

The contribution of hydrogen bonding to differential binding in catalysis by serine proteases is one of the few remaining uncertainties in the mechanism of these extensively studied enzymes. We may identify two mechanisms, each based on extrapolation of X-ray data, and each supported by a large body of physical and chemical data (*4*). These mechanisms differ primarily in their suppositions about hydrogen bonding of the substrate during the course of the reaction. The first, proposed for subtilisin by Robertus and co-workers (*5*), postulates the stabilization of transition states for acylation and deacylation by three hydrogen bonds formed between transition state and enzyme, all of which are absent in the Michaelis complex and acyl enzyme. Since these hydrogen bonds are presumably present in free, solvated substrate, this mechanism amounts to a relative destabilization of Michaelis complex by removal of hydrogen bonds, followed by relative stabilization of the transition state, in which hydrogen bonds are enzymically restored. Even at a modest rate of 3 kcal/mol per interaction, the differential hydrogen bonding elegantly accounts for a rate increase of 10^6–10^7.

On the other hand Fersht *et al.* (*6*) reached a different conclusion from their data on

0045-2068/78/0071-0069$02.00/0

chymotrypsin. They have proposed that although the analogous bonds are formed in the transition state, they are also present in the Michaelis complex and acyl enzyme. In this second mechanism hydrogen bonding makes no direct enthalpic contribution to catalysis. The two mechanisms are illustrated schematically in Fig. 1, along with a hypothetical third mechanism in which no hydrogen bonding exists.

In this paper we report the results of simple kinetic experiments with chymotrypsin and show how linear free energy arguments may be combined with these data to resolve the ambiguity between mechanisms.

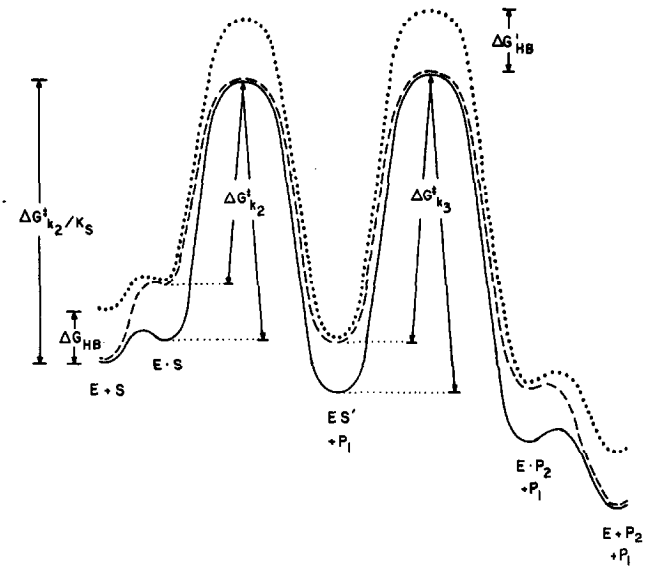

FIG. 1. Free energy surfaces for mechanisms of chymotrypsin-catalyzed hydrolysis of esters. Mechanism B (———), mechanism K (– – –), and a mechanism involving no hydrogen bonding (· · ·). E · S represents Michaelis complex, ES′ acyl enzyme.

MATERIALS AND METHODS

Substituted phenyl acetates were prepared by the methods of Shawali and Biechler (*7*). They exhibited melting points and boiling points in accord with reported values (*7*, *8*), and had ir and ^{1}H nmr consistent with their proposed structures. Bovine α-chymotrypsin was a 3× crystallized salt-free preparation from Sigma Chemical Co. When titrated by the cinnamoylimidazole method (*9*), it showed active site concentrations of 80–85% of the theoretical value. It was used without further purification. Acetonitrile was distilled from calcium hydride. Water was redistilled in glass vessels. Other materials were commercially available and were used without further purification.

Kinetic measurements were obtained either spectrophotometrically, by monitoring phenol release at 275 nm, or titrimetrically, by maintaining the pH of an unbuffered medium by adding sodium hydroxide from a pH-stat. The former method employed phosphate buffers; extrapolation to zero buffer concentration was used to determine the rate constant. Reactions were initiated by the addition of a small volume of ester

dissolved in acetonitrile, producing a reaction medium containing 1.9% (v/v) acetonitrile. Ionic strength was maintained at 0.1 by addition of sodium chloride.

Enzymic parameters were calculated using the unweighted nonlinear least squares method of Wilkinson (*10*), except in the case of the dihaloacetates, where first-order kinetics were observed and the ratio k_{cat}/K_m obtained directly from the first-order rate constant.

RESULTS AND DISCUSSION

The strategy used in this study is to vary the electronic properties of the substrate so as to change the amount of free energy available as a result of hydrogen bond formation. Accordingly we have attached strongly electron-withdrawing groups to the

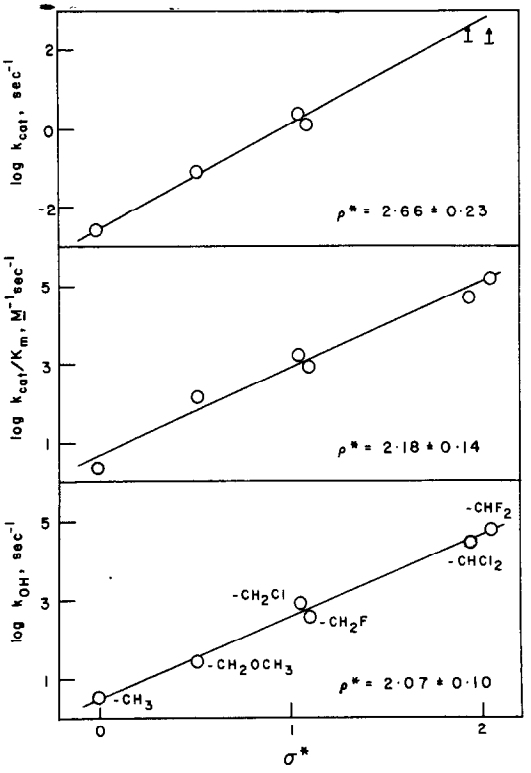

FIG. 2. Taft plots for chymotrypsin and hydroxide catalysis of hydrolysis of substituted phenyl acetates. Bars below the arrows represent lower limits on k_{cat}. Enzymic reactions are at pH 6.78. All reactions are at 25°C, 1.9% (v/v) CH_3CN, $\mu = 0.1$.

acyl moiety of a series of chymotrypsin substrates. The results are presented in Fig. 2 as a Taft (*11*) structure–reactivity correlation. The substituted phenyl acetates employed here as substrates all contain a reactive leaving group and exhibit rate determing deacylation. Under these conditions k_2/K_s corresponds directly to the experimental quantity k_{cat}/K_m, and k_3 to k_{cat} (*12*). As expected, both kinetic parameters increase as

electron withdrawal in the substrate increases, as does the rate of nonenzymic hydrolysis. A distinction between the mechanism proposed by Kraut (mechanism K) and that proposed by Blow (mechanism B), however, requires a quantitative analysis of these trends. What do the two different mechanisms predict about the behavior of the kinetic parameters as hydrogen bond energies change?

Effect on k_2/K_s

The mechanisms agree totally in their predictions about the rate constant k_2/K_s. As illustrated in Fig. 1, the free energy difference $\Delta G^{\ddagger}_{k_2/K_s}$ is identical for the two mechanisms, since in each case the transition state is fully hydrogen bonded. Thus both mechanisms predict that as electron density in the carbonyl oxygen of the substrate is decreased by electron withdrawing groups, changes in hydrogen bonding will raise both reactants and transition state in free energy. The resulting change in $\Delta G^{\ddagger}_{k_2/K_s}$ is $\delta(\Delta G'_{HB} - \Delta G_{HB})$, where δ represents the perturbation caused by electron withdrawal. Of course the perturbation will change $\Delta G^{\ddagger}_{k_2/K_s}$ in other ways, and the part of the change caused by hydrogen bonding is impossible to sort out experimentally.

However, one might expect $\delta(\Delta G'_{HB} - \Delta G_{HB})$ to be small. First, the quantity perturbed, $\Delta G'_{HB} - \Delta G_{HB}$, is itself expected to be small. Although it is likely that $\Delta G'_{HB} > \Delta G_{HB}$, since the transition state resembles an oxyanion, the difference is probably not great. Estimates of ΔG_{HB} and $\Delta G'_{HB}$ can be made from literature data. Free energies of formation of hydrogen bonds between simple alkyl esters and methanol are on the order of 0.2 kcal/mol (*13*). Studies with phenol as the hydrogen donor indicate that phenyl esters may form somewhat weaker bonds (*14*), while formamide, which approximates the enzymic hydrogen donor groups, is probably a slightly weaker donor than methanol (*15*). $\Delta G'_{HB}$ can be estimated less closely, as thermodynamic data of hydrogen bonding to anions are far less abundant. Singh *et al.* (*16*) found the free energy of hydrogen bond formation between iodide ion and methanol in CCl_4 solution to be 2.2 kcal/mol; this decreased to 1.1–1.5 kcal/mol for other simple alcohols. Taylor and Kuntz (*17*) report 1.5 and 2.0 kcal/mol for Br^-–H_2O and Cl^-–H_2O bonds, respectively, in chloroform solution; Benoit *et al.* (*18*) report 1.2 and 1.1 kcal/mol for Cl^-–H_2O and Cl^-–$HOCH_3$ in sulfolane solution. Data for oxyanions are extremely limited, but it has been observed that in CH_2Cl_2 picrate hydrogen bonds less strongly to phenol than does the much less basic chloride by 1.9 kcal/mol (*17*). Values of up to 5 kcal/mol are reported for hydrogen bonds to phenolates, with *m*-nitrophenolate a better acceptor than picrate by 4.7 kcal/mol (*19*); These, however, are for *p*-bromophenol, a rather good hydrogen donor. Although extrapolation is difficult, it seems unlikely that $\Delta G'_{HB} - \Delta G_{HB}$ should exceed a few kilocalories.

Second, since a perturbation of electron withdrawal will act in the same direction on ΔG_{HB} and on $\Delta G'_{HB}$, the net change, $\delta(\Delta G'_{HB} - \Delta G_{HB})$, will be even smaller.

The analysis is complicated, however, by considerations of unusual entropy factors which are likely to apply at the active site of an enzyme. In free solution, hydrogen bond formation with solvent is invariably accompanied by an entropy loss, presumably caused by greater order imposed on the polymeric configuration of the solvent (*20*). For particular classes of acceptors ΔS_{HB} is linearly correlated with ΔG_{HB} (*13*, *21*). In other words a more or less constant fraction of the enthalpy gained in hydrogen bond

formation is repaid as entropy cost. However, in some reported series (*22*, *23*) the entropy cost seems to be constant and independent of enthalpy gain; it is clear that over differing kinds of compounds the correlation is only approximate (*24*). Examination of the limited thermodynamic data on hydrogen bonding to anions (*16–18*) reveals no correlation whatsoever between ΔG_{HB} and ΔH_{HB}.

This entropy cost is important because it is usually assumed to be absent or severely reduced in enzymic reactions and to constitute a significant factor in enzymic catalysis (*25*, *26*). Particularly, as discussed for the serine protease case (*26*), if hydrogen bonding of free substrate costs entropy but hydrogen bonding of enzymic transition state does not, since the required hydrogen donors are already fixed in their proper positions, then the difference results in a contribution to the rate acceleration.

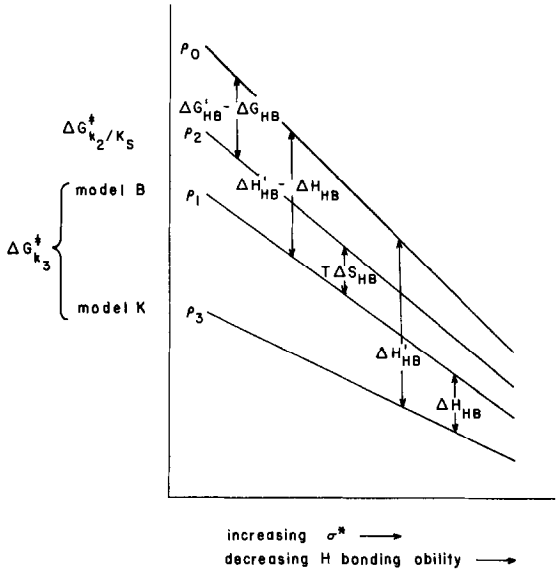

FIG. 3. Idealized structure–reactivity correlations for reactions assisted in various ways by hydrogen bonding as a function of electron withdrawal. See text for discussion.

For purposes of this work we can now note the following general predictions made by both mechanism K and mechanism B on the effect of electron withdrawal on the parameter k_2/K_s.

1. The primary effect of electron withdrawal will be to stabilize the development of negative charge in the transition state, independent of any effects on hydrogen bonding. A model would be the (hypothetical) gas-phase general base-catalyzed attack of water on a series of haloacetate esters of serine. This effect will be large, certainly larger than the $\rho^* = 2.07$ of Fig. 2 for the direct hydroxide attack on our series of phenyl esters. This is because the attack of water on esters is known to be more sensitive to electronic factors than is the attack of hydroxide (*27*) and also because the hydroxide reaction is in solution and subject to hydrogen bonding effects. Let us call the sensitivity of this hypothetical model reaction to electron withdrawal ρ_0, as shown in Fig. 3.

2. The effect of hydrogen bonding will be to diminish the sensitivity of k_2/K_s to electron withdrawal. The resulting sensitivity, represented by ρ_1 in Fig. 3, is thus smaller

than ρ_0. In other words as electron withdrawal increases the reaction rate increases, but part of this increase is offset by weaker hydrogen bonding. The amount of this offset is limited, however, by the size of $\Delta G'_{HB} - \Delta G_{HB}$ and by the fact that electron withdrawal decreases the strength of hydrogen bonding in both reactants and transition state.

3. To the extent that ΔS_{HB} is correlated with ΔH_{HB}, the sensitivity of k_2/K_s will increase to ρ_2, as shown in Fig. 3. This assumes $\Delta S'_{HB}$ is approximately zero or at least is constant for different esters. Clearly $\rho_1 \leq \rho_2 < \rho_0$, since the entropy lost in hydrogen bond formation is only a part of the enthalpy gain. If ΔS_{HB} is constant with electron withdrawal, then $\rho_1 = \rho_2$.

Effect on k_3

Mechanism K and Mechanism B vary with respect to their predictions about the kinetic parameter k_3. Although the mechanisms postulate identical transition states, the reactant (now the acyl enzyme) is stabilized by hydrogen bonds in mechanism B but not in mechanism K. The predictions of mechanism B about the sensitivity of k_3 to electron withdrawal are much the same as those about that of k_2/K_s. The reactants are similarly hydrogen bonded in each case. The only difference is the entropy factor, since the acyl enzyme will be hydrogen bonded to the enzyme, presumably at little or no entropy cost. Thus the prediction of mechanism B for the sensitivity of k_3 is best represented by ρ_1.

Mechanism K, however, supposes stabilization of only the transition state by hydrogen bonding. Thus $\Delta G^{\ddagger}_{k_3}$ is lowered from the hypothetical gas-phase model reaction by the full amount of hydrogen bonding energy. This is best estimated by $\Delta H'_{HB}$ as opposed to $\Delta G'_{HB}$; again the entropy factor is assumed to be small for hydrogen bonding from the enzyme. Since $\Delta H'_{HB}$ increases as the reaction rate decreases, the result is still further diminished sensitivity to electron withdrawal, designated ρ_3. Figure 3 shows that ρ_3, predicted by mechanism K, is less than ρ_1, predicted by mechanism B, by the amount $\delta(\Delta H_{HB})$; that is, the change in hydrogen bonding enthalpy in solution caused by electron withdrawal.

How much is this change expected to be? For hydrogen bonding to phenol, the change in H acceptor from CH_3CO_2Et to $ClCH_2CO_2Et$ reduces ΔH_{HB} by 1.84 and ΔG_{HB} by 0.49 kcal/mol (*14*). More recent work, based on a variety of independent measurement techniques, gives excellent correlation with the latter value (*28*). Several studies (*22*) show that the sensitivity of ΔH_{HB} or ΔG_{HB} to acidity of the hydrogen donor is nearly constant as the basicity of the hydrogen acceptor changes over 18 orders of magnitude. Therefore the *change* of 1.84 kcal/mol is a reasonable estimate when an enzymic amide group (*5*, *6*) is hydrogen donor, although the absolute magnitudes of hydrogen bond energies to both CH_3CO_2Et and $ClCH_2CO_2Et$ will be smaller for the enzyme than for phenol. If one makes the further assumption, which usually holds within a given class of base (*24*), that ΔH_{HB} is correlated with the pK_a of the conjugate acid and therefore with σ^*, one calculates $\rho_1 - \rho_3 = 1.3$. In other words, mechanism K predicts a value of $\rho^*_{k_3}$ smaller than that predicted by mechanism B by a significant and easily measurable amount.

In practice, of course, one simply measures a value for $\rho^*_{k_3}$, as we have done and have shown in Fig. 2. This must be compared to something, since the above analysis supplies only the difference between predictions of the two mechanisms. The value of $\rho^*_{k_2/K_s}$, also

shown in Fig. 2, can provide the necessary comparison. Mechanism B predicts $\rho_2 \geq \rho_1$, or $\rho^*_{k_2/K_S} \geq \rho^*_{k_3}$, with the difference small but attributable to entropy–enthalpy compensation in hydrogen bonding to the reactant in free solution. Mechanism K, however predicts $\rho^*_{k_2/K_S} > \rho^*_{k_3}$ by *at least 1.3* and by more if entropy–enthalpy compensation is important. The results, which show $\rho^*_{k_3}$ is if anything slightly greater than $\rho^*_{k_2/K_S}$, strongly support mechanism B.

Although there have been many structure–reactivity studies of chymotrypsin catalyzed reactions (*29*), none has been addressed to the question of differential hydrogen bonding. We have accordingly selected a series of substrates where strong electron withdrawal allows the best chance of detecting a difference in values of the kinetic parameters predicted to result from such bonding. As Fig. 2 shows, no such large difference could be found.

These conclusions should be tempered by remembering that the substrates studied here are nonspecific and presumably fit somewhat loosely into the active site of the enzyme. It is possible that as specificity increases, the substrate binds more rigidly with concomitant weakening of hydrogen bonding in the Michaelis complex and acyl enzyme. This would be consistent with the data of Williams (*30*) on aryl hippurates and with the general observation (*26*, *31*) that increasing substrate specificity is reflected in increases in k_2 and k_3 rather than in decreases in K_s. Such an effect would constitute a precise and elegant mechanism for the expression of substrate specificity in serine proteases and, given the ubiquity of the carbonyl group in biological chemistry, may be of general importance. Experiments with more specific substrates are currently underway in these laboratories to test this possibility.

ACKNOWLEDGMENTS

Acknowledgment is made of the donors of the Petroleum Research Fund, administered by the American Chemical Society, and of the New York University Committee on Institutional Grants, for partial support of this research.

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