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Rate Enhancement by Catalytic Groups in Enzymes. Imidazole Catalysis of the Hydrolysis of *N,O*-Diacetylserinamide as a Model for General Base Catalysis in Chymotrypsin

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An attempt to estimate the importance of general acid-base catalysis in enzymic catalysis has been made, using the hydrolysis of the ester group of *N,O*-diacetylserinamide as a model for the deacylation of acyl-chymotrypsins. General base catalysis of this reaction by imidazole is estimated to reduce the activation energy by at least 31 kJ mol^{-1}. The rate of reaction, however, is not greatly enhanced because of an unfavourable change in the entropy of activation from −132 to −197 J K^{-1} mol^{-1}. At about 300 K, a typical temperature for enzyme-catalysed reactions, the reduction in activation energy would cause a rate enhancement of about 3×10^5-fold if the unfavourable entropy change did not occur. For specific acyl-chymotrypsins the entropy of activation for deacylation is about −89 J K^{-1} mol^{-1}, allowing the full effect of general base catalysis by imidazole to be realized. It is, therefore, postulated that in the active site of an enzyme, a properly oriented imidazole side chain may catalyse the rate of a reaction 10^5-fold by general base catalysis.

The factors contributing to the great effect of enzyme catalysis on reaction rates have been the subject of active discussion among biochemists. Most recent discussion has concentrated on the enhancement of reaction rates by reduction in the magnitude of the entropy of activation $\Delta S^{\ddagger}$, either by the freezing out of rotational entropy, "orbital steering" (*1–5*), or by the freezing out of translational entropy (*6*). Both of these approaches involve freezing out of entropy in the reactants by binding to the enzyme, and both predict rate enhancements of the order of 10^4 or greater for a bimolecular reaction. Recent discussion of rate enhancement has mostly been limited to enhancement by binding two substrate molecules, and the role of catalytic groups in the active site of an enzyme has not been sufficiently considered.

This paper considers catalysis by reactive groups in the active site, other than those directly involved in covalent intermediate formation. The effect of these groups on the energy and entropy of activation is discussed. The contribution of general base catalysis

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to enzymic catalysis has been studied using imidazole catalysis of the hydrolysis of *N,O*-diacetylserinamide as a model for the deacylation of acyl-chymotrypsins.

RESULTS AND DISCUSSION

Energy and Entropy of Activation in Enzyme Catalysis

The role of catalytic groups has been briefly considered by Storm and Koshland (*1, 2*) who suggested that the reaction rate may be increased by a factor of 10^4 for each catalytic group correctly aligned in the active site. This approach implies that the principal contribution of a catalytic group is orientational and hence an enhancement of 10^4-fold could be attributed to a group which has only a minor role in the reaction and is only capable of enhancing the reaction rate, say, 10-fold. Furthermore such orientation effects (or effects from freezing of translational motions) would appear in the entropy term of Eq. (1); each correctly aligned catalytic group would increase exp $(\Delta S^{\ddagger}/R)$ by a factor of 10^4.

$$k_{\text{rate}} = \frac{kT}{h} \exp\frac{\Delta S^{\ddagger}}{R} \exp\frac{-\Delta E^{\ddagger}}{RT} \tag{1}$$

However, the effect of a catalytic group is to lower the energy of activation, $\Delta E^{\ddagger}$. The data in Table 1 indicate that the activation energies for enzyme-catalysed reactions are much less than for the uncatalysed reactions. The maximum possible rate

TABLE 1

ACTIVATION ENERGIES FOR UNCATALYSED AND ENZYME-CATALYSED REACTIONS[a]

Substrate	Enzyme	$\Delta E^{\ddagger}_0$ (kJ mol^{-1})	$\Delta E^{\ddagger}_{\text{enzyme}}$ (kJ mol^{-1})	Possible rate enhancement[b]
H_2O_2	Catalase	75	<8	$>10^{11}$
Casein	Trypsin	87	50	10^6
Sucrose	Invertase	107	<42	$>10^{11}$
β-Methyl glucoside	β-Glucosidase	137	51	4×10^{14}

[a] Data from A. White, P. Handler, and E. Smith, "Principles of Biochemistry," 4th ed, p. 233. McGraw-Hill, New York, 1968.

[b] Calculated using Eq. (2).

enhancements due to the reduction in $\Delta E^{\ddagger}$, calculated according to Eq. (2), are also given in Table 1. At 300 K the rate would increase by an order of magnitude for each 5.7 kJ mol^{-1} decrease in $\Delta E^{\ddagger}$, if $\Delta S^{\ddagger}$ remained unchanged.

$$\frac{k_{\text{cat}}}{k_0} = \exp\frac{\Delta S^{\ddagger}_{\text{cat}} - \Delta S^{\ddagger}_0}{R} \exp\frac{\Delta E^{\ddagger}_0 - \Delta E^{\ddagger}_{\text{cat}}}{RT}. \tag{2}$$

$\Delta S^{\ddagger}$ may become more negative if the introduction of a catalytic group increases the ground state entropy. The role of freezing of rotational or translational motions in the

enzyme–substrate complex is to ensure that the rate enhancement is not significantly diminished by such a decrease in $\Delta S^{\ddagger}_{u}$. A large part of the rate enhancement due to enzyme catalysis can be accounted for by the lowering of the activation energy (cf Table 1), and the problem that arises from this discussion is the explanation of how activation energies are reduced sufficiently to provide the observed magnitude of catalysis.

General Base Catalysis in Proteolytic Enzymes

Since many enzymes, particularly hydrolases, have acidic or basic groups at the active site, it has been suggested that acid–base catalysis may contribute to the rate acceleration by these enzymes. However, this type of catalysis has been considered (*7, 8*) to make only a small contribution to the overall catalysis, about a factor of 10. This figure is based on comparisons of uncatalysed and imidazole-catalysed hydrolyses of esters as models for the action of chymotrypsin.

The catalytic reaction for chymotrypsin involves the attack of a reactive serine hydroxyl group on the carbonyl carbon of the ester to form an acyl-enzyme intermediate, followed by hydrolysis of the acyl-enzyme (*9*). The hydrolytic step is usually rate limiting and is likely to be the step where enhancement effects are most easily seen. As a model for this step, the hydrolysis of the ester linkage of *N,O*-diacetylserinamide has been studied (*10*). The reaction is subject to general base catalysis by imidazole and the extent of this catalysis can be determined.

The pseudo-first-order rate constants for the hydrolysis of *N,O*-diacetylserinamide at three imidazole concentrations are given in Table 2. The rate constant for spontaneous

TABLE 2

KINETIC PARAMETERS FOR THE HYDROLYSIS OF *N,O*-DIACETYLSERINAMIDE (pH 7.07)

	$10^3 \times k_1$[a] (min^{-1})				
T (K)	0.25 M imidazole	0.5 M imidazole	1.0 M imidazole	$10^3 \times k_2$[b] (M^{-1} min^{-1})	$10^3 \times k_{H_2O}$[c] (min^{-1})
373	3.35	5.5	9.9	15.8	1.20
363	2.26	3.4	5.8	8.7	1.03
353	0.96	1.62	2.88	4.8	0.35
344	0.79	1.33	2.30	3.7	0.35

[a] Observed pseudo-first-order rate constant.
[b] Second-order rate constant for imidazole-catalysed hydrolysis.
[c] First-order rate constant for water-catalysed hydrolysis.

hydrolysis, k_{H_2O}, was obtained from the intercept of a plot of rate constant against imidazole concentration, and is given in Table 2. This represents neutral hydrolysis since the intercept differs little from pH 7.1 to pH 8.1 (*10*). The slope of this line was corrected for the fraction of imidazole in the base form, 0.54 at pH 7.07, to give the pseudo-second-order rate constant for the imidazole-catalysed reaction, k_2, in Table 2. Since the catalysed and uncatalysed reactions are of different order, the extent of

catalysis must be calculated at a particular imidazole concentration. At a free base concentration of 1 *M*, the rate enhancement is only about 13-fold.

This factor is not, however, a realistic measure of catalysis by imidazole. The neutral hydrolysis of esters usually involves general base catalysis by a second water molecule (*11, 12*). The reaction has a large negative entropy of activation but the high water concentration and lowered energy of activation allow this reaction to predominate over the uncatalysed reaction. Comparison of the imidazole-catalysed reaction with the observed water reaction would only give a comparison of general base catalysis by imidazole with that by water. The total effect of general base catalysis by imidazole on the hydrolysis of the ester can only be estimated by comparison of the imidazole-catalysed hydrolysis with the (bimolecular) unaided attack of water on the ester. Unfortunately, it is impracticable to measure the rate of this reaction, as the water-catalysed reaction is much faster, but the uncatalysed reaction is clearly more than 13-fold slower than the imidazole-catalysed reaction. The imidazole-catalysed reaction, however, is termolecular, and the formation of the transition state involves loss of entropy for the interacting substrate molecules as for the uncatalysed reaction, plus a further loss of entropy for the interaction of the imidazole molecule with the substrate molecules, mostly due to additional translational (*6*) or rotational (*2*) constraints in the transition state (cf *13, 14*). Therefore, the effect of imidazole on $\Delta E^{\ddagger}$ may be offset by a decrease in $\Delta S^{\ddagger}$ and the effects of imidazole on $\Delta E^{\ddagger}$ and $\Delta S^{\ddagger}$ should be separated.

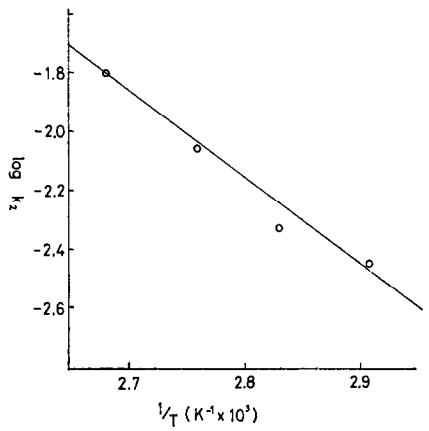

FIG. 1. Arrhenius plot for imidazole-catalysed hydrolysis of *N,O*-diacetylserinamide. The line was obtained by an unweighted least-squares treatment.

The activation parameters for the imidazole-catalysed reaction were obtained from an Arrhenius plot of log k_2 vs $1/T$ (Fig. 1). The energy of activation, obtained from the slope of the least-squares line, is 56 kJ mol^{-1} and the entropy of activation (at 1 *M* water), calculated from the intercept is, -197 J K^{-1} mol^{-1}. The activation parameters for the uncatalysed hydrolysis cannot be obtained directly because of the general base catalysis by water, but reasonable estimates can be obtained.

The entropy of activation for a reaction between two nonlinear molecules can be calculated from transition-state theory (*15*) to be in the range -134 to -153 J K^{-1} mol^{-1}.

A better estimate of $\Delta S^{\ddagger}$ for the uncatalysed reaction should, however, be obtained by comparison with a similar reaction (*15*) such as the alkaline hydrolysis of an ester, which is also bimolecular and has a similar mechanism (*16*). Although hydroxide ion and water differ greatly in reactivity, this difference will mainly appear in $\Delta E^{\ddagger}$, not $\Delta S^{\ddagger}$. Transition-state theory predicts that the values of $\Delta S^{\ddagger}$ for bimolecular reactions will be similar (*15*), and Bruice has shown that the values of $T\Delta S^{\ddagger}$ for a series of reactions of esters depend mainly on the kinetic order of the reactions (*13, 14*).

From the second-order rate constants for the alkaline hydrolysis of the methyl, ethyl, and isobutyl esters of acetic acid (*17*), $\Delta S^{\ddagger}$ was calculated to be about -113 J K^{-1} mol^{-1} (*18*). Since the ester linkage of *N,O*-diacetylserinamide has the same acyl group as these esters and a similar alcohol function to the isobutyl ester, it is reasonable to assume that $\Delta S^{\ddagger}$ for the alkaline hydrolysis of the serine ester will also be approximately -113 J K^{-1} mol^{-1}. The value of $\Delta S^{\ddagger}$ for the neutral hydrolysis will probably be about 19 J K^{-1} mol^{-1} more negative because the water molecule is not linear (*15*), giving an estimated $\Delta S^{\ddagger}$ of -132 J K^{-1} mol^{-1}. This correction is a small one in comparison with the total entropy changes, and will reduce our final estimate of the catalysis by imidazole. The value of -132 J K^{-1} mol^{-1} is, therefore, taken as a working estimate of the entropy of activation for the uncatalysed (bimolecular) neutral hydrolysis of *N,O*-diacetylserinamide.

This value and that for the imidazole-catalysed reaction are consistent with the observation by Bruice (*13, 14*) that at 298 K ($-T\Delta S^{\ddagger}$)/(kinetic order) for nucleophilic displacement on an ester is about 17–21 kJ mol^{-1}; this predicts that $\Delta S^{\ddagger}$ will be about -110 to -140 J K^{-1} mol^{-1} for a bimolecular reaction and about -165 to -210 J K^{-1} mol^{-1} for a termolecular reaction. The difference between the $\Delta S^{\ddagger}$ values for the catalysed and uncatalysed reactions corresponds to a decrease in rate of 2.5×10^3-fold because of the additional entropic constraints in the termolecular transition state. This factor, which may contain both rotational and translational contributions, is comparable with the factor of 10^4 postulated for the orientation of interacting groups (*1*), but it appears as a negative effect masking the true catalytic power of the imidazole.

Since the unaided attack of water is slower than the observed water-catalysed reaction, calculation from k_{H_2O} yields a minimum value of $\Delta E^{\ddagger}$ for the former reaction. Using Eq. (2) and the values of k_{H_2O} in Table 2, we estimate that $\Delta E^{\ddagger}$ for the uncatalysed reaction is at least 87 kJ mol^{-1}. This is at least 31 kJ mol^{-1} greater than for the imidazole-catalysed reaction, equivalent at 350 K to a rate ratio of 3×10^4-fold. At about 300 K, where chymotrypsin-catalysed reactions are normally studied, this reduction in $\Delta E^{\ddagger}$ by imidazole would correspond to an acceleration of about 3×10^5-fold, in the absence of entropic effects.

The activation parameters for the imidazole-catalysed and uncatalysed hydrolyses of *N,O*-diacetylserinamide may now be compared with those for the deacylation of acyl derivatives of α-chymotrypsin (Table 3). The values of $\Delta E^{\ddagger}$ indicate that most of the catalysis observed in the enzyme-catalysed reaction can be accounted for by general base catalysis by the imidazole side chain of histidine 57. The additional reduction in $\Delta E^{\ddagger}$ by the enzyme may be partly due to increased basicity of the imidazole group produced by participation of the carboxyl side chain of asp 102 (*19*), although this contribution is probably not very large (*20*).

The entropy of activation for acetyl-chymotrypsin is very large, comparable to that

TABLE 3

ACTIVATION PARAMETERS FOR THE HYDROLYSES OF *N,O*-DIACETYLSERINAMIDE AND ACYL DERIVATIVES OF α-CHYMOTRYPSIN

	ΔE^* (kJ mol^{-1})	ΔS^* (J K^{-1} mol^{-1})
N,O-Diacetylserinamide (uncatalysed)	>87	−132
N,O-Diacetylserinamide (imidazole catalysed)	56	−197
Acetyl-chymotrypsin[a]	40	−183[b]
N-Acetyltyrosyl-chymotrypsin[a]	43	−89[b]

[a] Data are from Bender et al. (21).
[b] At 1 *M* water.

for the imidazole-catalysed hydrolysis of diacetylserinamide. Since the acetyl group has neither an aromatic nor an amide function it is bound to the enzyme only by the covalent bond to serine 195. It is, therefore, subject to few rotational or vibrational constraints, and there is a large loss of entropy on proceeding to a relatively rigid transition state. For the *N*-acetyltyrosyl derivative, ΔS^* is lower than that estimated for the uncatalysed hydrolysis of diacetylserinamide. The aromatic side chain and amido function are bound strongly and specifically to binding sites in the enzyme, holding the ester function in the correct position and orientation (*21*, *22*). The reduced rotational and vibrational freedom of the acyl-enzyme ensures a low value of ΔS^* and the full-rate enhancement from the lowering of ΔE^* by the imidazole group is realised.

CONCLUSION

General base catalysis by imidazole can account for a large part of the catalysis of the deacylation of acyl-chymotrypsins. This contribution is much greater than previously considered (*7*). Such catalysis is also likely to occur in the acylation step of chymotrypsin-catalysed hydrolyses (*9*). However, catalysis will only be observed in the rate-determining step, which for esters is the deacylation step. Since hydrolyses catalysed by thiol proteases have a similar mechanism and also involve a histidine residue, general base catalysis by imidazole may also make a significant contribution for these enzymes.

The evidence presented in this paper suggests that a large part of enzymic catalysis is likely to arise from the reduction in activation energy by catalytic groups, in the case of proteolytic enzymes, at least, by acid-base catalysis. The enzyme is, however, so much better than solution catalysts because, although its energy of activation is favourable, solution catalysis incurs a very unfavourable entropic factor. Enzymic catalysis and solution catalysis both reduce ΔE^* (Fig. 2a) but ΔS^* for the enzyme-catalysed reaction is similar to that for the uncatalysed reaction (Fig. 2b). This ensures that the catalysis is not significantly offset by the large negative entropy of activation present in solution catalysis (Fig. 2b).

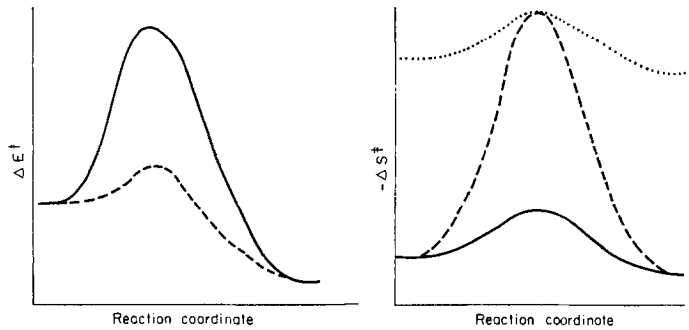

FIG. 2. The effect of catalysts on the energy and entropy of activation of a reaction. (a) Hypothetical energy profiles for a reaction. ——, uncatalysed. – – –, catalysed by enzyme or solution catalyst. (b) Hypothetical entropy profiles for a reaction. ——, uncatalysed, – – –, solution catalysis. · · · ·, enzymic catalysis.

EXPERIMENTAL

N-Acetylserinamide was prepared from DL-serine methyl ester (Sigma) by the method of Rothstein (*23*). The crude product (which contained a mixture of *N*-acetylserinamide and *N,O*-diacetylserinamide) was then acetylated according to Anderson et al. (*10*). Imidazole buffers were prepared by titration of imidazole with HCl to pH 7.07 and made up to ionic strength 0.46 with NaCl.

Hydrolyses of solutions of *N,O*-diacetylserinamide (1.5 mg/ml) were carried out in an oil bath at 100, 90, 80, and 71°C, using 0.5 ml samples in sealed tubes. The ester content of each sample was determined using a modified version of the ferric–hydroxamic acid method (*10*) with double quantities of all reagents. The absorbance of each sample was read on a Unicam SP 500 spectrophotometer. The pseudo-first-order rate constant at each buffer concentration and temperature was obtained from semilogarithmic plots of ester concentration against time.

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