Viscosity-Dependent Structural Fluctuations in Enzyme Catalysis†

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ABSTRACT: The effect of viscosity on the rate of catalysis of carboxypeptidase A has been tested. By use of the tripeptide carbobenzoxy-L-alanyl-L-alanyl-L-alanine [Z(L-Ala)$_3$] as substrate, it was shown that most of the effect on the hydrolysis rate caused by the presence of 30 or 40% methanol or glycerol in aqueous solution can be ascribed to a contribution of viscosity to the catalytic rate constant, k_{cat}. Arrhenius plots of k_{cat} in 30 and 40% glycerol or methanol are linear and almost parallel. When the rate constants are "corrected" for the viscosity of various media, the difference between the various Arrhenius plots is considerably reduced; it vanishes, within experimental error, when the effect of the dielectric constant of the solutions is taken into account as well. It is proposed that the viscosity of the medium can influence the rate-limiting step of the enzymic reaction, which is the rate of transitions over the energy barrier preceding product formation. According to the suggested mechanism, the enzyme–substrate complex can overcome this energy barrier by viscosity-dependent structural fluctuations. The quantitative agreement between the theory and the experimental results suggests that (a) due to the temperature dependence of the viscosity of the solution, the potential energy barrier of the reaction is about 5 kcal/mol lower than the observed activation energy and (b) information about the structural flexibility of the complex can be obtained by kinetic measurements.

The factors responsible for the powerful catalysis observed in enzymes have been extensively studied. These include induced fit (Koshland & Neet, 1968), entropy loss (Page & Jencks, 1970), substrate strain or destabilization (Jencks, 1975), orbital steering (Storm & Koshland, 1970), stereopopulation control (Milstein & Cohen, 1970), desolvation (Cohen et al., 1970), electrostatic catalysis (Warshel & Levitt, 1976), and a variety of other concepts which have been invoked in order to explain the large rate enhancements (up to a factor of 10^{13}) provided by enzyme catalysis.

Another aspect of this problem deals with the identification of the rate-limiting step of enzyme catalysis. The reaction rate at saturating substrate as well as at low product concentration is believed to be limited by the rate of transitions over some energy barrier leading to product formation. The diffusion of substrate to the active site can be shown to occur at rates too high to be rate limiting for most of the enzymic reactions (Eigen, 1974). On the other hand, substantial evidence has accumulated to suggest that in many cases conformational changes that lead to product release are the rate-limiting steps of the reaction (Cleland, 1975; Boyer, 1974).

Since some of the factors affecting the rate of catalysis may contribute to the energy barrier of the reaction, the above two fundamental aspects may be related when we answer the following questions: what is the mechanism by which the enzyme, or its enzyme–substrate complex, overcomes an energy barrier and what are the parameters which are involved in this process? A theoretical solution of the latter problem, which is supported by experiments, has not yet been found.

The modeling of a chemical reaction by Brownian motion in the presence of a potential energy barrier has been suggested by Kramers (1940), who gave an explicit expression for the absolute rate of transitions over the barrier. The latter includes structural parameters, which characterize the profile of the potential energy as a function of a one-dimensional "reaction coordinate", and the viscosity of the medium (see Theory). Kramers' theory is strongly supported by experiments, in which the rate of conformational transitions of polymers in solution has been measured as a function of the viscosity and the temperature of the solution (Mashimo et al., 1975; Mashimo, 1976; Bullock et al., 1974).

The possibility of applying Kramers' theory to the description of dynamic states of proteins involved in enzymic catalysis has been recently suggested by one of us (Gavish, 1978). This theory may provide an answer to the question of how enzymes overcome the energy barrier. The experimental test must involve measurements of the rate of catalysis as a function of the viscosity and the temperature of the medium. This can be done very conveniently by measurement of the activity of an enzyme in a range of mixed aqueous–organic solutions having different viscosities, while other factors, such as proton activity and ionic strength, are kept constant.

In this communication, we report our experiments to test the effect of viscosity on the rate of catalysis of carboxypeptidase A. The results are interpreted quantitatively in light of structural fluctuations, whose occurrence during enzymic catalysis has been suggested (Gavish, 1978) and whose frequency is inversely proportional to the viscosity of the medium. A mechanism is hereby proposed for overcoming the energy barrier of an enzymic reaction by such fluctuations.

Theory

Model for the Mechanism of Overcoming an Energy Barrier during Catalysis. Let us consider a reaction in which a substrate S is converted into a product P by the enzyme E. The last cleaves a specific bond in S during the process of conformation transition ES → EP (Figure 1a). We define as the "reaction coordinate" a distance between two atoms in the proximity of the scissile bond, the length of which is changed monotonically during the transition. It is assumed that at every given moment the length of the scissile bond uniquely determines the value of a *microscopic* potential energy of the complex (Laidler, 1950) (Figure 1b). It must be emphasized that this description is a phenomenological one and it provides no information about specific factors, which are likely to be involved in catalysis.

By use of the ideas of the Brownian theory of motion in a field of force, it has been suggested (Kramers, 1940; Gavish,

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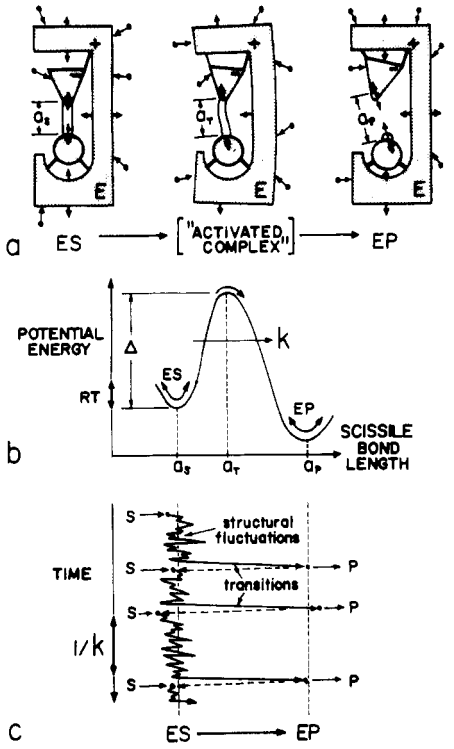

FIGURE 1: Transient strain model of enzymic catalysis (Gavish, 1978). (a) Discrete catalytic steps during the cleavage of a specific bond (unshaded) in a substrate S (heavily shaded), which forms a complex with the enzyme E to give a product P (heavily shaded). The binding of E to either S or P may include electrostatic interaction between charges (shown). The bond breaking occurs during the transition through the "activated complex", shown during its decay to EP (note the distortion in E and S). The latter dynamic process is the result of structural fluctuations. These originate from the collisions of the solvent's molecules (black circles with arrows) with the complex, accompanied by various modes of motion of parts of the complex through the solvent (small arrows), which acts to dissipate their kinetic energy. a_S, a_T, and a_P are the lengths of the scissile bond at the discrete states shown above. (b) Description of the catalysis by means of potential energy vs. scissile length. Structural fluctuations (double-edge arrows) possess an average thermal energy, RT. The conformational transitions ES → EP occur at rate k by overcoming an energy barrier of height Δ. (c) The evolution in time of structural fluctuations and conformational transitions. The horizontal dashed lines represent the assumption that after releasing the product the enzyme rapidly reaches its initial state. The steps of formation or breaking the complex (denoted by points) are assumed to be relatively rapid processes.

1978) that the dynamic state of the molecular structure of the enzyme–substrate complex is governed by its interaction with the solvent molecules through the action of two competing processes: random collisions and frictional forces. The net action of the former is to *increase* the kinetic energy of the various modes of motion of the structure, and that of the latter is to *dissipate* their energy by viscous damping. As a result of these processes the dynamic state of the complex may be described by structural fluctuations, whose randomness gives the structure a finite probability to reach any degree of strain and to overcome the energy barrier, i.e., the occurence of a conformational transition. These are the main features of the "transient strain" model of enzymic catalysis (Gavish, 1978). For more details see the legend of Figure 1.

The quantitative description of the process results in the expression (Kramers, 1940; Gavish, 1978)

$$k = (A/\eta) \exp(-\Delta/RT) \qquad (1)$$

where k and Δ are the rate of transitions over the energy barrier and its height in the direction S → P, respectively, R is the gas constant, T is the absolute temperature, and η is the viscosity of the solution. A is an explicit function of structural parameters which characterized the profile of the potential energy curve (Kramers, 1940; Gavish, 1978) (Figure 1b). It may serve as a measure of the structural flexibility of the complex, with respect to variations in the scissile bond length for the reaction ES → EP. It has been shown that A is directly proportional to the natural frequency of spring-like oscillations of ES in a vacuum. Thus, the more rigid the complex, the higher this frequency, which results in an increase of the rate of transitions over the energy barrier.

It can be shown that the structural fluctuations of ES around the length a_s of the scissile bond (Figure 1c) may be visualized as the motion of a mass connected to a spring embedded in a viscous liquid and which is acted upon by random forces (Imry & Gavish, 1974). The average mean square amplitude of these fluctuations corresponds to a content of thermal energy $RT \sim 0.6$ kcal/mol at room temperature.

The decrease of k with increasing η is the result of stronger friction forces. These forces diminish the larger amplitude structural fluctuations that are favorable for conformational transitions (Figure 1c).

When a chemical reaction involves electrostatic interaction between ions in the solution (Figure 1a), its contribution to the rate of catalysis is taken into account by addition of the quantity $-B/\epsilon$ to the height of the energy barrier (Laidler, 1950). ϵ is the dielectric constant of the solution. If two ions having valencies Z_1 and Z_2 are separated initially by the distance d_i and finally by d_f, B is given by the expression

$$B = 332 Z_1 Z_2 / d \qquad \text{(kcal/mol)} \qquad (2)$$

where d (in angstroms) is defined by the relation $1/d = 1/d_i - 1/d_f$. It was argued that the description of the effect by two electric charges interacting in a continuous and homogeneous medium is oversimplified [Kirkwood & Westheimer (1938); Sturtevant (1955); Laurence (1955)] due to local effects. Nevertheless, the contribution $\exp(-B/\epsilon RT)$ to the reaction between simple ions (Harned & Samaras, 1932) and for a few examples of enzyme-catalyzed reactions [Kaufman & Neurath (1949); Laidler & Bunting (1973)] has been verified. The value of $d/|Z_1 Z_2|$ was found to be a few angstroms. We shall use this phenomenological law in order to eliminate the possible contribution of ϵ to the reaction rate, with $d/|Z_1 Z_2|$ as an adjustable parameter.

The generalization of eq 1 for the effect of the dielectric constant on the rate of conformation transitions is the following:

$$k = (A/\eta) \exp(-\Delta/RT + B/\epsilon RT) \qquad (3)$$

or

$$\ln k + \ln \eta - B/\epsilon RT = \ln A - \Delta/RT \qquad (4)$$

Finally, the above discussion on the transition ES → EP (Figure 1) may equally apply to other processes in which barriers have to be overcome, such as substrate binding or product release.

Choice of Experimental System to Test the Theory. (1) Choice of Enzyme and Substrate. The following considerations were taken into account in the choice of the enzyme–substrate system in order for us to increase the chance of probing the predicted effects and of relating them to some specific steps during the catalysis. (a) A great deal of information should be known about the enzyme studied, including its three-dimensional structure. (b) The enzyme should consist of a single-polypeptide chain, i.e., a monomeric protein, so that no effects arising from subunit interactions might be involved in its catalytic action. (c) The catalytic process should

Table I: Kinetic Parameters for the Hydrolysis of $Z(Ala)_3$ by Carboxypeptidase A[a]

solvent	K_m (mM)	k_{cat} (s^{-1})
water	1.2	62
glycerol, 30% wt	2.0	53
methanol, 30% wt	1.8	76

[a] Conditions: 0.05 M Tris-HCl, 0.1 M NaCl, pH 7.85; temperature 26 °C.

be associated with conformational changes. (d) The substrate should be rather large in order to interact with an extensive portion of the protein. (e) One should be able to follow the reaction in the presence of the cosolvents which are used for changing the viscosity of the solution. (f) The reaction should not be diffusion-controlled.

We chose carboxypeptidase A (CPA) as the enzyme and carbobenzoxy-L-alanyl-L-alanyl-L-alanine [$Z(Ala)_3$] as the substrate. The reaction that was followed is the hydrolysis of the C-terminal peptide bond (Abramowitz et al., 1967). CPA has a molecular weight of 34 600. Its kinetic and structural features have been extensively studied (Lipscomb et al., 1968; Johansen & Vallee, 1975). It has been suggested that conformational changes occur upon binding of peptide substrates, and in particular there is evidence for a conformational change involving Tyr-248 of the enzyme (Johansen & Vallee, 1975; Lipscomb, 1973). $Z(Ala)_3$ is a tripeptide and is therefore able to make many binding interactions with the enzyme (Abramowitz et al., 1967). Furthermore, the anomalies which are observed in the hydrolysis of smaller substrates by CPA disappear with tripeptides (Auld & Vallee, 1970). It has been shown in the case of CPA that water cannot be replaced by methanol in the hydrolysis direction (Breslow & Wernick, 1977). The relatively low values of k_{cat} for this reaction (Table I) ensure that the reaction is not a diffusion-controlled one.

(2) *Choice of the Solvent System.* The following considerations were taken into account in this case. (a) The effect of the cosolvents on protein structure should be relatively small. (b) The observed pH in the mixed solvent should be a meaningful expression of the proton activity. (c) The presence of the cosolvent should affect as little as possible the pK of the functional groups in the enzyme. (d) The cosolvent molecules should be as small as possible and should be present in large enough concentration. This is desirable in order to approach the ideal situation of a continuous medium with respect to the time scale involved in catalytic action. (e) Parameters which may characterize the microstructure of the mixed solvent, such as the ratio of polar to nonpolar groups, should be kept as constant as possible.

Addition of organic solvents to aqueous solutions is known to change the microstructure of water in a rather complex manner (Frank, 1967). Such cosolvents are also known to affect the conformation of protein and to cause changes in enzyme–substrate interactions (Makinen & Fink, 1977). However, it has been shown that mixed aqueous solutions of methanol, glycerol, and several other organic solvents satisfy conditions a, b, and c and are therefore suitable for studies of enzyme catalysis and protein structure in the range from room temperature down to subzero temperatures (Douzou, 1976). Furthermore, even in the presence of large fractions of cosolvents such as methanol and glycerol, the protonic activity is not greatly affected and can be kept constant by the use of appropriate buffer systems (Hui Bon Hoa & Douzou, 1973). It has been demonstrated in general that solvents do not affect the pK values of amines (Bell, 1973). Furthermore, the pK values of carboxylic acid undergo very little change in the presence of methanol, glycerol, and ethylene glycol (Maurel et al., 1975; Tanford, 1968).

Conditions d and e combined with the following specific considerations favor the choice of methanol and glycerol as suitable cosolvents for our experiments, at concentrations of 30 and 40% by weight. (1) By using equal concentrations of methanol and glycerol, we maintain the same concentration of water and the same ratio between the alcoholic groups and the CH_2 groups. (2) Below 20% the microstructure of methanol–water solutions is changed significantly when we varied the methanol concentration (Frank, 1967). (3) The methanol–water solutions have equal viscosities, but different dielectric constants, at concentrations of 30 and 40% methanol (Bingham et al., 1913; Akerlöf, 1932). (4) Glycerol is one of the most viscous organic solvents.

Materials and Methods

Carboxypeptidase A was purchased from Sigma, type II, in the form of an aqueous suspension with toluene added and diisopropyl phosphate treated. The enzyme concentration was determined from its absorbance at 278 nm by use of a molar absorptivity of 6.42×10^{-4} cm^{-2} M^{-1} (Simpson et al., 1963). The substrate $Z(Ala)_3$ was a gift from Miles Yeda, Rehovot. The organic solvents, glycerol and methanol, were of spectroscopic grade.

All rate measurements were performed in 0.1 M NaCl, 0.05 M Tris-HCl buffer, pH 7.85, containing varying amounts of organic solvents, in a thermostated (±0.1 °C) cell component of a Gilford spectrophotometer. The initial velocity of substrate hydrolysis was followed by measurement of the decrease of absorbance at 225 nm. To 1.1 mL of substrate solution, 20 μL of a stock solution of enzyme (prepared freshly every day) was added to give a final concentration of 8.4×10^{-7} M in 3-mL cuvettes with spacers. The light path obtained was 0.2 cm. In most rate measurements in the various mixed solvents, the substrate concentration was 9 mM. K_m determinations were performed over the concentration range of 0.5–10 mM substrate. Maximal k_{cat} and K_m values were calculated from a least-squares treatment of Lineweaver–Burk plots. The tested solvents were water, 30% methanol, 40% methanol, 30% glycerol, and 40% glycerol (percentage by weight).

Results

Effect of Organic Solvents on K_m and k_{cat}. Linear Lineweaver–Burk plots were obtained in all solvent systems tested. A comparison of the kinetic parameters in water, 30% glycerol, and 30% methanol at 26 °C is given in Table I. It is shown that the K_m value increases slightly as the fraction of the organic solvent increases; k_{cat} increases with increasing methanol concentration, but decreases with increasing glycerol concentration.

Temperature Dependence of the Rate of Catalysis. The Arrhenius plots with respect to the hydrolysis rate in the five tested solutions at a substrate concentration of 9 mM are given in Figure 2. The straight lines were obtained by a least-squares treatment of the data. For a given temperature, the experimental error in determination of the rate for a specific solvent is ±(5–7)% when the Arrhenius plot is assumed to be a straight line. Figure 2 shows that in the region 10–35 °C the best-fit straight lines are nearly parallel and the activation energy was deduced from their slope, $E_a \sim 13.5$ kcal/mol, which falls in the range of values found for other substrates (Lumry et al., 1951). The following efficiency of catalysis was observed for the solvents tested: methanol (40%) > methanol

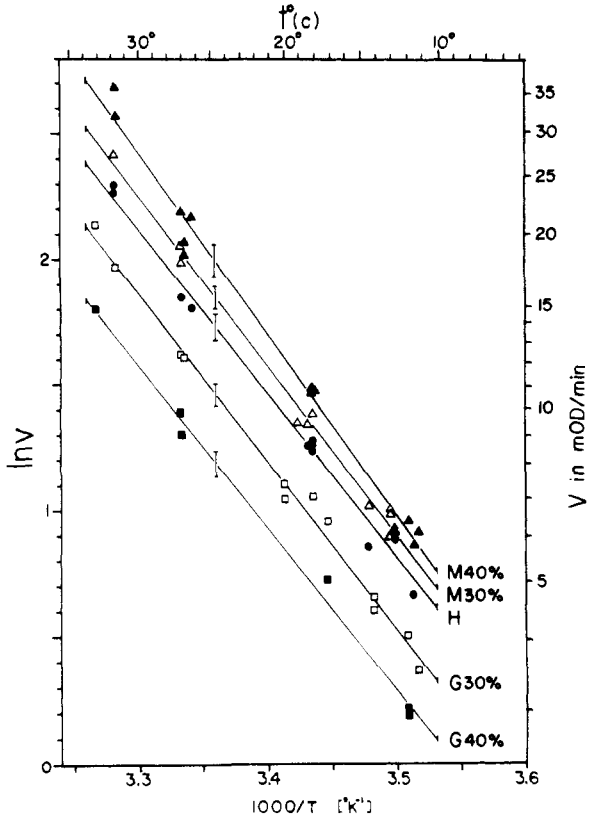

FIGURE 2: Rate of catalysis in different solvents. Arrhenius plots for the hydrolysis rate of $Z(Ala)_3$ carboxypeptidase A in 0.05 M Tris-HCl, 0.1 M NaCl, pH 7.85. The solvents are aqueous solution (H), filled circles; methanol (M), 40%, filled triangles; methanol (M), 30%, empty triangles; glycerol (G), 30%, empty squares; and glycerol (G), 40%, filled squares (percentage by weight). The rate, v, is expressed in "milli-optical density" (MOD) units per minute. The lines are the best-fitted and the standard deviation is shown.

(30%) > water > glycerol (30%) > glycerol (40%).

Discussion

As stated in the introduction, the enzymic rate constant (k_{cat}), determined under conditions of excess of substrate, can be identified with k, the rate of transitions over the energy barrier of the rate-limiting step. Therefore, eq 4 can be written in the following form:

$$\ln k_{cat} = -\ln \eta + B/\epsilon RT + \ln A - \Delta/RT \qquad (5)$$

Let us assume that (a) Δ and B are independent of the type of cosolvent and (b) ϵ and η possess their bulk values. Since the right-hand side of eq 5 is independent of the type of cosolvent, we can compare theory and experiment in either of the two ways described below.

Solvent Contribution to the Rate of Catalysis. This is given by the expression $\ln \eta + B/\epsilon RT$. Taking the values of η and ϵ from the literature[1] and choosing B as an adjustable parameter, we obtain the Arrhenius plots of $-\ln \eta$, $B/\epsilon RT$, and $-\ln \eta + B/\epsilon RT$ for three values of B shown in Figure 3a, 3b, and 3c–e, respectively. A few conclusions may be drawn from Figure 3. (a) For $d/|Z_1Z_2| \gtrsim 2$ Å, over a wide range of temperatures, the contributions of the mixed solvents to the observed rate obey the order

methanol (40%) > methanol (30%) > glycerol (30%) > glycerol (40%)

A comparison between Figure 3a and 3b reveals that the left-hand inequality is due to dielectric constant effect, whereas the right-hand one originates from viscosity effect. (b) The temperature dependences of η and ϵ give rise to contributions to the observed activation energies. These contributions are $W = \partial \ln \eta/\partial(1/RT)$ and $E_{el} = -\partial(B/\epsilon RT)/\partial(1/RT)$, respectively. Since the Arrhenius lines in Figure 3a and 3b are linear and nearly parallel, W and E_{el} appear to be independent of temperature and almost insensitive to the presence of the cosolvents. For the mixed solvents, we obtain $W \sim 5.5$ kcal/mol and $E_{el} \sim 0.5$ kcal/mol. These results are valid for many solvents (Akerlöf, 1932; Frenkel, 1955).

The experimental results shown in Figure 2 are compatible with the above two predictions concerning the relative positions and the parallelism of the Arrhenius lines for the mixed solvents. The deviations of the aqueous solution is discussed below.

The fact that the substrate concentration was finite in these measurements, i.e., measurement of v instead of V_{max}, gives rise to a small and unessential correction in the relative positions of the Arrhenius lines. This is due to the fact that the K_m values in the mixed solvents are not very different from each other (Table I).

Eliminating the Viscosity and the Dielectric Constant Contribution to the Observed Rate. Equation 5 predicts that, by subtraction of the solvent contribution $-\ln \eta + B/\epsilon RT$ from $\ln k_{cat}$, the experimental data will follow a Gaussian distribution around a single Arrhenius line. Elimination of firstly the viscosity contribution to $\ln k_{cat}$ (Figure 4) and secondly the dielectric constant contribution (Figure 5) leads in both cases to a single distribution of data in the case of mixed solvents. This distribution (Figure 5) becomes narrowest for the best-fitted value of $d/|Z_1Z_2| = 5$ Å and $Z_1Z_2 < 0$. The corresponding standard deviations are 0.12 (Figure 4) and 0.08 (Figure 5). The latter is comparable to the experimental error. The goodness of fit test supports the assumption that the data, corresponding to the mixed solvents in Figure 5, originate from a normal distribution around a single Arrenhius line. We may conclude that the dielectric constant effect is small but significant.

The single Arrhenius line of Figure 5, obtained for the mixed solvent, is linear and contributes, in accordance with eq 5, $\Delta \sim 8.0$ kcal/mol to the observed activation energy (Figure 2). The results support our primary assumption that A and Δ are independent of the type of cosolvent and in our case appear to be independent of temperature as well.

The Arrhenius line corresponding to the aqueous solution is positioned *below* the data of the mixed solvents.

Behavior of the Aqueous Solution. Let us assume the general validity of eq 5 for A and Δ, which are insensitive to the presence of the cosolvent. We shall try to isolate the origin of the deviations of the data for the aqueous solution from the general behavior of the other solutions (Figure 5). This is done by elimination of the various factors that can be shown not to contribute to this phenomenon. In this category we find (a) the difference between v and V_{max} which slightly increases the deviation and (b) corrections to the quantity B/ϵ due to local behavior of ϵ (Harned & Samaras, 1932). Factor b has been shown to increase the value of B/ϵ for $d/|Z_1Z_2| < 4$ Å and $Z_1Z_2 < 0$ (Laidler, 1950). Since we found $d/|Z_1Z_2| = 5$ Å and $Z_1Z_2 < 0$ for the mixed solvents, this effect might be relevant only for the aqueous solution, but in this case it increases the deviation.

[1] See Bingham et al. (1913) and Washburn (1929) for the viscosity and Akerlöf (1932) for ϵ. The presence of salt in concentrations of about 0.1 M can be shown to have only a minute effect on the viscosity (Washburn, 1929) and causes a small shift in the dielectric constant (Bockris & Reddy, 1970), which has a negligible effect on the predicted results.

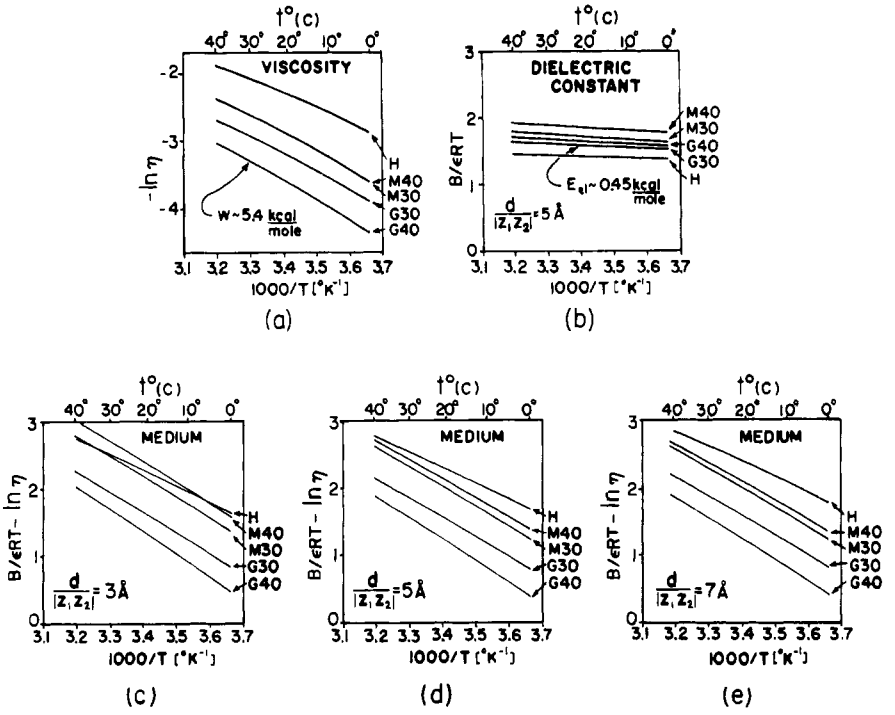

FIGURE 3: Solvent contribution to the reaction rate. Arrhenius plots for the contribution of the viscosity, η, and the dielectric constant, ϵ, to the reaction rate, as given by eq 5. The values of η and ϵ have been taken from the literature (Maurel et al., 1975; Tanford, 1968; Simpson et al., 1963) and are related to homogeneous media. The temperature dependence of η and ϵ contributes W and E_{el} to the activation energy of the reaction, respectively. (a) η contribution, (b) ϵ contribution for $Z_1Z_2 < 0$ and $d/|Z_1Z_2| = 5$ Å (eq 2), (c–e) the combined contributions of η and ϵ for $Z_1Z_2 < 0$ and at three values of $d/|Z_1Z_2|$. The solvents are pure water (H), glycerol (G), and methanol (M) in concentrations of 30 and 40% by weight.

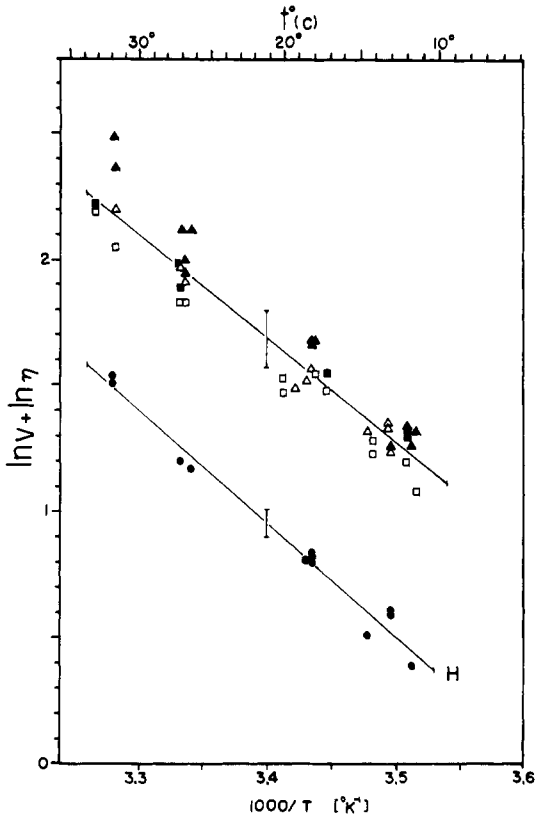

FIGURE 4: Elimination of the viscosity contribution from the observed rate. This is achieved by subtracting the values of $-\ln \eta$ given in Figure 3a from the data given in Figure 2 (eq 5). The two best-fitted Arrhenius lines shown here correspond to the data of the four mixed solvents (above) and to that of the aqueous solution (below).

The value of the viscosity, η, in eq 5 must be taken in the region at which the enzyme–substrate complex interacts with the solvent, i.e., near the protein surface. Proteins are known to induce order among the water molecules near their surfaces (Richards, 1977). An increase in the solvent viscosity, relative to its bulk value, is expected to be the result of such an induced order. The multiplicity of hydrogen bonding occurring at sufficiently high concentrations of methanol and glycerol (Auld & Vallee, 1970) is expected to weaken such a local effect. We therefore expect the aqueous solution to behave as though possessing high apparent values of η. Although the last conclusion is compatible with the observed deviation, a more detailed study is required in order to accept or to refute this explanation.

Relevance of the Results to the Mechanism of Catalysis by Carboxypeptidase A. The quantitative results for the hydrolysis of $Z(Ala)_3$ by this enzyme are summarized below. The activation energy of the reaction was found to be $E_a \sim 13.5$ kcal/mol. It was composed of three contributions: $W \sim 5$ kcal/mol due to the viscosity of the solution (Figure 3a), $E_{el} \sim 0.5$ kcal/mol due to electrostatic interactions (Figure 3b), and $\Delta \sim 8$ kcal/mol, which is the height of the potential energy barrier (Figure 5). If the electrostatic interaction during the reaction corresponds to two opposite charges Z_1e and Z_2e, we find that the sign of E_{el} might be interpreted as the result of *decreasing* the distance between the ions from d_i to d_f during the reaction. $1/A$, which characterizes the structural flexibility (eq 1), was found to be 1.2×10^{-6} cm s^2 g^{-1} (eq 3) and thus corresponds to a highly flexible structure (Gavish, 1978).

Let us try to correlate these results with the known facts about the structure of carboxypeptidase A and its interaction with substrates. A 2-Å resolution X-ray structure of carboxypeptidase A in its free form or in a form liganded to a dipeptide substrate has revealed that conformational changes occur during the binding (Lipscomb et al., 1968). Whether or not, as suggested by the structure of crystalline carboxy-

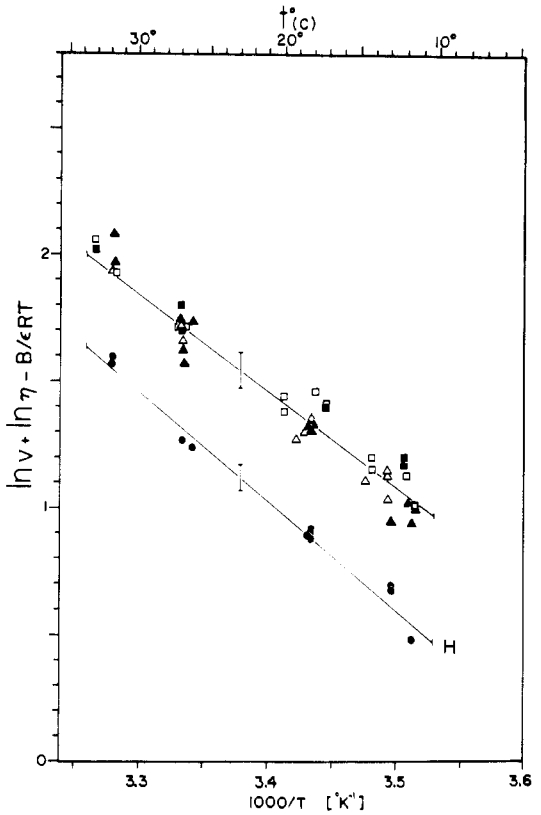

FIGURE 5: Elimination of the solvent contribution from the observed rate. This is achieved by subtraction of the values of $B/\epsilon RT$ given in Figures 3b from the data given in Figure 5 (eq 5). The two best-fitted Arrhenius lines shown here correspond to the data of the four mixed solvents (above) and to that of aqueous solution (below). The standard deviations are shown.

peptidase A, the changes involve movement of the hydroxyl group of Tyr-248 from an outer position toward the substrate (Abramowitz et al., 1967) or, as suggested by solution studies, the Tyr-248 moves out of the substrate pocket during the catalytic process (Lipscomb et al., 1968), it is generally accepted that in the enzyme, upon substrate binding, the conformation of the active site is altered. Our suggestion that the enzyme–substrate complex shows a high structural flexibility is compatible with the above picture.

The electrostatic interactions that occur during the binding of the substrate seem to be essential for catalysis by causing a strain in the scissile bond (Lipscomb et al., 1968; Warshel & Levitt, 1976). This is compatible with our observations concerning the direction of the dielectric constant effect. Nevertheless, we avoid any conclusion from the value of $d/|Z_1Z_2|$ due to its ambiguous interpretation (see the discussion following eq 2).

In view of the cooperative motions of Tyr-248, and possibly Arg-145 and Glu-270 and their proposed mode of binding from both sides of the scissile bond, it is tempting to speculate about the nature of the structural fluctuations. We suggest them to be cooperative motions of the above groups, giving rise to charge fluctuations around the scissile bond and resulting in time-dependent variations in its length (Figure 1a,b). The latter are the "transient strains" which we believe to be essential for the catalysis.

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References

Abramowitz, N., Schechter, I., & Berger, A. (1967) *Biochem. Biophys. Res. Commun. 29*, 862.

Akerlöf, G. (1932) *J. Am. Chem. Soc. 54*, 4125.

Auld, D. S., & Vallee, B. L. (1970) *Biochemistry 9*, 602.

Bell, R. P. (1973) *The Proton in Chemistry*, Chapman and Hall, London.

Bingham, E. O., White, G. F., Thomas, A., & Cadwell, J. L. (1913) *Z. Phys. Chem., Stoechiom. Verwandschaftsl. 83*, 641.

Bockris, J. O'N., & Reddy, A. K. N. (1970) *Modern Electrochemistry*, Vol. 1, Chapter 2, Plenum Press, New York.

Boyer, P. D. (1974) in *Dynamics of Energy-Transducing Membranes* (Ernster, Estabrook, & Slater, Eds.) pp 389, Elsevier, Amsterdam.

Breslow, R., & Wernick, D. L. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 1303.

Bullock, A. T., Cameron, G. G., & Smith, P. M. (1974) *J. Chem. Soc., Faraday Trans. 2 70*, 1202.

Cleland, W. W. (1975) *Acc. Chem. Res. 8*, 145.

Cohen, S. G., Vaidya, V. M., & Schultz, R. M. (1970) *Proc. Natl. Acad. Sci. U.S.A. 66*, 249.

Douzou, P. (1976) *Trends Biol. Sci. 1*, 25.

Eigen, M. (1974) in *Quantum Statistical Mechanics in the Natural Sciences* (Mintz, S. L., & Widmayer, S. M., Eds.) p 15, Plenum Press, New York.

Frank, F. A. (1967) in *Physico-Chemical Processes in Mixed Aqueous Solvents* (Frank, F., Ed.) p 50, Heinemann Educational Book, London.

Frenkel, J. (1955) *Kinetic Theory of Liquids* Chapter 4, Dover Publications, New York.

Gavish, B. (1978) *Biophys. Struct. Mech. 4*, 37.

Harned, H. S., & Samaras, N. N. T. (1932) *J. Am. Chem. Soc. 54*, 1.

Hui Bon Hoa, G., & Douzou, P. (1973) *J. Biol. Chem. 248*, 4649.

Imry, Y., & Gavish, B. (1974) *J. Chem. Phys. 61*, 1554.

Jencks, W. P. (1975) *Adv. Enzymol. Relat. Areas Mol. Biol. 43*, 219.

Johansen, J. T., & Vallee, B. L. (1975) *Biochemistry 14*, 649.

Kaufman, S., & Neurath, M. (1949) *J. Biol. Chem. 180*, 181.

Kirkwood, J. G., & Westheimer, F. H. (1938) *J. Chem. Phys. 6*, 506–513.

Koshland, D. F., Jr., & Neet, K. C. (1968) *Annu. Rev. Biochem. 37*, 359.

Kramers, H. A. (1940) *Physica (Utrecht) 7*, 284.

Laidler, K. J. (1950) *Chemical Kinetics* Chapter 5, Tata McGraw-Hill, Bombay.

Laidler, K. J., & Bunting, P. S. (1973) *The Chemical Kinetics of Enzyme Action*, 2nd ed., pp 216–220, Clarendon Press, Oxford.

Laurence, D. J. R. (1955) *Discuss. Faraday Soc. No. 20*, 255.

Lipscomb, W. N. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 3797.

Lipscomb, W. N., Hartsuck, J. A., Recke, G. N., Jr., Quiocho, F. A., Bethge, P. H., Ludwig, M. L., Steits, T. A., Muirhead, H., & Coppola, J. C. (1968) *Brookhaven Symp. Biol. No. 21*, 24.

Lumry, R., Smith, E. L., & Glantz, R. R. (1951) *J. Am. Chem. Soc. 73*, 4330.

Makinen, M. W., & Fink, A. L. (1977) *Annu. Rev. Biophys. Bioeng. 6*, 301.

Mashimo, S. (1976) *Macromolecules 9*, 91.

Mashimo, S., Iwasa, Y., Chiba, A., & Shinohara, K. (1975) *J. Phys. Soc. Jpn. 39*, 1529.
Maurel, P., Hui Bon Hoa, G., & Douzou, P. (1975) *J. Biol. Chem. 250*, 1376.
Milstein, S., & Cohen, L. A. (1970) *Proc. Natl. Acad. Sci. U.S.A. 67*, 1143.
Page, M. I., & Jencks, W. P. (1970) *Proc. Natl. Acad. Sci. U.S.A. 68*, 1678.
Richards, F. M. (1977) *Annu. Rev. Biophys. Bioeng. 6*, 151.
Simpson, R. T., Riordan, J. F., & Vallee, B. L. (1963) *Biochemistry 2*, 616.
Storm, D. R., & Koshland, D. E., Jr. (1970) *Proc. Natl. Acad. Sci. U.S.A. 66*, 445.
Sturtevant, J. M. (1955) *Discuss. Faraday Soc. No. 20*, 254.
Tanford, C. (1968) *Adv. Protein Chem. 23*, 122.
Warshel, A., & Levitt, M. (1976) *J. Mol. Biol. 103*, 227.
Washburn, E. W., Ed. (1929) *International Critical Tables*, Vol. V, p 12, McGraw-Hill, New York.

Analysis of Protein–Protein Relationships in 30S Ribosome Assembly Intermediates Using Protection from Proteolytic Digestion†

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ABSTRACT: Treatment of the intact bacterial ribosome with proteolytic enzymes results in little or no digestion of many of the component proteins [Craven, G. R., & Gupta, V. (1970) *Proc. Natl. Acad. Sci. U.S.A. 67*, 1329]. In contrast, when the proteins are released from the constraints of ribosome structure, they become completely susceptible to proteolytic attack. We have attempted to exploit these observations in an effort to determine the precise steps in ribosome assembly which result in a conversion of the structures of the various proteins from a proteolysis sensitive to a resistant form. Thus, a total of 11 30S ribosome assembly intermediate complexes of proteins and 16S RNA were prepared and digested with trypsin or chymotrypsin. The kinetics of digestion of each protein in the complex were followed by polyacrylamide gel electrophoresis. By a comparison of the digestion pattern of two complexes differing only by the presence of a single protein, it was possible to deduce several specific protective effects of one protein on its neighbor in the complex. On the basis of these studies, we propose nine protein–protein protective effects. The possible relevance of these interrelationships to other well-established proximity relationships is discussed.

The architectural organization of the proteins in the ribosome remains as one of the most difficult unsolved problems in molecular biology. This is not, however, due to lack of effort. Numerous investigators have constructed sophisticated, imaginative, and potentially powerful approaches toward the elucidation of protein–protein distance relationships in the bacterial ribosome. The approaches include the use of bifunctional reagents to covalently cross-link neighboring proteins (e.g., Lutter et al., 1972; Shih & Craven, 1973; Sommer & Traut, 1976; Peretz et al., 1976; Expert-Bezancon et al., 1977), electron microscopy of ribosomes labeled with specific antibodies (Lake, 1977; Tischendorf et al., 1975), energy transfer between pairs of proteins labeled with different fluorescent dyes (Huang et al., 1975), neutron scattering of deuterated protein pairs (Langer et al., 1978), and protection from chemical iodination (Changchien & Craven, 1977). In summary, the combination of these techniques, and others not mentioned here, has produced information about over 80 protein pairs in the 30S ribosome (for reviews, see Changchien & Craven, 1977; Gaffney & Craven, 1978). Unfortunately, despite this impressive catalogue of data, no definitive model of the 30S particle has been presented. Apparently more information is required before a consistent model can be deduced. With this goal in view, we have developed another approach to the determination of protein–protein proximity relationships.

Our approach is to compare the proteolytic sensitivity of various specific ribosomal protein–16S RNA complexes. We have constructed a series of complexes intermediate in the pathway of 30S ribosome assembly. The protein composition of the complexes was selected to allow the direct comparison of two complexes which differed only by the presence of a single protein. This has made it possible to investigate the relative alterations in sensitivity to mild proteolytic digestion induced by the presence of a given protein in a ribonucleoprotein particle. Using either trypsin or chymotrypsin under controlled conditions, we have found that the addition of a new protein to a complex sometimes results in a dramatic change in the susceptibility of one or more other proteins. A number of these effects involve a pair of proteins which have been implicated as close neighbors in the intact 30S ribosome by a variety of independent experiments. We propose that the protection of one protein from enzymatic attack by the presence of a second protein may be due to the physical proximity of the two proteins. We also present arguments defending the hypothesis that proteins situated close together at early stages in ribosome assembly remain in proximity throughout assembly and are found as near neighbors in the final ribosome structure.

Experimental Procedures

Preparation of 30S Ribosomal Subunits and Purification of Ribosomal Proteins. Ribosomes were isolated from *E. coli* MRE 600, and 30S ribosomal subunits were purified from 70S ribosomes by zonal centrifugation as described by Craven & Gupta (1970). Ribosomal proteins were extracted from the purified 30S subunits with 67% acetic acid and further

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