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Energetics of enzyme catalysis

(lysozyme/enzyme mechanism/dielectric effects in enzymes and in solutions/relationship between protein folding and catalysis)

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ABSTRACT **Quantitative studies of the energetics of enzymatic reactions and the corresponding reactions in aqueous solutions indicate that charge stabilization is the most important energy contribution in enzyme catalysis. Low electrostatic stabilization in aqueous solutions is shown to be consistent with surprisingly large electrostatic stabilization effects in active sites of enzymes. This is established quantitatively by comparing the relative stabilization of the transition states of the reaction of lysozyme and the corresponding reaction in aqueous solution.**

Enzymes are the catalysis of many biological processes. They can accelerate the rate of chemical reactions by more than 10 orders of magnitude relative to the corresponding rates in solutions. How can enzymes make the activation barrier of a given reaction lower than the corresponding barrier in solution? Several explanations have been given in the past (1–2). One explanation implies that enzymes can exert strong steric strain on the ground states of their substrates and thus decrease the relative activation energy (3). However, recent theoretical (4–7) and experimental studies (8) have indicated that strain cannot be the most important factor (see also ref. 2). Another explanation uses entropy as a crucial effect in enzymatic reactions (9). Although this proposal might be correct in bond formation reactions, it cannot explain catalytic effects in the basic reactions of bond breaking. Because many enzymatic reactions involve polar transition states, it was suggested that enzymes can decrease the activation barrier by electrostatic stabilization of these states (5, 10, 11). However, experimental studies of model compounds in aqueous solution seemed to indicate that electrostatic stabilization cannot be an important factor (12, 13). Thus, despite extensive studies, there is no quantitative explanation for the crucial energetic factors in enzyme catalysis.

The understanding of the energetics of enzymatic catalysis is handicapped by a lack of quantitative understanding of chemical reactions in solution. Without evaluation of the energy contributions to reactions in solution, the difference between an enzymatic reaction and the corresponding reaction in solution cannot be examined. This is especially true of ionic reactions in which macroscopic dielectric theories do not allow the role of electrostatic stabilization to be accurately assessed.

Recent theoretical studies of the energy balance of chemical reactions in solutions (14) and in the active sites of enzymes (5) show that electrostatic stabilization is the most important factor in enzymatic catalysis even though it may play but a minor part in solution. This point is established quantitatively here by comparing the relative stabilizations of the transition states of the catalytic reaction of lysozyme and of the corresponding reaction in aqueous solution.

Beyond the macroscopic dielectric concepts

Most theories of electrostatic interactions consider interacting charges as embedded in a continuum with the bulk dielectric constant ϵ_0. The application of such "continuum" theories to the active sites of enzymes is questionable. In fact, such theories cannot be used even for quantitative studies of the interaction between ions in aqueous solutions, especially when the distance between interacting ions is similar to the size of the solvent molecules (for discussion, see ref. 14). If charge stabilization is to be studied quantitatively, the microscopic nature of the solute solvent system must be taken in account. This can be done by the following microscopic models (5, 14).

Model for Calculating Electrostatic Interactions in Proteins. The electrostatic energy of a group of charges inside a given region of a protein (e.g., the active site) is composed of the following contributions: (*i*) the charge–charge interactions which are given by:

$$V_{QQ} + V_{Qq} = \sum_{i>j} Q_i Q_j / r_{ij} + \sum_{ik} Q_i q_k / r_{ik} \qquad [1]$$

in which Q and q designate, respectively, charges inside and outside the given region, and r is the distance between the charges; and (*ii*) the inductive interactions between the charges of the protein and the polarizable electrons of the protein atoms. These contributions are evaluated by assigning induced dipoles to all the protein atoms and calculating the self-consistent magnitudes and directions of these dipoles in the presence of the protein charges (for details, see ref. 5). The inductive energy is then given by:

$$V_{\text{ind}} = -\frac{1}{2}\left[\sum_{i,k} Q_i \mu_k \mathbf{r}_{ik}/r_{ik}^3 + \sum_{j,k} q_j \mu_k \mathbf{r}_{jk}/r_{jk}^3\right] \qquad [2]$$

in which μ_k are the induced dipoles of the system.

Model for Calculating Electrostatic Interactions in Water. The electrostatic energy of charges in water is evaluated by representing the water molecules as point dipoles attached to the centers of soft spheres and minimizing the solute–solvent energy with respect to the orientation and position of these dipoles (14). Because the calculations are limited to only a few solvation shells, these are surrounded by a surface of dipoles in the orientation and position of the bulk water.* This model was used successfully to calculate the known solvation enthalpies of various ions and the dissociation energies of different acids (14). Here, the free energies (rather than the enthalpies) of solvation at 300 K are estimated by calibrating the model with the free energies of solvation of different ionized acids. Such microscopic approaches allow us to compare the electrostatic energy balance in polar liquids to that in enzymes and to search for hidden concepts at the microscopic level which have eluded macroscopic theories.

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Abbreviations: see footnote, Table 1.

* The interaction with the surface dipoles prevents the dipoles in the internal solvation shells from excess polarization toward the solute charges.

Consider the separation of charges in polar liquids as presented in Fig. 1 *upper*, which shows the sum of the solute and solute–solvent electrostatic energies to be nearly constant for charge separations from 3 Å to infinity, because the charge–charge interaction energy is almost compensated for by the solvation energy. This effect arises because the solvent molecules have so many degrees of freedom that the force created by charge separation from $\bar{r}$ to $r + \Delta r$ can be compensated for by their small reorientation and compression, without significant change in the total energy of the system. Because hard-core repulsion prevents charged groups approaching closer than 3 Å, large electrostatic attraction cannot occur in aqueous solutions.

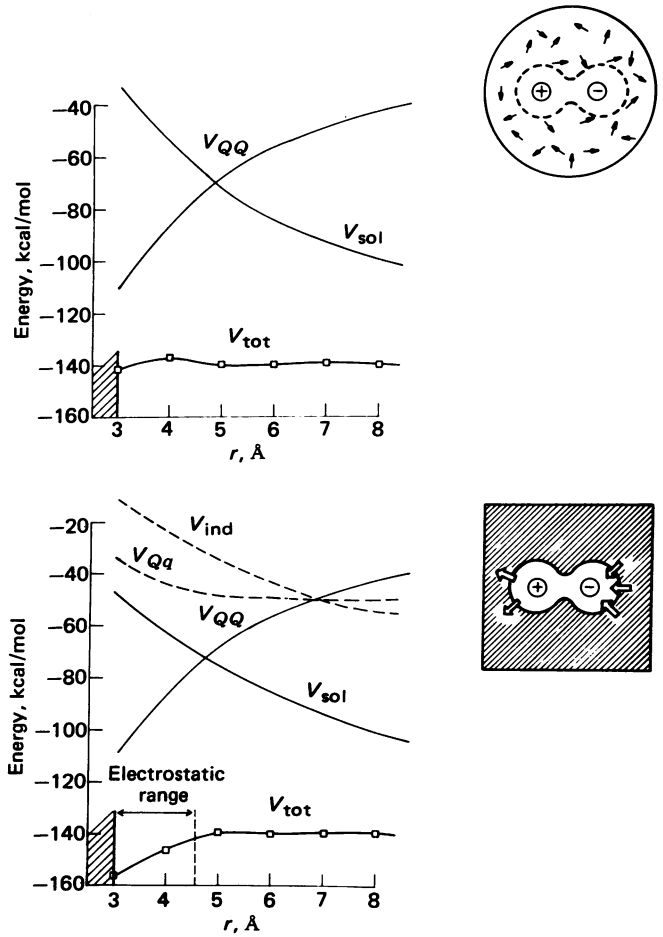

FIG. 1. Energetics of charge separation in aqueous solutions (*Upper*) and in an hypothetical enzyme (*Lower*). r is the distance between charges. V_{QQ}, V_{sol}, and V_{tot} are, respectively, the charge–charge interaction energy, the solvation energy, and the total energy. V_{Qq} is the interaction between the solute charges and the enzyme charges, which are represented here by dipoles. V_{ind} is the interaction between the solute charges and the induced dipoles of the enzyme. V_{sol} in the enzyme case is the sum of V_{Qq} and V_{ind}. The calculations for the enzyme case were done with the indicated permanent dipoles embedded in a cubic lattice of induced point dipoles (with the average density and polarizability of the lysozyme atoms). The cavity shape was determined by eliminating from the enzyme lattice any point within 2.8 Å from either the solute charges or the enzyme permanent dipoles. The size of the enzyme permanent dipoles was chosen so that the solvation energy for $r = \infty$ is the same in the enzyme and in aqueous solution. The orientation and position of the enzyme dipoles were kept constant relative to the corresponding nearest neighbor charge. The figure demonstrates that in aqueous solution there is no accessible range of strong electrostatic interaction (the region of $r <$ 3 Å is forbidden for nonbonded charges). In the enzyme, on the other hand, there is a significant accessible range of effective electrostatic interaction.

Can a system with fewer degrees of freedom provide larger charge stabilization than water? Consider first an isolated charge: according to macroscopic dielectric theories the solvation of a group with net charge Q is given by:

$$V_{sol} \simeq -166\, Q^2(\epsilon_0 - 1)/(\epsilon_0 \bar{a}) \text{ kcal/mol} \quad [3]$$

in which $\bar{a}$ is the radius of the solvent cavity (in Å) and ϵ_0 is the bulk dielectric constant. Because, in enzymes, $\epsilon_0 \approx 2$, the "solvation" will be only about half of that in aqueous solution. However, my microscopic model shows that, by placing two or three fixed dipoles (e.g., hydrogen bonds) around the charged group, an enzyme can give as much solvation as an aqueous solution. The total solvation energy is then given by two contributions: (*i*) the interaction between the enzyme charges (the fixed dipoles) and the charged group (V_{Qq}); and (*ii*) the interaction between the induced dipoles of the enzyme atoms and the charged group (V_{ind}). The sum of V_{Qq} and V_{ind} turns out to be close to the solvation energy of the same charged group in water. This is consistent with the facts that enzymes can bind ions (15) and that the pK_as of acidic groups in active sites of enzymes are similar to those in aqueous solutions (16).

Given that enzymes can stabilize isolated charges as much as aqueous solutions, one finds that pairs of opposite charges can be stabilized by enzymes much more than by aqueous solutions. This point is demonstrated in Fig. 1 *lower* where the stabilization of the isolated charges in an hypothetical enzyme is calibrated (by the proper selection of permanent dipoles) so as to be equal to the corresponding solvation in aqueous solution. When this is done, the total energy (V_{tot}) in the enzyme is not constant but falls with decreasing distance between the charges, allowing for a whole range of charge stabilization effects in the range 4–3 Å (note that the solvation energy in this range is larger than the corresponding solvation energy in bulk water). Similar charge stabilization effects arise for other charge distributions including the one described in Fig. 2. Enzyme can stabilize ion pairs and other charge distributions more than water because, in contrast to water, the enzyme dipoles are kept oriented toward the charges even when the field from the charges is small (small r).

Charge stabilization as a major factor in enzyme catalysis

To determine whether the above concepts are relevant to a real enzyme–substrate system, I examined the catalytic reaction of lysozyme. The accepted mechanism for the rate-limiting step in this reaction (Fig. 3) consists of general acid catalysis by transfer of a proton from glutamic acid-35 and stabilization of the transition state by ionized aspartic acid-52 (3). The activation energy of this process is ≈18 kcal/mol (1 cal = 4.18 J). In solution the corresponding activation energy for general acid catalysis of the substrate by an attached carboxylic acid is ≈25 kcal/mol (Table 1). The difference between the two activation energies corresponds to a rate enhancement of $\approx 10^6$ which cannot be accounted for by current concepts of enzyme catalysis.

In order to obtain reliable energies the following approach was used: the intramolecular energies in forming charged species *in vacuo* were taken from gas phase and solution experiments (12, 17–19) (see ref. 14) rather than from quantum mechanical calculations (5), and the entropy contributions were estimated from solution experiments. In this way only charge–charge interactions and solvation energies were calculated in comparing enzyme and solution reactions.

Table 1 compares the activation energy in the enzyme ($A^-N^+G^-$–enzyme) to the corresponding activation energy of general acid catalysis in solution (N^+G^-–water), so that the

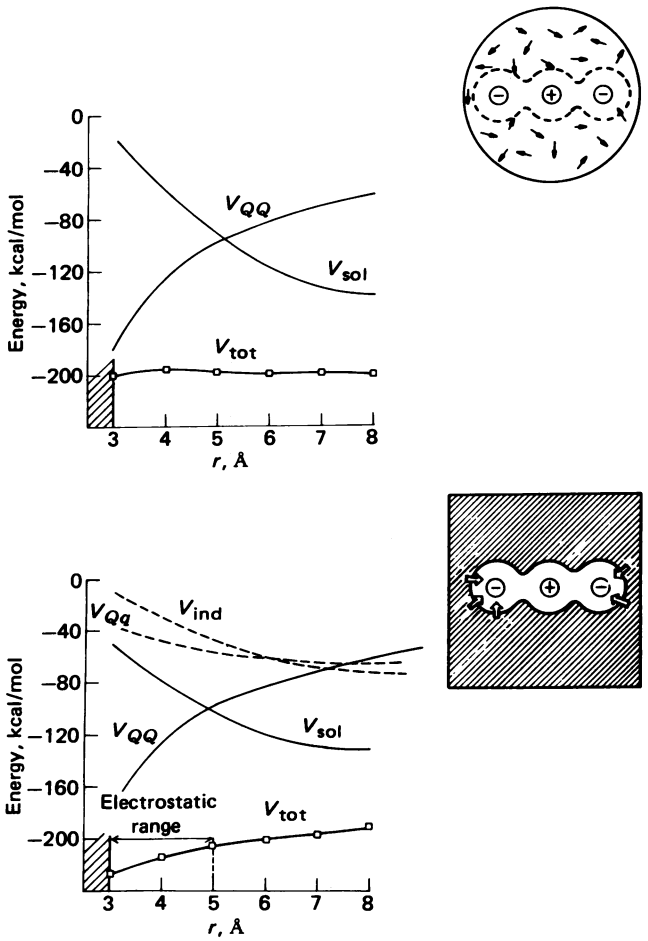

FIG. 2. Charge separation energy of the (−) (+) (−) configuration in aqueous solution (*Upper*) and a hypothetical enzyme (*Lower*). *r* is the (−) (+) distance for a symmetric separation in which the positive charge is kept fixed and the two negative charges are displaced in opposite direction. Notation as in Fig. 1.

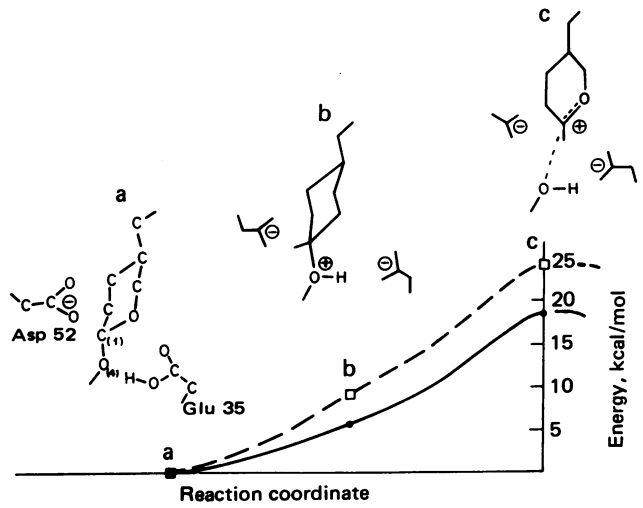

FIG. 3. Schematic description of the catalytic bond cleavage of the sugar residue in the D subsite of lysozyme. The figure presents the following configurations. (a) Ground state of the enzyme substrate complex. (b) O-4 of the substrate is protonated by the proton of Glu-35. (c) C-1—O-4 is broken, forming a planar carbonium ion intermediate. The figure also presents the estimated energy of the system (relative to the ground state energy) along the reaction coordinate: —, reaction in the enzyme active site; - - - -, reaction in aqueous solution. The reaction energies were estimated by using the calculated solvation energies and experimental data for sugar hydrolysis (17) and acid dissociation in solutions as well as related gas phase measurements (18, 19). More details about this estimate as well as a description of calculations will be given elsewhere.

substrate is the same in both cases, but the enzyme case includes the charge stabilization by Asp-52. The interaction with Asp-52 decreases the enthalpy of activation relative to the solution by about 12 kcal/mol. However, in solution the free energy of activation is lower than the corresponding enthalpy by about 5 kcal/mol. This is due to the change in entropy of the torsion and bending modes when the C—O bond between the two fragments of the transition state is loosened, whereas in the active site of the enzyme the transition state fragments are immobile, so that free energy and enthalpy are nearly equal. Due to this entropic factor the calculated difference in enthalpy of activation is decreased to a difference of ≈7 kcal/mol in free energy as compared to an experimental estimate of ≈7 kcal/mol.

These results do not indicate clearly the origin of the difference between the reaction in the enzyme and in aqueous solution, because it might be argued that the effect of Asp-52 could be reproduced in solution by a negative charge fixed near the N^+G^- system and that the observed rate difference is due only to the entropy involved in fixing the charge. It seems preferable to avoid this argument and related questions of effective concentration at this stage and to deal with well-defined energy questions. This can be done by comparing the enzyme reaction to a reaction of a model compound with all the enzyme active groups including Asp-52 (see Fig. 4). The energy of the transition state of such a model compound is estimated in the $[A^-N^+G^-]$-water entry. The step $[N^+G^-]$ in water → $[A^-N^+G^-]$ in water corresponds to the ionization of a second carboxylic group in water and its movement toward the positive carbon. To a first approximation, this process does not change the energy of the system, because I have shown above that the motion of oppositely charged ions toward each other in aqueous solution does not change it either. This is also consistent with experimental studies of model compounds that have not shown significant rate enhancement in the presence of ionized groups (12). $[A^-N^+G^-]$-Lysozyme and $[A^-N^+G^-]$-water allow comparison of the total free energy of two identical solutes in two different "solvents," water and the active site of the enzyme (Fig. 4). In this way the catalytic energy of the enzyme is reformulated as purely a difference in solvation energies. The calculations show that the solvation energy in $[A^-N^+G^-]$-enzyme is ≈12 kcal/mol larger than in $[A^-N^+G^-]$-water. After allowing for the entropy contribution of 5 kcal/mol, this difference comes down to the 7 kcal/mol observed experimentally. Fig. 4 illustrates the physical analogy between the two cases considered here and the earlier examples represented in Fig. 2. Therefore, catalysis by lysozyme may be regarded as a specific manifestation of the general ability of enzymes to stabilize polar transition states more than bulk water, because they can combine the effects of permanent and induced dipoles in a more favorable manner (Figs. 2 and 4).

Why is bulk water not such a good solvent?

In order to understand why the solvation energy of ionic transition states is larger in active sites of enzymes than in bulk water, I studied the solvation of ion pairs by clusters of water molecules (14). The calculations show that the solvation energy of an ion pair by a small number of water molecules (20–100 molecules) is greater by ≈10 kcal/mol than the corresponding solvation by bulk water where the dipoles of the first few solvation shells cannot be strongly polarized toward the solute charges because they interact with the surrounding randomly oriented bulk dipoles. The constraints of a randomly oriented

Table 1. Calculated and observed energy contributions (in kcal/mol) of different configurations related to the reaction of lysozyme*

	Enzyme†						Water			
Configuration	Δ	V_{QQ}	ΔV_{Qq}	ΔV_{ind}	ΔV_{sol}	ΔF_{tot}	Δ	V_{QQ}	ΔF_{sol}	ΔF_{tot}
$[A^-](H^+)$	77-7‡	0	−31	−40	−71	−1 (−1)	77-7‡	0	−71 (−70)	−1 (−1)
$[G^-](H^+)$	77-7‡	0	−20	−45	−65	5 (6)	77-7‡	0	−71 (−70)	−1 (−1)
$[N^+G^-]$	165	−84	−13	−35	−48	33	160§	−84	−49	27 (25)
$[A^-N^+G^-](H^+)$	242-7‡	−131	−27	−57	−84	20 (18)	237§-7‡	−131	−72	27 (25)

* A, G, and N designate respectively Asp-52, Glu-35, and the *N*-acetylglucosamine residue; (H^+) designates a solvated H_3O^+ in the bulk water. The acids are simulated here by formic acid type systems. The configurations in each row are identical for enzyme and water. All energies are relative to the corresponding nonpolar configurations (e.g., the energy of $[A^-N^+G^-](H^+)$ is relative to that of [AH N GH]). For enzyme, $\Delta F_{tot} = \Delta + V_{QQ} + \Delta V_{sol}$; for water, $\Delta F_{tot} = \Delta + V_{QQ} + \Delta F_{sol}$. V_{QQ}, V_{Qq}, V_{ind}, and V_{sol} are defined in the legend of Fig. 1. Δ is the experimental estimate of the enthalpy of forming the indicated ionized configuration (including the proton transfer to H_2O) in vacuo at infinite separation between its components. When a configuration involves (H^+), the corresponding Δ also includes the solvation free energy of H_3O^+ in the bulk water (−100 kcal/mol) (18). The Δ values for A^- and G^- include the enthalpy of proton transfer from HCOOH to H_2O (−177 kcal/mol) (18) and the solvation free energy of H_3O^+. The Δ value for $[A^-N^+G^-]$ includes the gas phase enthalpy of proton transfer from HCOOH to glucose (estimated from refs. 18 and 19 as ≈147 kcal/mol), the enthalpy of dissociation of the protonated glucose (estimated from analysis of results of refs. 12, 17, 18, and 19 as ≈18 kcal/mol), the enthalpy of proton transfer from HCOOH to H_2O, and the solvation free energy of H_3O^+ in bulk water. The experimental estimates for the different energies are given in parentheses. The estimate of the solvation energy of the enzyme transition state $[A^-N^+G^-]$ in water is a lowest limit obtained by estimating the energies of the solvated compounds at infinity and bringing them together to the solvent cavity (see text).

† The solvation of charged groups by the enzyme is calibrated by fitting the calculated and observed pK_as of A^- and G^-. The average polarizabilities used for evaluation of V_{ind} are 0.4 Å^3 for hydrogen atoms and 0.9 Å^3 for all other atoms. All atoms within the cutoff radius of 10 Å are included in the calculations, and the water molecules around the enzyme are represented by Langevin type model (5). In the case of Asp-52 the surrounding protein groups (especially Asn-59) give the −31 kcal/mol V_{qQ}. The stabilization of Glu-35 involves, in addition to the protein groups, one water molecule in the cavity between the O-6 of the sugar E residue, Try-111, and Glu-35. The energy of transferring this water molecule from the bulk water to the active site is taken into account.

‡ The indicated configuration involves solvation of H^+ in the bulk water (in the $[A^-N^+G^-]$ case this is the Asp-52 proton). This corresponds to the process $AH \rightarrow H^+ + A^-$ in which A is the given acid and the equation $-\Delta F_A \equiv RT \ln([A^-][H^+]/[AH])$ determines the A^- concentration. Because the concentration of H^+ is given by the pH of the bulk water we could write (for small concentration of AH) $\Delta F_A - 1.38\,pH = -RT \ln([A^-]/[AH])$. Thus, the apparent ΔF for 50% dissociation of AH can be defined as $\Delta F^*_A = \Delta F_A - 1.38\,pH$. This gives the indicated correction of −7 kcal/mol at pH 5.0.

§ The Δ column for $[N^+G^-]$ and $[A^-N^+G^-]$ in the water case includes the estimate of 5 kcal/mol for free energy contribution from the motion of the substrate fragments in the water cavity (see text).

polar surface (see the section on microscopic model above and ref. 14) do not exist in small clusters of water molecules, which means that even the most primitive enzyme could achieve charge stabilization by enveloping a hydrated substrate in an hydrophobic pocket. In general, the active sites of enzymes separate the bulk water from the reaction region, which allows them to stabilize charges either by their dipoles alone or in combination with several water molecules.

Supporting evidence

My conclusions are supported by the following. (*i*) The solvation energies in the active site of lysozyme were extrapolated by calibration from the pK_as of its acidic groups and then applied to the (−)(+)(−) configuration. Similarly the solvation energies in aqueous solution were calibrated from the pK_as of different acids in solutions. (*ii*) The absence of significant charge stabilization effects in solution is consistent with the weak dependence of the ΔpK_as of dicarboxylic acids and zwitterions on the distance between the ionized groups (14) (see also ref. 2, page 49, for related evidence). (*iii*) The proposal that enzymes can stabilize polar systems better than water is supported by the existence of ion pairs (salt bridges) in hemoglobin (20) and other systems (21). Ion pairs are not stabilized in aqueous solutions. (*iv*) Charge stabilization is a common element in many enzymatic reactions. This includes the serine proteases (22), carboxypeptidase (23), thermolysin (24), and alcohol dehydrogenases (25, 26). (*v*) Modern attempts to design model compounds for enzymatic reactions (27) are based mainly on removal of the bulk water from the reacting region. Such model compounds display very large rate enhancement. (*vi*) Chemical reactions in micelles (28) also show very large rate enhancement relative to the corresponding reactions in bulk water. This seems to be due to the formation of small water clusters inside hydrophobic pockets, which stabilize polar transition states (see above). This evidence is merely circumstantial, but it reinforces the results of the calculations presented above.

Concluding remarks

This work replaces the macroscopic concepts of charge stabilization in aqueous solution and in enzymes by new microscopic models. It is shown that experiments concerned with the stabilization of ions in solutions are not directly relevant to the corresponding stabilization in the active sites of enzyme. In aqueous solutions, large electrostatic attraction does not arise because any force created by moving opposite charges toward each other is balanced by reorientation of the solvent dipoles. Enzymes, on the other hand, can stabilize varied constellations of charges by fixing their dipoles in appropriate orientations.

The crucial contribution of charge stabilization is demonstrated by a study of the energetics of the catalytic reaction of lysozyme. Arguments about the entropy of bringing catalytic groups together are avoided by comparing the enzymatic reaction to a reaction of a model compound containing all of the enzyme's catalytic groups. The effect of charge stabilization is shown to account for most of the difference between the activation energy of the enzymatic reaction and the reaction of the corresponding model compound in water.

Earlier authors had attributed charge stabilization effects to the low dielectric constant of the interior of enzymes surrounding their active sites (10, 11). However, the present work shows the explanation to be more subtle. That is, the energy of formation of an ionic transition state from a nonpolar ground

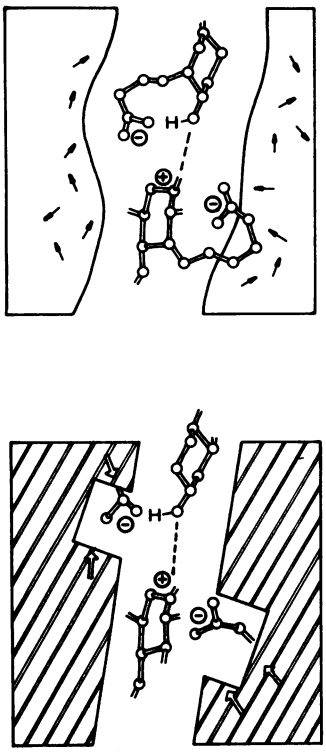

FIG. 4. Transition states of the reaction of lysozyme (*Upper*) and the corresponding hypothetical reaction in water (*Lower*). The "solvation" of the transition state is larger in lysozyme than in water. This is due to the optimal orientation of the permanent dipoles of the enzyme. See Fig. 2 for a general consideration of a related case.

state is $(\Delta + V_{QQ}) + \Delta F_{sol}$ (see Table 1 for notation). Because, for a given configuration, the solvation energy (ΔF_{sol}) is larger in water than in nonpolar solvents but $\Delta + V_{QQ}$ is the same, the activation energy of ionic reactions will be *higher* in nonpolar solvents than in water. On the other hand, as was demonstrated here, the active site of enzymes can provide more solvation energy than can bulk water. Examination of the reason why the solvation energy of polar transition states is smaller in aqueous solutions than in active sites of enzymes indicates that bulk water prevents optimal interaction of the first few solvation shells with the solute charges. The solvation energy of a polar transition state can be increased by surrounding the substrate and several water molecules by a pocket that expels the bulk water. Enzymes, in general, stabilize polar transition states by expelling the bulk water and using their permanent dipoles either alone or in combination with several water molecules.

Stabilization of polar transition states by enzymes requires configurations with parallel permanent dipoles. In consequence, the ground states of enzyme–substrate complexes have significant electrostatic strain energy. This is stored in the secondary, tertiary, and quaternary interactions which determine the unique structure of the enzyme and impose the necessary orientation on its dipoles. A chemist can probably obtain the same effect by locking parallel dipoles together in one molecule (e.g., a crown ether), using the chemical bond energy to compensate for the tendency of the dipoles to be antiparallel.

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