Biochemistry

 Volume 28, Number 9 May 2, 1989

Perspectives in Biochemistry

How Do Serine Proteases Really Work?†

A. Warshel,* G. Naray-Szabo,‡ F. Sussman, and J.-K. Hwang
Department of Chemistry, University of Southern California, Los Angeles, California 90089-0482
Received December 27, 1988; Revised Manuscript Received February 16, 1989

ABSTRACT: Recent advances in genetic engineering have led to a growing acceptance of the fact that enzymes work like other catalysts by reducing the activation barriers of the corresponding reactions. However, the key question about the action of enzymes is not related to the fact that they stabilize transition states but to the question to *how* they accomplish this task. This work considers the catalytic reaction of serine proteases and demonstrates how one can use a combination of calculations and experimental information to elucidate the key contributions to the catalytic free energy. Recent reports about genetic modifications of the buried aspartic group in serine proteases, which established the large effect of this group (but could not determine its origin), are analyzed. Two independent methods indicate that the buried aspartic group in serine proteases stabilizes the transition state by electrostatic interactions rather than by alternative mechanisms. Simple free energy considerations are used to eliminate the double proton-transfer mechanism (which is depicted in many textbooks as the key catalytic factor in serine proteases). The electrostatic stabilization of the oxyanion side of the transition state is also considered. It is argued that serine proteases and other enzymes work by providing electrostatic complementarity to the *changes* in charge distribution occurring during the reactions they catalyze.

One of the most basic problems in biochemistry is the understanding of the molecular origin of enzyme catalysis [for a recent review, see Kraut (1988)]. The enormous advances in site-directed mutagenesis of enzymes [for review, see Knowles (1987)] and the production of catalytic antibodies (Tramontano et al., 1986; Jacobs et al., 1987) have led to a renewed interest in this question. Unfortunately, however, it seems that the problem is sometimes being reduced to the statement that "enzymes work because they work". That is, it is now repeatedly stated that Pauling (1946) was right and that enzymes work because they stabilize the transition state of the reacting system. Although a great insight has been provided by the pioneering statement about transtion-state stabilization (Pauling, 1946), those who have accepted the main premises of transition-state theory knew clearly that enzymes (as well as other catalysts) work by reducing the activation free energies of the corresponding reactions. This is in fact the figure drawn in countless textbooks [e.g., Dickerson et al. (1984)]. Thus the real question and the real puzzle do not boil down to whether or not enzymes reduce activation barriers but to how do they accomplish it? This problem is far from being trivial and cannot be resolved quantitatively by saying that enzymes provide "templates" that bind the transition state. That is, an enzyme can reduce the free energy difference between the ground state and the transition state by 10 kcal/mol, relative to the same system in water. Apparently, it is hard to account for such an enormous effect by a consistent interpolation of related factors from chemical reactions in solutions to enzyme active sites. For example, an optimal complement between the van der Waals interactions of an active site and the substrate's geometry cannot account for more than 2 kcal/mol if the flexibility of the enzyme is taken into account (Warshel & Levitt, 1976). In fact, many proposals attempted to address this crucial question [for a review, see Page (1987)] but only now with the emergence of genetic engineering can one distinguish between different proposals and assess their merits.

This paper explores what is probably the most extensively studied enzyme family (the serine protease family) and demonstrates the feasibility of quantitative examination of different

† This work was supported by Grant GM-24492 from the National Institutes of Health and by Contract N00014-87-K0507 from the Office of Naval Research.
* To whom correspondence should be addressed.
‡ Permanent address: CHINOIN Pharmaceutical and Chemical Works, P.O. Box 110, H-1325 Budapest, Hungary.

0006-2960/89/0428-3629$01.50/0

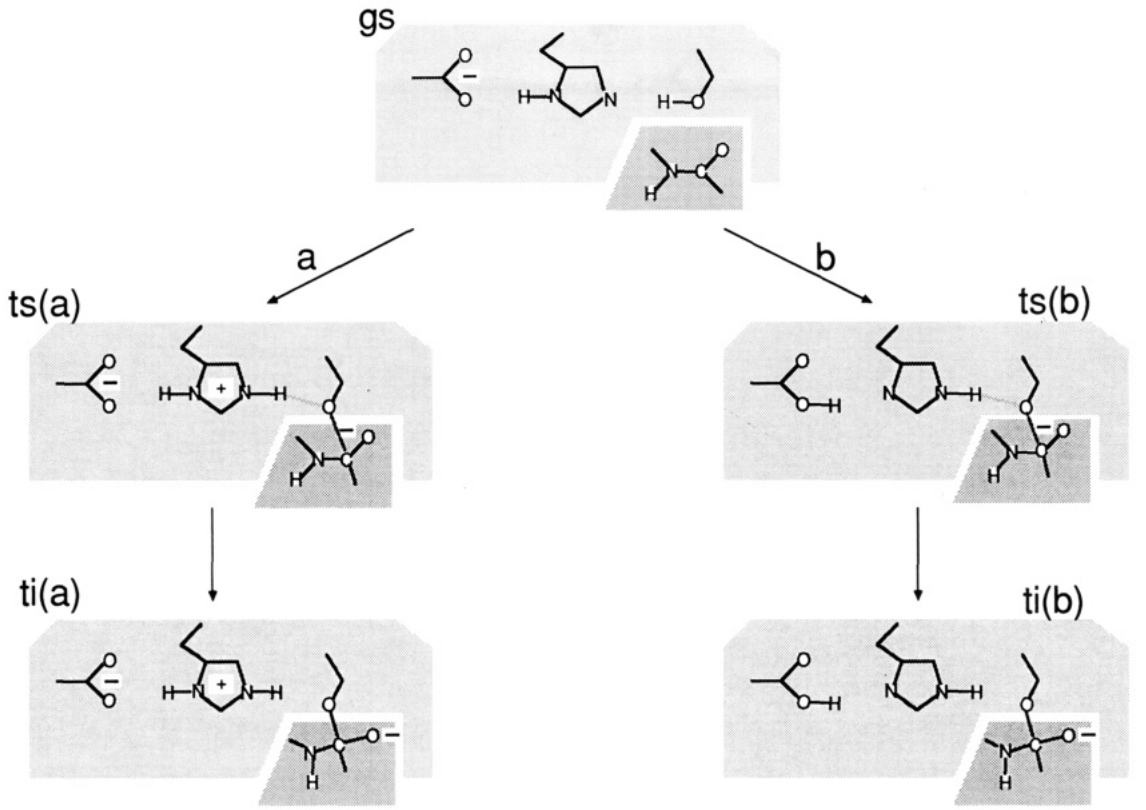

FIGURE 1: Schematic drawings displaying the possible reaction mechanisms for the formation of the tetrahedral intermediate (ti) in serine proteases. Route a, electrostatic catalysis; route b, double PT mechanism. gs, ts, and ti denote ground state, transition state, and tetrahedral intermediate state, respectively. The figure is merely a schematic diagram, and the actual structure of the transition state is given in Figure 3 (see caption of Table I for more details on the transition state). The shaded substrate region does not indicate planarity but only designates the substrate region.

proposals for the catalytic action of enzymes. The proposal that electrostatic free energy is the key factor in enzyme catalysis (Warshel, 1978, 1981; Naray-Szabo & Bleha, 1982) gains further support in this specific test case.

The central machinery of serine proteases is composed of the three invariant residues Asp_c–His_c–Ser_c (where c denotes a catalytic residue), which are referred to as the *catalytic triad.* These residues catalyze the hydrolysis of peptide bonds in proteins and peptides by a mechanism whose rate-limiting step can be formally described as

$$Im + R'—OH + X—C(=O)R \rightarrow Im^+—H + R'OC(O^-)(R)X \quad (1)$$

where Im and R′—OH designate His_c and Ser_c, respectively. The origin of the great catalytic activity of this system and the specific role of Asp_c have been the subject of continuous debate since the early structural studies of serine proteases [for reviews, see Kraut (1977), Stroud et al. (1977), Markley (1979), and Schowen (1988)].

The pioneering work of Blow and co-workers (Matthews et al., 1967; Blow et al., 1969; Henderson et al., 1971), which was the first to reveal the structure of this catalytic system, suggested an electronic rearrangement of the form Asp_c^- His_c $Ser_c \rightarrow Asp_c$ His_c Ser_c^- as a factor that increases the nucleophilicity of Ser_c. This implied that the Asp_c His_c pair has a zero net charge at the transition state but did not specify explicitly its internal charge distribution and protonation state. However, the bonding diagram drawn in the original work could be interpreted as assuming the (Asp_c–H His_c) rather than the (Asp_c^- H–His_c^+) tautomeric form. In fact, subsequent workers [e.g., Hunkapillar et al. (1973)] interpreted the electronic rearrangement idea as an explicit mechanism that involves a concerted transfer of two protons (from Ser_c to His_c and from His_c to Asp_c, as described in Figure 1b). This mechanism [see discussion in Markley (1979)], which is now depicted in many textbooks, will be called here the double proton-transfer (double PT) mechanism. The acceptance of this mechanism in the chemical community might have been motivated by the recognition that ion pairs are not stable in nonpolar environments so that Asp_c^- His_c^+ must be less stable than Asp_c His_c in nonpolar active sites [see Dewar (1986)]. This, however, overlooks the fact that the active site around Asp_c is very polar (see below). Some workers [e.g., Polgar and Bender (1969), Rogers and Bruice (1974), and Brayer et al. (1978)] have objected to the double PT mechanism and more recent nuclear magnetic resonance experiments (Bachouchin & Roberts, 1978; Markley, 1979) as well as neutron diffraction experiments (Kossiakoff & Spencer, 1981) gave evidence against ground-state proton transfer but could not exclude a transfer at the transition state.

Most quantum mechanical calculations that consider only the catalytic triad without its surrounding protein [e.g., Scheiner et al. (1975), Umeyama et al. (1973), and Dewar and Storch (1985)] supported the double PT mechanism. One such calculation (Kollman & Hayes 1981) that considered the effect of Ser_c concluded that Asp_c^- His_c^+ Ser_c^- is more stable than Asp_c His_c Ser_c^- in the gas phase. Calculations that attempted to include the effect of the protein active site on the catalytic triad (Umeyama et al., 1981; Warshel et al., 1982; Naray-Szabo, 1982, 1983; Warshel & Russell, 1986) contradicted the double PT mechanism, but these latter calculations [with the exception of Warshel et al. (1982) and Warshel and Russell (1986)] did not include the key effect of the solvent around the protein and did not calibrate the intrinsic energy of the ion pair on reliable experimental information. This prevented a quantitative assessment of the

Table I: Electrostatic Energy Changes Calculated by the PDLD[a] Method for the Native (N) and Mutant (M) Enzymes

	native			mutant			
	ts	gs	$\Delta G^{\ddagger}_{N} - \lambda^{\ddagger}$	ts	gs	$\Delta G^{\ddagger}_{M} - \lambda^{\ddagger}$	$\Delta\Delta G^{\ddagger}_{M\rightarrow N}$
				Trypsin			
V_{QQ}	−95.3	−36.5	−58.8	−47.5	−9.8	−37.7	21.1
$V_{Q\mu}$	−42.5	−28.2	−14.3	−25.2	−10.9	−14.3	0.0
$V_{Q\alpha}$	−4.4	0.0	−4.4	−13.2	0.0	−13.2	−8.8
G_{Qw}	−11.7	−14.8	3.1	−14.6	−10.8	−3.8	−6.9
sum	−153.9	−79.5	−74.4	−100.4	−31.5	−69.0	5.4[b] (6.1)[c]
				Subtilisin			
V_{QQ}	−97.5	−35.7	−61.8	−46.4	−6.4	−40.0	21.8
$V_{Q\mu}$	−61.5	−53.5	−8.0	−12.0	−4.0	−8.0	0.0
$V_{Q\alpha}$	−0.7	0.0	−0.7	−8.7	0.0	−8.7	−8.0
G_{Qw}	−22.6	−17.9	−4.7	−32.3	−18.2	−14.1	−9.4
sum	−182.3	−107.1	−75.2	−99.4	−28.6	−70.8	4.4 (6.0)[a]

[a] Energies are in kilocalories per mole. Experimental values, $\Delta\Delta G^{\ddagger}_{exp} = -1.38[\log (k^{M}_{cat}/k^{N}_{cat})]$, are in parentheses. The experiments are pH dependent, and the results quoted for subtilisin (Carter & Wells, 1988) were obtained at pH = 8.6, while in the case of trypsin the experimental result was obtained from the hydroxide-independent rate constant [see Craik et al. (1987) for details]. ts and gs refer to transition and ground states, respectively. $\lambda^{\ddagger}$ is the nonelectrostatic contribution to the formation of the transition state (see eq 4). For other notations, see text. The three-dimensional coordinates were taken from X-ray diffraction studies of trypsin (Huber et al., 1974) and subtilisin (Robertus et al., 1972). Atomic charges of region 1 were taken from Warshel and Russell (1986) except for the ti, whose charges were modified to −0.5, 0.7, and −1.2, respectively, for the serine oxygen, the carbonyl carbon, and the carbonyl oxygen [the set of Warshel and Russell (1986) has zero charge on the serine oxygen of ti]. The transition-state charges (−0.70, 0.58, −0.88) and structure were obtained from the EVB calculations of Warshel et al. (1988), weighting the charges of the $O^{-}C{=}O$ and $O{-}C{-}O^{-}$ resonance forms by 0.4 and 0.6, respectively (the sensitivity of the calculations to the contributions of the two resonance structures can be judged by comparing the calculations for ts and ti in Figure 2). The weighting factors used for locating the transition state are obtained by the EVB procdure as described in Warshel et al. (1988). The O···C bond length at the transition state is around 2.2 Å, and the out-of-plane deformation of the carbonyl carbon is around 50°. The grid size and spacing for the Langevin dipoles, modeling the water molecules, were 12.0 Å and 3.0 Å, respectively. [b] The calculated value does not include the contribution of 1.8 kcal/mol (see text) associated with the rotation of His_c frosm its ground-state improper configuration (Sprang et al., 1987) to the configuration that is adequate for accepting a proton from Ser_c. The later configuration was used in the PDLD calculations. [c] This value is taken by using k_{enz} of Table II in Craik et al. (1987).

feasibility of the double PT mechanism.

It should also be mentioned that the search for catalysis by the double PT mechanism is a subject of intensive effort in biomimetic chemistry, and various model compounds that were meant to mimic the catalytic triad have been designed in recent years [e.g., Mallick et al. (1984) and Cram et al. (1986)].

The emergence of genetic engineering allows one to determine the contribution of individual residues to the catalytic activity of an enzyme. Recent site-directed mutagenesis of trypsin (Sprang et al., 1987; Craik et al., 1987) and subtilisin (Carter & Wells, 1988) has indicated clearly that Asp_c is important since its removal leads to a reduction of 4 orders of magnitude in k_{cat}. However, while these experimental findings establish that Asp_c contributes around 6 kcal/mol to the reduction of the activation free energy of amide hydrolysis, they cannot tell us how this effect is being obtained. To examine this point, one should determine the free energy contribution associated with Asp_c for different possible mechanisms. Such an examination is presented in this work.

Microscopic Calculations of the Electrostatic Contribution of Asp_c

In order to clarify the role of Asp_c, one has to consider the following alternatives (cf. Figure 1): (i) Asp_c is left in its ionized form and serves to stabilize the ionic transition state (route a), (ii) Asp_c is used to accept a proton from His_c in the transition state, thus creating by a double PT-type mechanism a neutral Asp_c His_c pair (route b), and (iii) Asp_c helps in orienting His_c in a proper position to interact with the substrate contributing entropically to the rate acceleration.

The examination of the possible electrostatic role of Asp_c is far from being trivial. Attempts to calculate the electrostatic interaction between Asp_c^{-} and the His_c^{+} t^{-} pair [where t^{-} designates the $(OCO)^{-}$ fragment] by conventional macroscopic models may amount to *assuming* the effect of Asp_c rather than calculating it. That is, one can use a Coulomb's law type expression for the interaction

$$\Delta G = -332[1/(R_1\epsilon) - 1/(R_2\epsilon)] \qquad (2)$$

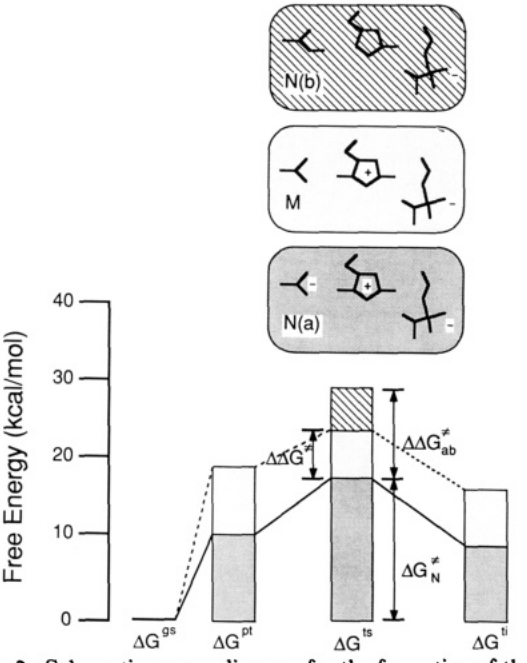

FIGURE 2: Schematic energy diagram for the formation of the tetrahedral intermediate in trypsin. For notations, see eq 5. Numerical values for ΔG^{i}_{N} (i = pt, ts, ti) were taken from earlier calculations (Warshel & Russell, 1986). $\Delta G^{ts}_{N(b)}$ denotes the activation energy in the double PT mechanism, $\Delta\Delta G^{\ddagger}_{ab} = \Delta G^{ts}_{N(b)} - \Delta G^{ts}_{N(a)}$, with $\Delta G^{ts}_{N(a)} = \Delta G^{ts}_{N}$. Subscripts a and b refer to routes a and b in Figure 1.

where R_1 and R_2 are respectively the distances (in angstroms) between the charge center of Asp_c^{-} and that of His_c^{+} and t^{-}, respectively, ϵ is the assumed dielectric constant, and the free energy ΔG is given in kilocalories per mole. R_1 and R_2 of around 3.4 and 6.3 Å reproduce the actual charge–charge interaction (as given by the V_{QQ} of Table I), but more sophisticated calculations should also consider the electrostatic energy in the ground state (where His_c is uncharged). A dielectric constant of about 40 [which is the typical value for

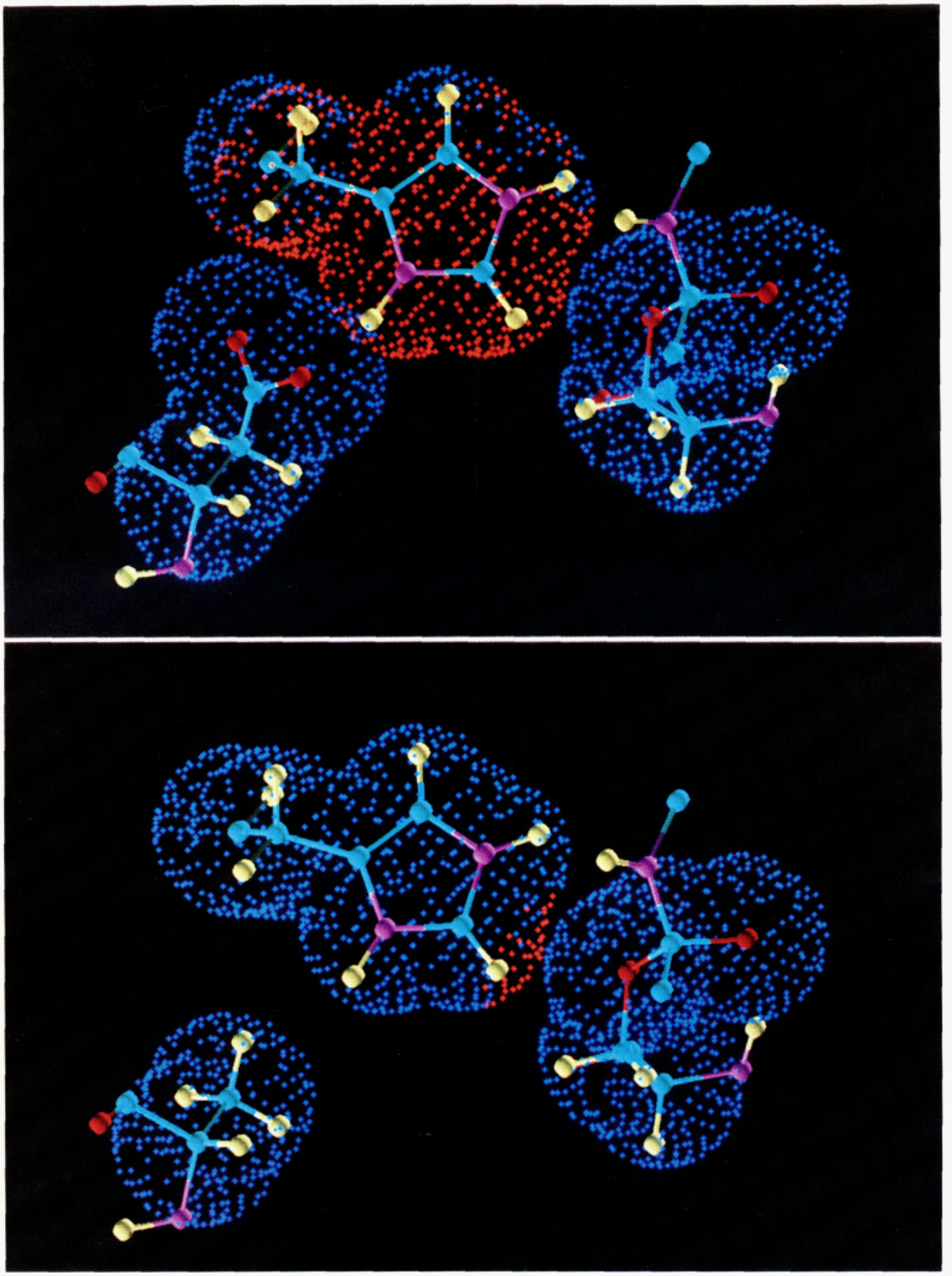

FIGURE 3: Electrostatic potential from the protein at the sites of Asp_c^- and the (His_c^+ t^-) transition state for the native (top) and mutant (bottom) subtilisin. The Asp_c, His_c, and t^- are located respectively at the left, center, and right of the figure. The electrostatic potential surface at 1.5 Å from the atoms of a given residue (for example, the His_c^+) is calculated by summing all the contributions from *other* residues that are within 7 Å of the given residue. Note that the contribution to the electrostatic potential on each residue is from its surrounding environment and not from the residue itself. Positive and negative potentials are given in blue and red, respectively. The figure shows that the large negative potential at the site of His_c, in the native enzyme, disappears upon substitution of Asp_c by Ala_c. This demonstrates that Asp_c plays a major role in stabilizing the (His^+ t^-) transition state.

charge–charge interactions between surface groups in proteins; see Warshel and Russell (1984)] will give $\Delta G \simeq 1$ kcal/mol, which is negligible compared to the ~6 kcal/mol observed effect of Asp_c. In fact, experiments in model compounds in solutions that gave such small effect (Rogers & Bruice, 1974) could serve as evidence that electrostatic stabilization by Asp_c cannot be important. Similar results would be obtained by the modified Tanford–Kirkwood method that places the ionized groups on the surface of the protein (Matthew et al., 1979). On the other hand, assuming a dielectric constant of around 2 for the entire system will give $\Delta G \simeq -22$ kcal/mol (or $\Delta G = -12$ kcal/mol if the energy of the neutral ground state is taken into account), which is consistent with the observed effect. The effect of Asp_c will be overestimated even

if the solvent around the protein is modeled consistently with a large dielectric constant and a discretized continuum approach, while leaving the protein interior with $\epsilon = 2$. Furthermore, Asp_c will not be ionized in such a model. Only semimacroscopic models that explicitly include the protein polar groups (see eq 9 at the end of this paper) can reproduce the correct energetics, but the value of the relevant ϵ cannot be obtained from macroscopic considerations. Similar problems will arise with quantum mechanical studies that neglect the effect of the protein-induced dipoles and the solvent around the protein [e.g., Umeyama et al. (1981) and Naray-Szabo (1983)].

From the above discussion it appears that using a macroscopic model with an assumed dielectric constant cannot tell us what the actual role of Asp_c is (unless we known from experience with related systems what ϵ to use). It appears that the most effective way to determine the actual electrostatic interaction between Asp_c^- and His_c^+ is to use a microscopic model, which avoids the need of a dielectric constant altogether (in the microscopic world there is no need for a dielectric constant since all interactions are taken into account explicitly). The simplest and the first model used for microscopic calculations of electrostatic energies in proteins (Warshel & Levitt, 1976) is the protein dipoles langevin dipoles (PDLD). This model [see Russell and Warshel (1985)], which is now implemented in the convenient program POLARIS, is based on the assumption that the electrostatic energy of the average structure of the system is a good approximation for the electrostatic free energy of the system. The crystallographic coordinates are taken as the average protein structure, and the electrostatic contributions associated with the permanent residual charges and induced dipoles of the protein atoms are taken into account explicitly (the polarization of each induced dipole is evaluated self-consistently by considering the field from the charges of the system and other induced dipoles). The average polarization of the solvent around the protein is simulated by using a grid of polarizable point dipoles whose response to the local field is evaluated by a Langevin-type relationship (which is calibrated by fitting the model to microscopic simulations of solvated ions). The actual calculations are implemented by dividing the reacting system into the following three regions: (i) The catalytic triad (region 1), whose atoms are represented by residual charges ($\mathbf{Q}^i$) that change during the course of the reaction, proceeding from the ground state (gs) via a proton-transfer stage (pt) and the transition state (ts) to the tetrahedral intermediate (ti); (ii) the rest of the protein environment (region 2), which is represented by a standard set of permanent residual charges and atomic induced dipoles; (iii) the water molecules surrounding the active site (region 3), which are modeled by Langevin-type dipoles. More details on the definition and characteristics of these regions are given in Russell and Warshel (1985).

The total electrostatic free energy of a given catalytic triad charge distribution, $G(\mathbf{Q}^i)$, is estimated as the sum of the Coulombic interaction energy within region 1 (V_{QQ}), the interaction between the charges of region 1 and the permanent dipoles of region 2 ($V_{Q\mu}$), the energy of polarization of the protein-induced dipoles by the charges of region 1 ($V_{Q\alpha}$), and the free energy of polarization of the surrounding water molecules (G_{Qw})

$$G(\mathbf{Q}^i) = V^i_{QQ} + V^i_{Q\mu} + V^i_{Q\alpha} + G^i_{Qw} \quad (3)$$

Using the charge distributions of the ground state and state i, evaluated in earlier works by the empirical valence bond (EVB) method (Warshel & Russell, 1986), we can approximate the corresponding free energy difference by

$$\Delta G^i = G(\mathbf{Q}^i) - G(\mathbf{Q}^{gs}) + \lambda^i \quad (4)$$

where λ^i is the nonelectrostatic contribution associated with the formation of state i from the ground state. The change in activation energy as a result of a given mutation is given by

$$\Delta\Delta G^{\ddagger}_{M\rightarrow N} = \Delta G^{\ddagger}_M - \Delta G^{\ddagger}_N \sim [G_M(\mathbf{Q}^{ts}) - G_N(\mathbf{Q}^{ts})] - [G_M(\mathbf{Q}^{gs}) - G_N(\mathbf{Q}^{gs})] \quad (5)$$

where N and M designate the native and mutant enzymes, respectively.

The results of the calculations are summarized in Table I and Figure 2. The calculated values of $\Delta\Delta G^{\ddagger}$ are in a semiquantitative agreement with the corresponding observed values. According to these calculations (which consider only electrostatic effects) the replacement of Asp_c by a neutral residue results in destabilization of the transition state by more than 4 kcal/mol. Apparently, as illustrated in Figure 2, the transition state involves an ion pair between His_c^+ and t^-. Asp_c^- is located in an optimal position to stabilize the positive charge on His_c. This electrostatic effect can be examined in a very qualitative way by evaluating the electrostatic potential on the (His_c^- t^-) pair from the rest of the protein. As shown in Figure 3, the replacement of Asp_c by a noncharged residue leads to a major reduction in the negative potential on His_c, destabilizing the transition state.

The results of the PDLD calculations might still be considered by the reader as an accidental success, since nonelectrostatic contributions were not included. To establish the validity of these results, we repeated the calculations using the more sophisticated (and much more expensive) combination of the empirical valence bond (EVB) and the free energy perturbation (FEP) methods (Warshel & Sussman, 1986; Warshel et al., 1988). This approach uses the detailed all-atom model for the solvent and evaluates (at least in principle) the rigorous free energy of the ground state of a model Hamiltonian [for details, see Warshel et al. (1988)], allowing one to evaluate both the total $\Delta\Delta G^{\ddagger}$ and its electrostatic component. The results of EVB/FEP calculations, summarized in Table II, are quite similar to those obtained by the PDLD and to the corresponding experimental results.

Energetics of the Double PT Mechanism

The agreement between the calculated and observed results strongly supports the role of Asp_c as a key element in the electrostatic stabilization of the transition state. It is, however, important to examine other options. Let us first consider option 2, namely, that Asp_c is involved in the double PT mechanism (route b in Figure 1). This important option could not be explored quantitatively by early quantum mechanical calculations [e.g., Umeyama et al. (1983) and Naray-Szabo (1983)] since (as will be shown below) the actual difference between the two options is smaller than the error associated with the neglect of the surrounding water molecules and with the errors associated with calculations of the intrinsic gas-phase energy of the reacting fragments. However, one can explore the *difference* between route b and route a without the uncertainty associated with quantum mechanical calculations of large systems. That is, the energetics of the double PT mechanism can be obtained from the difference between the activation barriers of route b and route a by $\Delta G^{\ddagger}_b = \Delta G^{\ddagger}_a + \Delta\Delta G^{\ddagger}_{ab}$. If $\Delta\Delta G^{\ddagger}_{ab}$ is positive, then the double PT mechanism is not important. The free energy $\Delta\Delta G^{\ddagger}_{ab}$ is basically the free energy associated with a proton transfer from His_c to Asp_c at the transition state. This free energy can be evaluated in two

Table II: Summary of Results of the EVB–FEP Calculations[a]

enzyme	energy	$\Delta\Delta G^{\ddagger}$	$\Delta\Delta G^{\ddagger}_{exp}$
trypsin	electrostatic	5.4 ± 1.6	
	total	6.2[b] ± 1.6	6.1[c]
subtilisin	electrostatic	3.6 ± 1.2.	
	total	4.1 ± 1.2	6.0[d]

[a] $\Delta\Delta G^{\ddagger}$ denotes the differences between the activation free energies of the native and the mutant enzymes [the transition-state structure is the same as obtained in Warshel et al. (1988)]. All energy values are in kilocalories per mole. Thes EVB–FEP calculations were performed by our molecular simulation package MOLARIS. The parameters used are given in Warshel et al. (1988). The reacting systems were represented by the EVB formalism with resonance structures that describe the ground state, proton transfer state, oxyanion state, and other relevant states [for more details, see Warshel and Russell (1986) and Warshel et al. (1988)]. The calculations involved in initial equilibration of 3 ps at 300 K, followed by free energy calculations in which the systems were driven by the EVB mapping potential in 20 discrete steps from the ground state, via the proton-transfer step to the oxyanion state. Each of the mapping steps involved 0.5-ps equilibration and 0.5-ps data collection. The differences between the forward and backward integration was less than 3 kcal/mol, and the average value was taken ass the calculated $\Delta\Delta G$. The calculated $\Delta\Delta G$ values changes by less than 0.2 kcal/mol with longer integration time. [b] The calculated value includes a contribution of 1.8 kcal/mol from the free energy associated with rotating His_c from its improper ground-state orientation to the configuration that is adequate for accepting a proton from Ser_c (see text). [c] Using k_{enz} of Craik et al. (1987). [d] Carter & Wells, 1988.

steps. First we estimate the free energy difference for the reference reaction in water. This is done by evaluating the free energy of proton transfer from His to Asp in water at the transition-state configuration $r^{\ddagger}$. The corresponding thermodynamic cycle can be expressed as the free energy of taking the fragments of $[Asp^{-}–His^{+}–t^{-}]$ from $r^{\ddagger}$ to infinity, transferring the proton from His to Asp at $r = \infty$, and then bringing the fragments back from infinity to $r^{\ddagger}$. The free energy for the proton-transfer process is evaluated by using the expression (Warshel & Russell, 1984)

$$\Delta G^{w}_{PT}(\infty) = 1.38[pK_a(His) - pK_a(Asp)] \simeq 4 \text{ kcal/mol} \quad (6)$$

The sum of the electrostatic free energies of taking the fragments of $[Asp^{-}\cdots His^{+}\cdots t^{-}]$ from $r^{\ddagger}$ to ∞ and bringing them back to $r^{\ddagger}$ with the charge distribution of $[Asp\cdots His\cdots t^{-}]$ can be evaluated by

$$\Delta\Delta G^{w}_{el}(a\rightarrow b, r=r^{\ddagger}) \simeq \Delta(332\sum_{ij} Q_i Q_j / r_{ij}\epsilon) = \Delta V^{\ddagger}_{QQ}(a\rightarrow b, r=r^{\ddagger})/\epsilon \simeq 2 \pm 1 \text{ kcal/mol} \quad (7)$$

where V_{QQ} designates charge–charge interaction in the vacuum and r_{ij}'s are the distances (in angstroms) between the atoms of the different fragments and where we use a uniform dielectric constant ($\epsilon = 40$). The approximation associated with using a large ϵ for charge–charge interaction in water is completely justified on the basis of countless experimental facts (Warshel & Russell, 1984), and the error associated with changing ϵ from 40 to 80 is around 1 kcal/mol. The same result obtained from eq 7 can be obtained by the reader from eq 2 using just one effective charge center for each fragment. Combining ΔG^{w}_{PT} and $\Delta\Delta G^{w}_{el}$ completes our thermodynamic cycle and gives an overall estimate of 6 kcal/mol for $\Delta\Delta G^{\ddagger}_{ab}$ in water (Figure 4a). This indicates that the double PT mechanism is quite unfavorable in an aqueous medium [see also Rogers and Bruice (1974)]. Replacing the solvent cage by the protein environment *increases* the energy loss by an additional 6 kcal/mol [as estimated by the PDLD calculations of Warshel and Russell (1986)], yielding a value of 12 kcal/mol for $\Delta\Delta G^{\ddagger}_{ab}$ (Figure 4b). This estimate indicates that the double PT mechanism is strongly unfavorable as compared

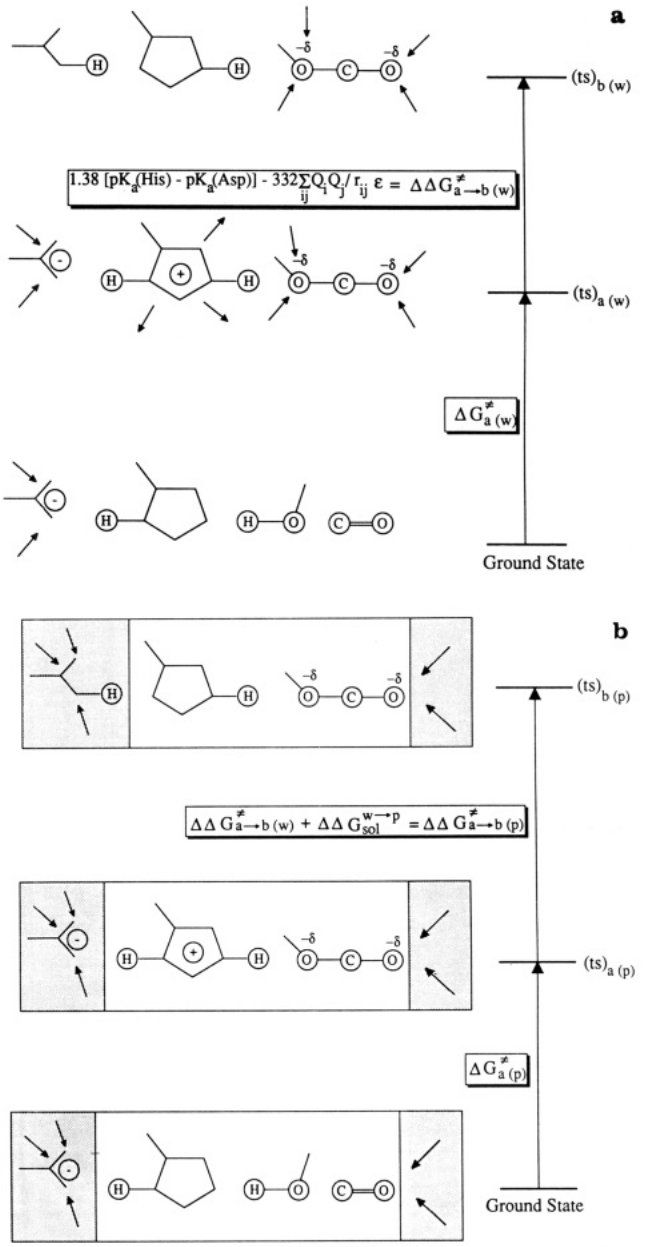

FIGURE 4: (a) Energetics of the double PT mechanism in water. The figure gives the energy difference between the transition states of routes a and b in terms of the free energy of forming the ionized fragments at infinite separation in water (the pK_a term) and the electrostatic energy associated with bringing the fragments together. Note that the dielectric constant ϵ is larger than 40. (b) Energetics of the double PT mechanism in the protein active site. This energetic is given by taking the corresponding free energy in water (a) and adding the free energy associated with moving the relevant fragments from water to the protein active site, $\Delta\Delta G^{w\rightarrow p}_{sol}$, where w and p designate water and protein, respectively.

to the electrostatic one. To realize this from a simple intuitive point of view, it is important to recognize that the ionized form of Asp_c is even more stable in the protein active site than in water, as is apparent from its observed pK_a being equal to 3 in chymotrypsin and the fact that this group is stabilized by three hydrogen bonds [cf. Brayer et al. (1978) and Warshel and Russell (1986) for discussion]. The more stable the negative charge on Asp_c, the less advantageous a proton transfer from His_c to Asp_c would be.

Let us now consider option iii for the catalytic rate acceleration, namely, that Asp_c contributes to catalysis by fixing His_c in an exact orientation for accepting a proton from Ser_c. The X-ray study of the $Asp_c \rightarrow Asn_c$ mutant of trypsin reveals

two configurations of His_c, where in one of them the histidine is rotated out of the catalytic site. The population ratio of this rotated conformation to the one where His_c is inside the active site is 1:2. The corresponding increase in ground-state entropy [R ln (3/2)] increases $\Delta\Delta G^{\ddagger}_{M\rightarrow N}$ by less than 0.3 kcal/mol. However, the configuration with His_c in the correct position appears to stabilize a tautomer that is unable to accept a proton from Ser_c [see Sprang et al. (1987)]. Thus one has to consider the free energy associated with the 180° inversion of His_c in the active site of the Asn_c mutant as a part of the free energy contribution to $\Delta G^{\ddagger}_{M}$. The calculated value of this contribution is around 2 kcal/mol (see Table II). This is a significant contribution that is, however, not entropic in nature but simply the free energy required to move an incorrectly oriented group to the correct orientation. Also note that the incorrect orientation of the Asn_c mutant of trypsin is not expected in the Ala_c mutant of subtilisin. Nevertheless, one can still argue that the actual role of Asp_c is to fix His_c in a very exact catalytic orientation. This possibility cannot be completely excluded by the present calculations, which do not give converging results for $\Delta\Delta S$. Yet the fact that the electrostatic contribution to $\Delta\Delta G^{\ddagger}$ (Table II) accounts for almost all the calculated effect of the mutation can be used to argue that other catalytic effects should be small.

This paper argues that Asp_c is ionized at the ti state, and the reader might wonder how this is related to the original charge relay system (Blow et al., 1969). This original proposal considered two resonance structures at pH 8 [they are **1** and **4** in Figure 2 of Warshel and Russell (1986)], and one more [**2** in Warshel and Russell (1986)] could also be added. The relative contribution to each resonance structure depends strongly on the nuclear coordinates. For example, the Asp_c^- His_c^+ is much less stable than the Asp_c His_c when the proton is closer to Asp_c than to His_c, and the reverse occurs when the proton is closer to His_c (Figure 5). Both resonance structures contribute to the energy of the transition state, but the calculations of Warshel and Russell (1986) indicate that the Asp^- His^+ resonance structure gives a much larger contribution (for the lower energy position of the proton) and that Asp_c is almost fully charged at the transition state.

The present work argues that the removal of Asp_c leads to large electrostatic destabilization of the transition state. Thus one might expect a large effect of this removal on the pK_a of His_c (in the absence of substrate). However, the situation in the two cases is entirely different: In the transition state the water molecules are largely removed from the active site, and the "dielectric constant" for the $Asp_c^-\cdots(His_c^+\ t^-)$ interaction is much lower than that of the Asp_c^- His_c^+ interaction in the absence of the substrate. That is, in the latter case the water molecules on the Ser_c side of His_c^+ can compensate for the removal of Asp_c^- by increasing their solvation effects. Furthermore, if the ionized His_c^+ is unstable, it will be displaced out to water, as is indeed the case in one of the conformers of the mutated trypsin (Craik et al., 1987).

It is important to mention that the effect of Asp 102 was estimated experimentally before the emergence of genetic engineering. This pioneering study (Henderson, 1971; Henderson et al., 1971) involved methylation of His_c, which reduced k_{cat} by more than 3 orders of magnitudes. The resultsant rate reduction was attributed to the incorrect orientation of His_c and to the absence of the polarizing effect of Asp_c.

Some readers might feel that the arguments presented here are valid only for a mechanism that involves a full proton transfer from Ser_c to His_c, while the actual mechanism in serine proteases might involve a concerted proton transfer from

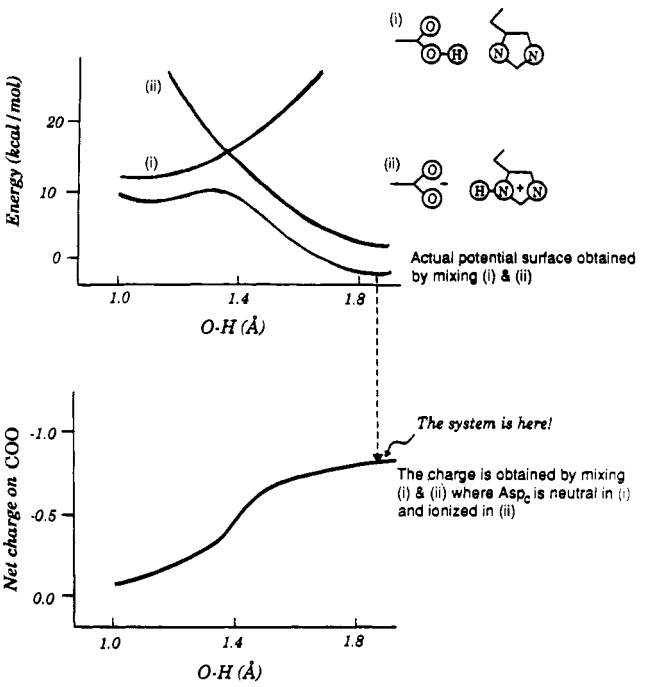

FIGURE 5: Plots showing the relationship between electronic distribution and nuclear coordinates for the Asp_c His_c system at the transition state of the catalytic reaction of serine protease. The upper figure demonstrates that the Asp_c^- His_c^+ configuration is less stable than the Asp_c His_c configuration when the proton is closer to Asp_c than to His_c, while it becomes more stable when the proton is closer to His_c. The lower figure gives the charge on Asp_c as a function of the proton position [this charge is obtained by mixing (i) and (ii)]. As seen from the figure, both resonance structures can contribute to the actual potential surface and charge distribution, but at the transition state the minimum of (ii) is lower than that of (i) [as found by Warshel and Russell (1986) and the present considerations] and the Asp_c^- His_c^+ is the most important configuration, so that Asp_c is almost fully charged.

Ser_c to the amide nitrogen. Although our calculations do not support such a mechanism, they give a linear correlation between the electrostatic stabilization of the fully ionic resonance structures used here and the corresponding stabilization of the transition state of the concerted mechanism. Thus, the ionized Asp_c would also provide a major electrostatic stabilization effect to the concerted mechanism.

What Stabilizes the Tetrahedral Intermediate?

The previous sections concentrated on the electrostatic role of Asp_c and did not address the stabilization of t^-. This issue, which was addressed in our earlier works [e.g., Hwang and Warshel (1987)], is quite instructive. That is, as was noted in early structural studies of serine proteases (Henderson, 1970; Robertus et al., 1972), the oxyanion intermediate, t^-, is stabilized by hydrogen bonding from the so-called "oxyanion hole" of the protein. However, this important discovery has not been emphasized as a major catalytic factor since the X-ray structure by itself could not be used to determine the actual energy contribution associated with the oxyanion hole. As much as anyone knew at that time, the energy of interaction between hydrogen bonds to a charge inside a protein could be the same as between hydrogen bonds to a charge in water (which would mean no catalytic advantage of the oxyanion hole). In fact, only microscopic theoretical calculations [e.g., Warshel et al. 1982)] could estimate the effect of the oxyanion hole until the emergence of genetic engineering experiments on this system (Wells et al., 1986; Bryan et al., 1986). These experiments and FEP calculations (Hwang & Warshel, 1987; Rao et al., 1987) indicated that one hydrogen bond from the

oxyanion hole contributes about 5 kcal/mol to the stabilization of t^-, and similar results have been found in related cases [e.g., Wilkinson et al. (1984)]. This electrostatic stabilization effect is not a univeral effect for hydrogen bonds in proteins; it only occurs in sites designed to stabilize a charge for a specific purpose. The electrostatic stabilization of the oxyanion is provided by the folding energy of the protein that is invested in aligning the dipoles of the oxyanion hole (i.e., the hydrogen bonds) toward the carbonyl oxygen. Using prealigned dipoles can minimize the "reorganization energy", usually associated with the polarization of solvent molecules around charges. That is, in water we invest approximately half of the gain in solvent–charge interaction, $V_{Q\mu}$, on bringing the solvent molecules to a configuration where the solvent–solvent interaction, $V_{\mu\mu}$, is unfavorable. A "solvent" that is already polarized in the right direction does not have to pay the reorganization energy and can stabilize charges more than water does [see Warshel (1978)].

It has been frequently implied that the tetrahedral geometry of the t^- system is somehow very important. In fact, some books do not draw negative charge on t^- and only emphasize its tetrahedral geometry, perhaps assuming that the protein stabilizes the transition state by being a template to the tetrahedral geometry. This overlooks the fact that the change of the carbonyl carbon of the substrates from the sp^2 to the sp^3 geometry is mainly the result of change in intrinsic bonding structure and not the effect of the surrounding active site. In fact, the change in the geometry around the carbonyl carbon is associated with less than 0.3-Å displacement of each atom, and the flexible enzyme can accommodate these changes for less than 1 kcal/mol strain energy. For related discussion on strain in lysozyme, see Warshel and Levitt (1976).

Can We Use Semimacroscopic Electrostatic Models To Study Enzyme Catalysis?

Macroscopic models with assumed dielectric constants could not be used as a predictive tool before the emergence of both genetic engineering and microscopic electrostatic calculations (see Microscopic Calculations of the Electrostatic Contribution of Asp_c). However, at the current stage when we can evaluate the actual electrostatic energy of a given transition state, it is tempting to look for a macroscopic model that reproduces this energy. Accomplishing such a task is not so simple, as the macroscopic nature of the enzyme–water system is not defined uniquely by any dielectric model [see Warshel and Russell (1984)]. Yet, a useful and consistent macroscopic approximation can be obtained by "scaling" the PDLD model. That is, we can take the following two-step procedure: First, assign a uniform internal dielectric constant (ϵ_p) to the protein and then surround the protein by a very large sphere of the same dielectric, which is then surrounded by water ($\epsilon_w = 80$). The free energy of the relevant charges (Q) in this "extended protein" is given by the sum of the energy (ΔG_1) of transferring the charges from water to ϵ_p and the energy of bringing these charges to their actual positions in the protein (ΔG_2). This sum is given by

$$\Delta G_1 + \Delta G_2 = 166\sum_i (Q_i^2/a_i)/(1/\epsilon_p - 1/\epsilon_w) + 332\sum_{ij} Q_i q_j/r_{ij}\epsilon_p \quad (8)$$

where a_i is the cavity radius of ith charge and the q_j's are all the protein residual charges, as well as other charges not included in region 1. The first term in this equation can be obtained in a more quantitative way by defining the cavity radius a_i, using the solvation free energy of the given charge in water [$\Delta G^i_{sol,w} = -166(Q_i^2/a_i)$]. The second term in eq 8 is given by the PDLD model as $(V_{QQ} + V_{Q\mu})/\epsilon_p$, where the contribution of $V_{Q\alpha}$ is incorporated in ϵ_p [see Figure 13 of Warshel and Russell (1984)]. In the second step we evaluate the energy (ΔG_3) associated with changing back the surrounding of the protein from ϵ_p to ϵ_w. This quantity is also given by the PDLD model as $\Delta G_3 = \Delta G_{Qw}(1/\epsilon_p - 1/\epsilon_w)$. Thus we obtain the estimate

$$\Delta G_1 + \Delta G_2 + \Delta G_3 = [(\sum_i -\Delta G^i_{sol}) + \Delta G_{Qw}](1/\epsilon_p - 1/\epsilon_w) + (V_{QQ} + V_{Q\mu})/\epsilon_p \quad (9)$$

This macroscopic expression is more precise than the corresponding microscopic expression (although its results might be less accurate). That is, in the microscopic approaches one evaluates the electrostatic free energy as the sum of two very large numbers (ΔV_{QQ} and ΔG_{sol}) that compensate each other as shown in Figure 16 of Warshel and Russell (1984). On the other hand, in the macroscopic world one *assumes* the compensation by scaling the energy associated with moving the charges by $1/\epsilon_p$ and obtains small numbers for large ϵ_p. Adopting eq 9, one can find that $\epsilon_p = 4$ reproduces the microscopic results of Table I. Unfortunately, the parameter ϵ_p is not a universal parameter. For example, in studying the catalytic effect of lysozyme, one has to use $\epsilon_p = 7$ in eq 9 to reproduce the results of microscopic calculations (Warshel, 1978). It is also important to note that the dielectric constant that should be used in eq 9 is entirely different from the one that *should* have been used in traditional macroscopic methods [e.g., Tanford and Kirkwood (1957)], which describe the protein as a uniform medium with a single dielectric constant, that should also represent the effect of the protein polar groups. Such models, which are much more consistent with the macroscopic philosophy than the formulation of eq 9, require the use of $\epsilon_p \simeq 80$ to reproduce the actual energetics of the Asp_c^- His_c^+ ion pair, since they do not take into account the protein polarity in an explicit way.

It seems to us, in view of the above discussion, that the semimacroscopic formulation of eq 9 (which has not been reported before) can provide a useful estimate of electrostatic effects in enzyme active sites, if ϵ_p is evaluated by microscopic simulations. It is instructive to note that the ΔG_{Qw} contribution in eq 9 can be evaluated by discretized continuum approaches [e.g., Gilson and Honig (1987) and Sternberg et al. (1987)] but the $V_{Q\mu}$ term must be evaluated explicitly.

Concluding Remarks

In summary, we have concluded on the basis of both sophisticated and simple free energy calculations that the buried aspartate contributes to catalysis by stabilizing the charges of the His^+ t^- transition state. The overall rate acceleration appears to be associated with two elements: the ionized Asp_c that stabilizes the ionic transition state from the His_c side and the oxyanion hole that stabilizes the t^- side. The stabilization by the oxyanion hole is manifested by hydrogen bonding to t^-, and since hydrogen bonding is mostly electrostatic in nature (Umeyama & Morokuma, 1977), we can state that the rate acceleration in serine proteases is largely due to electrostatic stabilization of the (His^+···t^-) transition state. This point can be best realized by considering the fact that the formation of the transition state involves a major change in charge distribution that can be described as a formation of an ion pair from a neutral ground state (see Figure 1). The easiest way to reduce the relevant free energy difference is by stabilizing the charge distribution of the ion pair. This fact led to the evolutionary constraint that results in the design of the negatively

charged Asp_c and the dipolar oxyanion hole. It is important to realize in this respect that Asp_c^- would not be kept ionized without its stabilizing hydrogen bonds [see Warshel and Russell (1986)], which can be called the "aspartate hole". The enzyme, in fact, stabilizes the (Asp^-···His^+···t^-) transition state by a preorganized cryptate-like network of oriented hydrogen bonds (Warshel, 1981). Thus, it would be interesting in this respect to destroy the aspartate hole by mutating Ser 214 in trypsin.

ACKNOWLEDGMENTS

We are grateful to Dr. J. Wells and Dr. P. Carter for making their results available before publication.

REFERENCES

Bachouchin, W. W., & Roberts, J. D. (1978) *J. Am. Chem. Soc. 100*, 8041–8047.
Brayer, G. D., Delbaere, J. T. J., & James, M. N. G. (1978) *J. Mol. Biol. 124*, 261–283.
Bryan, P., Pantoliano, M. W., Quill, S. G., Hsiao, H. Y., & Poulos, T. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 3743–3745.
Blow, D. M., Birktoft, J. J., & Hartley, B. S. (1969) *Nature 21*, 337–340.
Carter, P., & Wells, J. A. (1988) *Nature 332*, 564–568.
Craik, C. S., Roczniak, S., Largeman, C., & Rutter, W. J. (1987) *Science 237*, 909–913.
Cram, D. J., Lam, P. Y.-Y., & Ho, S. P. (1986) *J. Am. Chem. Soc. 108*, 839.
Dewar, M. J. S. (1986) *Enzymes 36*, 8–20.
Dewar, M. J. S., & Storch, D. M. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 2225–2229.
Dickerson, R. E., Gray, H. B., Darensbourg, M. Y., & Darensbourg, D. J. (1984) *Chemical Principles*, p 873, Benjamin/Cummings, Menlo Park, CA.
Gilson, M. K., & Honig, B. H. (1987) *Nature 330*, 84–86.
Henderson, R. (1970) *J. Mol. Biol. 54*, 341–354.
Henderson, R. (1971) *Biochem. J. 124*, 13–18.
Henderson, R., Wright, C. S., Hess, G. P., & Blow, D. M. (1971) *Cold Spring Harbor Symp. Quant. Biol. 36*, 63–70.
Huber, R., Kukla, D., Bode, W., Schwager, P., Bartels, K., Deisenhofer, J., & Steigemann, W. (1974) *J. Mol. Biol. 89*, 73–101.
Hunkapillar, M. W., Smallcombe, S. H., Whitaker, D. R., & Richards, J. H. (1973) *Biochemistry 12*, 4732–4743.
Hwang, J.-K., & Warshel, A. (1987) *Biochemistry 26*, 2669–2673.
Jacobs, J., Schultz, P. G., Sugasawara, R., & Powell, M. (1987) *J. Am. Chem. Soc. 109*, 2174–2176.
Knowles, J. R. (1987) *Science 236*, 1252–1258.
Kollman, P. A., & Hayes, D. M. (1981) *J. Am. Chem. Soc. 103*, 2955–2961.
Kossiakoff, A. A., & Spencer, S. A. (1981) *Biochemistry 20*, 6462–6473.
Kraut, J. (1977) *Annu. Rev. Biochem 46*, 331–358.
Kraut, J. (1988) *Science 28*, 533–540.
Mallick, I. M., D'Souza, V. T., Yamaguchi, M., Lee, J., Chalabi, P., Gadwood, R. C., & Bender, M. L. (1984) *J. Am. Chem. Soc. 106*, 7252.
Markley, J. L. (1979) in *Biological Applications of Magnetic Resonance* (Shulman, R. G., Ed.) pp 397–461, Academic Press, New York.
Mattew, J. B., Hanania, G. I. H., & Gurd, F. R. N. (1979) *Biochemistry 18*, 1919–1928.
Matthews, B. W., Sigler, P. B., Henderson, R., & Blow, D. M. (1967) *Nature 214*, 652.
Naray-Szabo, G. (1982) *Int. J. Quantum Chem. 22*, 575–582.
Naray-Szabo, G. (1983) *Int. J. Quantum Chem. 23*, 723–728.
Naray-Szabo, G., & Bleha, M. (1982) in *Progress in Theoretical Organic Chemistry* (Csizmadia, I. G., Ed.) Vol. 3, pp 267–336, Elsevier, Amsterdam.
Page, M. I. (1987) in *Enzyme Mechanisms* (Page, M. I., & Williams, A., Ed.) pp 1–13, Royal Society of Chemistry, London.
Pauling, L. (1946) *Chem. Eng. News 263*, 294–297.
Polgar, L., & Bender, M. L. (1969) *Proc. Natl. Acad. Sci. U.S.A. 64*, 1335–1342.
Rao, S. N., Singh, U. C., Bash, P. A., & Kollman, P. A. (1987) *Nature 328*, 551–554.
Robertus, J. D., Kraut, J., Richard, A. A., & Birktoft, J. J. (1982) *Biochemistry 11*, 4293–4303.
Rogers, G. A., & Bruice, T. C. (1974) *J. Am. Chem. Soc. 96*, 2473–2481.
Russell, S., & Warshel, A. (1985) *J. Mol. Biol. 185*, 389–404.
Rutter, W. J., & Craik, C. S. (1987) *Science 237*, 905–909.
Showen, L. R. (1988) in *Principles of Enzyme Activity* (Liebman, J. F., & Greenberg, A., Eds.) Vol. 9, Molecular Structure and Energetics, VCH Publishers, Weinheim, FRG.
Sprang, S., Standing, T., Fletterick, R. J., Finer-Moore, J., Stroud, R. M., Xuong, N.-H., Hamlin, R., Rutter, W. J., & Craik, C. S. (1987) *Science 237*, 905–909.
Sternberg, M. J. E., Hayes, F. R. F., Russell, A. J., Thomas, P. J., & Fersht, A. R. (1987) *Nature 330*, 86–88.
Stroud, R. M. (1974) *Sci. Am. 231*, 74–88.
Stroud, R. M., Kossiakoff, A. A., & Chambers, J. L. (1977) *Annu. Rev. Biophys. Bioeng. 6*, 177–193.
Tanford, C., & Kirkwood, J. G. (1957) *J. Am. Chem. Soc. 79*, 5333–5339.
Tramontano, A., Janda, K. D., & Lerner, R. A. (1986) *Science 234*, 1566–1570.
Umeyama, H., & Morokuma, K. (1977) *J. Am. Chem. Soc. 99*, 1316–1322.
Umeyama, H., Imamura, A., Nagato, C., & Hanano, M. (1973) *J. Theor. Biol. 41*, 485–502.
Umeyama, H., Nakagawa, S., & Kudo, T. (1981) *J. Mol. Biol. 150*, 409–421.
Warshel, A. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 5250–5254.
Warshel, A. (1981) *Biochemistry 20*, 3167–3177.
Warshel, A., & Levitt, M. (1976) *J. Mol. Biol. 103*, 227–249.
Warshel, A., & Russell, S. T. (1984) *Q. Rev. Biophys. 17*, 283–422.
Warshel, A., & Sussman, F. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 3806–3810.
Warshel, A., & Russell, S. T. (1986) *J. Am. Chem. Soc. 108*, 6569–6579.
Warshel, A., Russell, S., & Weiss, R. M. (1982) in *Biomimetic Chemistry and Transition-State Analogs* (Green, B. S., Ashani, Y., & Chipman, D., Eds.) pp 267–273, Elsevier, Amsterdam.
Warshel, A., Sussman, F., & Hwang, J.-K. (1988) *J. Mol. Biol. 201*, 139–159.
Wells, J. A., Cunningham, B. C., Graycar, T. P., & Estell, D. A. (1986) *Philos. Trans. R. Soc. London, A No. 317*, 415–423.
Wilkinson, A. J., Fersht, A. R., Blow, D. M., Carter, C., & Winter, G. (1984) *Nature 307*, 187–188.