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Articles

Electrical Potentials in Trypsin Isozymes†

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ABSTRACT: Cow and rat trypsin differ in net charge by 12.5 units yet have the same enzymatic mechanism. The role of electrical potentials in the catalytic mechanism of these trypsin isozymes is investigated by using the finite difference Poisson-Boltzmann method. The calculations reveal that the active sites are effectively shielded from surface charge, thus making it possible for the two enzymes to have essentially identical potentials in their catalytically important regions. The potentials in both active sites are dominated by local interactions arising both from partial charges and from the negative charge on Asp-102. The latter is found to stabilize the transition state by about 4 kcal/mol, a value that is consistent with the extent of reduced catalytic activity in the variant Asn-102 trypsin, in which the negative charge is absent. The calculations predict that Asp-102 is ionized and that His-57 is neutral in the resting state of the enzyme. In contrast to their negligible effect on catalytic activity, the cumulative effect of surface charges is found to raise the pK of the N-terminal α-amino group of Ile-16 in the rat enzyme by about 1.5 units relative to that of cow trypsin. This charged amino acid forms an ionic bond with Asp-194, which stabilizes the active conformation of the enzyme. An increase in pK of Ile-16 thus provides a possible explanation for the retention of activity of rat trypsin at high pH. The results of this study could not have been obtained from an electrostatic model based on Coulombic potentials.

Electrostatic fields around the surface of proteins have been shown to play an important role in molecular recognition and binding. For example, the positive potential of DNA polymerase aids in the assembly of the protein-DNA complex (Warwicker et al., 1985). The electric field of Cu, Zn superoxide dismutase is responsible for enhanced diffusion rates of its superoxide substrate to the active site (Koppenol, 1981; Klapper et al., 1986; Sharp et al., 1987) while the electric field of cytochrome *c* plays a similar role in the interaction of this protein with cytochrome *c* peroxidase (Koppenol & Margoliash, 1982; Northrup et al., 1988). These fields, which extend out into solution, result from the asymmetric distribution of charged side chains on the protein surface. They do not appear to be due to a single amino acid but, rather, arise from the cumulative effects of a number of residues. It is of interest to consider whether surface charges might also play a role in influencing function in the interior of proteins, particularly in active sites.

In considering this question in "protein design", it is important to distinguish the effects of specific charged groups that are part of the active site from the aggregate effects of surface charge some distance from the active site. That charged residues near the active site might be functionally important has been suspected for some time. For example, it was suggested over 30 years ago that charged amino acids in the retinal binding site play a role in determining the colors of visual pigments (Kropf & Hubbard, 1958). Quantum mechanical calculations and experiments on artificial pigments have identified the location of these groups with respect to the retinal chromophore (Honig et al., 1976; Honig et al., 1979).

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In lysozyme, the electrostatic potential due to Asp-52 was suggested some time ago as a factor that might stabilize the transition state in the catalytic mechanism (Vernon, 1967). A theoretical analysis of the lysozyme structure provided a description of electrostatic contributions to the catalytic mechanism of this enzyme and questioned the use of simple Coulombic potentials (Warshel & Levitt, 1976). In contrast to these cases, it has been more difficult to establish theoretically whether electrostatic effects due to surface charge are also felt in active sites. Part of the problem has been the need to account for the effects of solvent screening, including ionic strength effects, on long-range electrostatic interactions.

In the past few years it has been shown that finite difference solutions to the Poisson–Boltzmann equation (Warwicker & Watson, 1982; Klapper et al., 1986) (the FDPB method) provide an accurate means of calculating electrostatic interactions in proteins [see, e.g., Gilson and Honig (1987) and Sternberg et al. (1987)]. A number of FDPB calculations of the electrical potentials in the active sites of enzymes have been reported. In a study of actinidin and papain electrostatic fields due to charged amino acids were evaluated and suggested to be the basis for the reactivity characteristics of these enzymes (Pickersgill et al., 1988). A similar conclusion was reached for lysozyme where a sharp potential gradient was found across the active sites of lysozymes with dissimilar amino acid sequences (Dao-pin et al., 1989). It was suggested that a significant part of this field arises from a clustering of positive charges on one lobe of the protein and to the focusing of field lines through the active site. These results appear to argue that surface charge, as well as specific active-site residues, can play an important role in catalysis. In this paper we study the relative magnitude of these two effects in the catalytic mechanism of trypsin. In the context of this work we also consider the catalytic role of Asp-102 in serine proteases as well as the mechanism of inactivation of serine proteases at high pH.

Trypsin, in common with many other enzymes, exists as multiple isozymes. Two of the principle isozymes secreted by the pancreas are termed anionic and cationic, reflecting their different migration in electric fields. The biochemical origin of this behavior is a net difference of 12.5 units of charge (assuming all histidines bear a charge of 0.5) in the amino acid sequences; however, the physiological basis for the different isozymes of trypsin is not known. The existence of high-resolution structures for both a cationic (bovine) (Bode & Schwager, 1975) and anionic (rat) (Sprang et al., 1987) trypsin allows us to consider how strikingly different charge distributions affect the active sites of two proteins with identical catalytic mechanisms.

The catalytic mechanism of trypsin is believed to involve the transfer of a proton from the active-site serine to the active-site histidine, followed by nucleophilic attack of the serine oxygen on the carbonyl carbon of the substrate [see, e.g., Kraut (1977), Steitz and Shulman (1982), and Bachovchin (1986)]. This produces an intermediate with a tetrahedral carboxylic carbon and a negative charge located on the substrate oxygen, the oxyanion. Both the transfer of the proton and the formation of the tetrahedral intermediate involve energetic barriers although the latter step is believed to be rate limiting [Bachovchin, 1986; see also the recent discussion by Warshel et al. (1989) for a detailed energetic analysis of the catalytic mechanism]. The oxyanion is stabilized by the main-chain amide groups of residues 193 and 195 that, together with bound water molecules, form the "oxyanion hole" (Henderson et al., 1971).

In order to investigate how charged amino acids might influence the catalytic mechanism, we have carried out FDPB calculations of the electrical potential of both cationic and anionic trypsin. We have focused on the effects of both the active-site Asp-102 and surface charges that are not part of the active site. The results obtained for the effects of Asp-102 provide a basis for explaining the observed reductions in catalytic rates in variant serine proteases that lack this residue. The results are used to address mechanistic issues that are specific to trypsin and, in addition, to consider the more general questions of the role of surface charges in influencing catalytic rates. The results are also compared to those obtained by Warshel et al. (1989) on the same system using an independent approach to the calculation of electrostatic interactions.

In addition to studying the electrical potential under physiological conditions we have also investigated the different responses of bovine and rat trypsin to high pH. Kinetic analysis has shown that bovine cationic trypsin inactivates at about pH 9.5 while rat anionic trypsin is reported to retain activity even at pH 10.5–11 (Craik et al., 1987). The activity of the homologous enzyme chymotrypsin has been characterized at pH values ranging from 4.5 to 10.5 (Fersht, 1972). Chymotrypsin exists in an active and inactive form whose equilibrium is controlled by a group with an apparent pK of about 8.8. There is strong evidence that this group corresponds to the N-terminus α-amino group of Ile-16 which, in its protonated form, forms a salt bridge with Asp-194 (Fersht, 1972). This ion pair is thought to control the conformational switch that converts the inactive zymogen to active enzyme. Deprotonation of the amino group weakens the salt bridge and shifts the equilibrium toward an inactive form. The same hypothesis can also account for the inactivation of cow trypsin at high pH but appears inconsistent with the fact that rat trypsin, in common with pig elastase (Geneste & Bender, 1969), is active above pH 10.

A number of explanations might account for the differences between cow trypsin and chymotrypsin on the one hand and rat trypsin and pig elastase on the other. First, it is possible that the pK of Ile-16 is identical in all four proteins but that, in rat trypsin and elastase, other energetic factors shift the equilibrium toward the active form of the enzyme even when Ile-16 is deprotonated. A second possibility is that Ile-16 has an elevated pK in rat trypsin and elastase. Finally, it is conceivable that electrostatic factors other than deprotonation of the α-amino group of Ile-16 lower the activity of chymotrypsin and cow trypsin at high pH. In order to investigate possible electrostatic contributions to the differences between cow and rat trypsin, we have calculated the potential at the α-amino nitrogen of Ile-16 in both enzymes. We have also calculated the potentials due to the various tyrosines and lysines in rat and cow trypsin since it is presumably these residues that change their ionization state in the pH range of interest.

Methods

The details of the FDPB method have been described in a number of recent publications (Gilson et al., 1988; Jayaram et al., 1989). Charges are assigned to the center of any atom whose contribution to the total electrostatic potential is to be studied, and these charges are treated as embedded in a low dielectric medium consisting of the volume enclosed by the solvent-accessible surface of the protein. The surrounding solvent is treated as a continuum of dielectric constant 80 with an electrolyte behaving according to the Poisson–Boltzmann equation. The protein and the surrounding solvent are mapped onto a cubic lattic which in most applications is of dimension 65 Å. In this work the Poisson–Boltzmann equation is solved

with the DelPhi program. Rotational averaging and focusing (Gilson et al., 1988) were used to improve the precision of the results. Recent enhancements in the DelPhi program have made it possible to obtain results in less than 30 s of CPU time on a CONVEX C2 computer (Nicholls and Honig, unpublished).

In order to obtain the contributions of each charged atom (i) to the electrical potential ϕ_j at a particular site (j) in the protein, it is not necessary to calculate the electric field for each atom in separate computations. Rather, the DelPhi program is run once for each site of interest on the assumption that a unit charge is located at that site. Since, in the linear Poisson–Boltzmann equation, $q_i\phi_j = q_j\phi_i$, the effect of each charged atom is determined by obtaining the potential induced by the site of interest at the charged atom and then multiplying this potential by the appropriate charge. Thus, for example, the potential induced by each lysyl side chain at the oxyanion hole is obtained by calculating the potential due to a negative charge placed at the oxyanion hole at the location of each lysine Nϵ atom and then multiplying this value by the charge assigned to the Nϵ atom.

The internal dielectric constant to be assigned to the protein is an important variable for certain interactions. Extensive comparisons between theory and experiment should ultimately make it possible to determine an optimal value for this parameter. However, in our current work we have relied primarily on theoretical considerations in assigning a value of the dielectric constant to the protein. It should be pointed out in this regard that most of the FDPB calculations reported to date have been applied to electrostatic interactions between groups that are near the protein surface or that are fairly far apart. Such interactions take place "through solvent" rather than "through protein" and are thus relatively insensitive to the dielectric constant assigned to the protein (Gilson & Honig, 1987; Sternberg et al., 1987). However, when short-range interactions are considered, such as between the catalytic triad in the active site of serine proteases, the results may be quite sensitive to the value of the dielectric constant assigned to the protein. Our current procedure for assigning the dielectric constant is based on the following considerations [see Gilson and Honig (1986) for a detailed discussion of the issues].

The dielectric constant of polar molecules can be partitioned into distinct contributions from electronic polarization and from the nuclear reorientation of the atoms in dipolar groups. When a structure that is assumed to be fixed or in a time-averaged conformation is analyzed, nuclear motion is not a factor so that its effects should not be incorporated into the dielectric constant. In such cases the potential induced by each charged or polar group can be calcualted explicitly. Only electronic polarization is not accounted for at an atomic level, and consequently, its screening effects must be incorporated into the dielectric constant. A dielectric constant of 2 appears to be a good average value to describe electronic polarization on the basis of the high-frequency dielectric constant of organic molecules.

In applications where the protein conformation is expected to change somewhat, for example as a consequence of site-directed mutagenesis or the formation of a tetrhedral intermediate, it is necessary to account for the screening effects of nuclear reorientation. If the coordinates of the native and perturbed proteins are known (for example, from two separate crystal structure determinations or from model building), a dielectric constant of 2 should still be used because all charges and permanent dipoles in the native and perturbed structure can be considered explicitly. However, in cases where the change in protein conformation is not known, it is still important to account for the response of the permanent dipoles of the protein to changes in the electrical potential. In such case we have argued that using a dielectric constant of 4 rather than 2 is a reasonable means of accounting for reorientation of the dipolar groups [Gilson & Honig, 1986; see also discussion in Harvey (1988)]. We have used an internal dielectric constant of 4 for all the calculations reported in this work because we are considering phenomena, such as the formation of a tetrahedral intermediate, where the protein is expected to relax as a consequence of some change in its charge distribution. Since we are not explicitly describing the conformational changes in the protein that occur during the course of catalysis or as a result of mutagenesis, we account for them in an average was by increasing the dielectric constant from 2 to 4.

Atomic coordinates for trypsins complexed with benzamidine were obtained from the Brookhaven Data Bank (1RTN and 3PTB, respectively) (Bernstein et al., 1977). Rat anionic and cow cationic trypsin are homologous. Out of the 223 residues in each chain 165 (64%) are identical. The low dielectric region was defined by the locations of all the protein atoms including the benzamidine inhibitor and all the observed waters. Since the rat protein has more reported waters than the cow, the waters in the two systems make somewhat different contributions to the shape of the dielectric boundary. The sensitivity of our results to this effect was studied by carrying out calculations in which bound waters were excluded from the low dielectric region. As will be shown, the conclusions of this study are not sensitive to the assumptions made regarding the placement of waters in the crystal structure.

In this work (using PDB notation), each arginine was assumed to have a charge of 0.5 at the nucleus of NH1 and NH2 and each lysine a charge of 1 at the Nϵ nucleus, histidines except for the active-site histidine were assigned a charge of 0.25 on each Nδ and Nϵ atom, aspartate and glutamate residues had a charge of −0.5 on each of the carboxylate oxygens, the N-terminal amino group had a charge of 1 on the nitrogen, and the C-terminal carboxy group had a charge of −0.5 on each oxygen. The active-site histidine had a net charge of either 0 or +1, in which case each nitrogen was assigned a charge of 0.5. In the one case where the contribution of uncharged groups was considered, the AMBER partial charge set (Weiner et al., 1984) was used. The effect of high pH was obtained by assuming that one or more lysines were neutralized while individual tyrosines were assigned a charge of −1 located at the oxygen. With the charge set used in this work cow trypsin has a charge of +10 at neutral pH while rat trypsin has a net charge of −2.5.

Results

Potential Contours. Figure 1 displays space-filling models of cow and rat trypsin coded so that all surface points of negative potential are shown in red and all points of positive potential are shown in blue. The active-site His and Ser residues are green. The patterns illustrated in the figure arise from all charged residues in rat and bovine trypsin (including the benzamidine inhibitor but excluding the active-site histidine). It is clear that, despite significant differences elsewhere in the protein, the pattern around the active site is very similar in both proteins.

This may be seen more clearly in Figure 2 which displays ±0.5 and −6 kcal/mol contours of the electrical potential in a two-dimensional slice through the proteins that contain the active-site residues: Asp···His···Ser from left to right. In both cow and rat trypsin the active-site histidine is in a region of

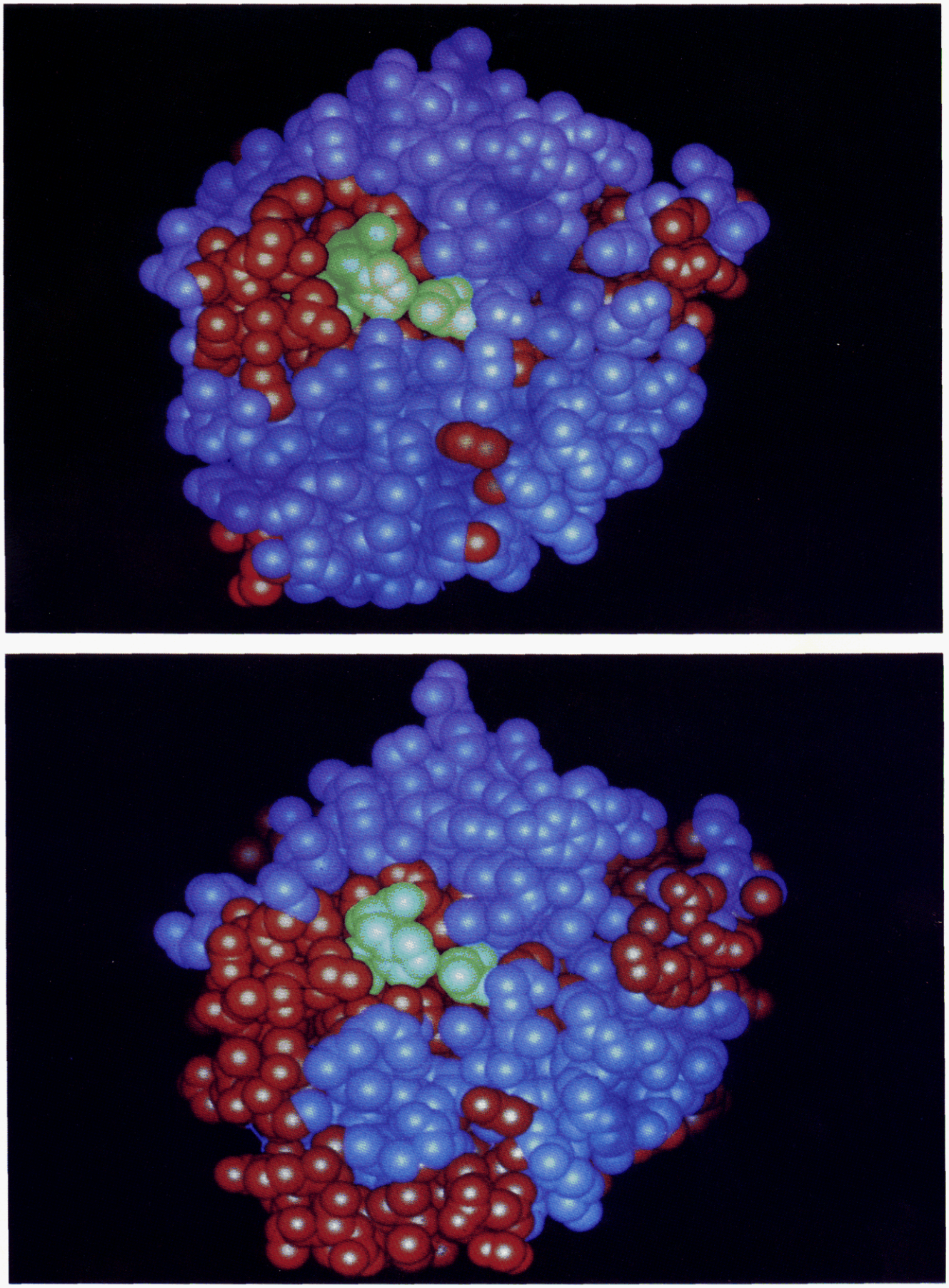

FIGURE 1: van der Waals surfaces colored according to electrostatic potentials. The potentials are generated by all charged amino acids and the benzamidine inhibitor, excluding the active-site His-57. Surface points of negative potentials are colored red; points with positive potential are drawn in blue. The active-site His and Ser residues are drawn in green but they correspond to points of negative potentials. (Top) Cow trypsin. (Bottom) Rat trypsin. The figure was generated with the Insight program distributed by Biosym Technologies.

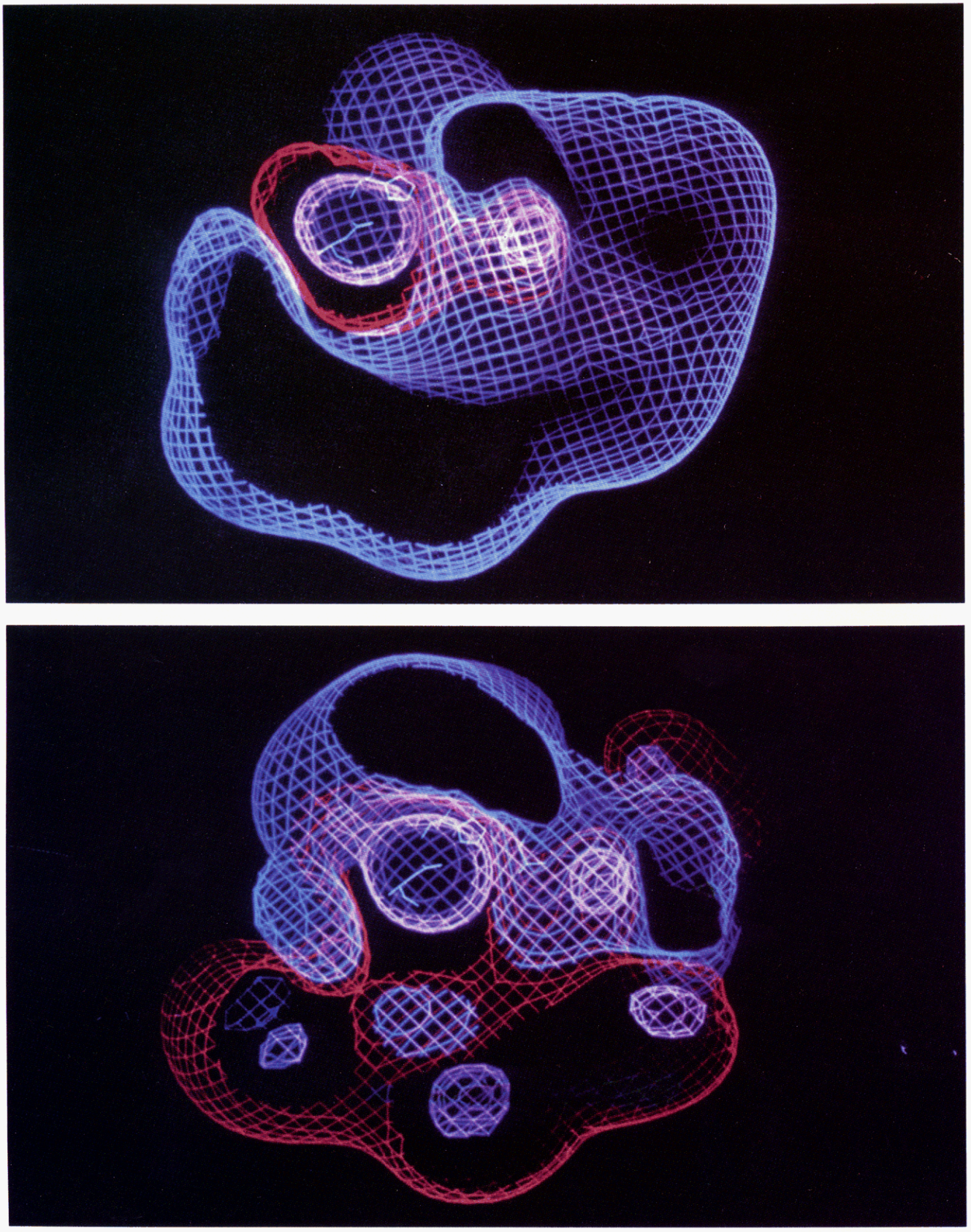

FIGURE 2: Electrostatic potential contours. The figure displays potential contours in a two-dimensional slice (of finite thickness) through the active site of trypsin. The active-site residues are drawn in green. Regions within a blue contour have electrostatic potentials greater than +0.5 kcal/mol, regions within a red contour have potentials less than −0.5 kcal/mol, and regions within a purple contour have potentials less than −6 kcal/mol. (Top) Cow trypsin. (Bottom) Rat trypsin. The figure was generated with the Insight program distributed by Biosym Technologies.

quite negative potential arising from Asp-102 while Ser-195 and the oxyanion hole (directly below Ser-195) are in regions lower negative potential. The strikingly sharp potential gradient through the active site, quantified in Table I, results primarily from Asp-102. In other regions, the potential generated by bovine trypsin is generally more positive than that

Table I: Electrical Potentials in the Active Site of Trypsin[a] (kcal/mol)

	cow			rat		
source	oxyanion	Ser-Oγ	His-Nε	oxyanion	Ser-Oγ	His-Nε
(1) surface charges[b]	−1.76	−2.06	−0.27	−2.03	−2.16	−0.1
(2) +inhibitor[c]	+0.41	+0.94	+1.02	+0.41	+0.86	+1.3
(3) Asp-102	−1.67	−3.74	−5.83	−1.77	−3.38	−6.5
(a) no crystal H_2O	−1.12	−2.63	−4.08	−1.26	−2.54	−4.9
(4) all partials[d]	+6.10	+6.33	−0.13	+5.00	+5.54	−1.3
(5) surface + inhibitor[e]	+35.4	+35.5	+37.3	−7.06	−6.67	−0.2
(6) Asp-102[e]	−8.85	−11.54	−15.78	−8.67	−10.77	−15

[a] ε(protein) = 4; ionic strength = 0.144 M; ion exclusion radius is 2 Å. [b] Charges on Lys, Arg, Glu, Asp, and His residues assigned as in text. [c] All charges in row 1 plus charge on inhibitor. [d] Only partial charges, i.e., no charged resdues. [e] Potentials calculated with Coulomb's law and a dielectric constant of 4 everywhere.

of rat trypsin, as expected from the different net charges on the two proteins.

Effects of Surface Charge. The values of the potentials at the mechanistically important locations in the active sites of both bovine and rat trypsin are given in Table I. The data in the table clearly show that the electrical potentials are very similar in both active sites. The first row in the table contains the summed potential of all the charged residues in the protein excluding the charge on the benzamidine and Asp-102 (and His-57, whose effects are never calculated explicitly since it is a participant in the catalytic mechanism). The straightforward expectation would have been for major differences in the active-site potential produced by surface charges since there is a difference of 12.5 atomic units between the net charge of rat and bovine trypsin. Nevertheless, to within the accuracy of the calculations, it is striking that the potential in the active sites due to non-active-site residues is essentially identical in both proteins. The potential in the active site of both proteins is weak and negative, which is surprising for cow trypsin given its net positive charge. The sum of rows 1 and 2 yields the net potential in the active site due to real charges except Asp-102. The potential is close to zero throughout the active site.

In order to understnad the source of this effect, we have also calculated the electrostatic potentials in the active site that are obtained from Coulomb's law. Potentials are reported with a uniform dielectric constant of 4, which is the value we have assumed for the interior of the protein. In contrast to the FDPB results, Coulomb's law with a dielectric constant of 4 predicts that the surface charges produce a large positive potential in bovine trypsin and a smaller negative potential in rat trypsin. If a larger dielectric constant than 4 (e.g., 40—divide rows 5 and 6 by 10) was used in conjunction with Coulomb's law, the prediction would still be that the two active sites are in very different regions of electrostatic potential: about +3.5 kcal/mol in the active site of cow trypsin and ranging from 0 to −0.7 kcal/mol across the active site of rat trypsin. Moreover, the use of a high dielectric constant would predict a stabilizing effect of Asp-102 of only about 0.7 kcal/mol, much less than the prediction of row 3, which is in good agreement with the experimental value (see discussion below). Thus, neither a high nor a low dielectric constant can reproduce the predictions of the FDPB calculations. Rather, it is necessary to treat the protein as a region of low dielectric constant (i.e., low polarizability) surrounded by a high dielectric solvent.

The conservation of the electrostatic potential in the active site of trypsin isozymes is a direct consequence of the dielectric properties of the protein–solvent system. The low dielectric protein essentially insulates the active site from most of the surface charge by forcing the electric field to go through solvent where screening is effective. A similar effect has been noted in a study of charge-induced pK shifts in subtilisin (Gilson & Honig, 1987; Sternberg et al., 1987).

Catalytic Mechanism. As discussed in the previous section, the net effect of the surface charge is to produce essentially zero electrostatic potential in the active site. Thus, the actual potential in the active site results from the partial charges residing on the polar atoms and from Asp-102. Asp-102 produces a strong negative potential in both active sites and, more importantly, a significant potential gradient between the oxyanion hole, the serine and the histidine. The potentials shown in Figures 1 and 2 correspond to the sum of the surface charge and Asp-102 effects. The potential in the active site is due primarily to Asp-102 while the potentials in other regions are due to the other charged amino acids.

One might expect the two active sites to have essentially identical geometries and hence identical potentials due to Asp-102 and the partial charges. The small differences that are obtained are due to experimental differences in the atomic coordinates of the two proteins, to inaccuracies in the calculations (a conservative estimate is that the calculations are reliable to within 10%), and to the different number of bound waters located in the crystal structures of the two proteins. Small differences in the coordinates are primarily responsible for differences in the partial charge potentials since these are due primarily to hydrogen bonding, which involves interactions over distances of an angstrom or two. Even differences of 0.1 Å in the crystal coordinates will lead to potential differences for interactions over small distances.

The first step in the catalytic mechanism of amide hydrolysis of trypsin is the transfer of a proton from the Oγ of Ser-195 to the Nε2 of His-57. As can be seen in Table I, the arrangement of partial charges of the catalytic triad and the neighboring atoms facilitates this process, but the transfer is further enhanced by the electrical gradient due to the negative charge on Asp-102. The negative charge appears to be crucial, as suggested by the lack of activity of Ser-195 toward diisopropyl phosphate in the Asp-102 to Asn-102 trypsin variant (Craik et al., 1987). Diisopropyl phosphate is a very potent covalent inactivator or Ser-195 in natural trypsin and reacts via nucleophilic attack of the serine-Oγ on the phosphorus atom of the inhibitor.

As discussed above, the postulated state of the enzyme in the tetrahedral intermediate involves a negative charge in the oxyanion hole and a positive charge on the histidine. This charge distribution is stabilized by the partial charges in the protein, particularly those that form the oxyanion hole. In addition, Asp-102 provides a strong stabilizing interaction due primarily to its effect on the active-site histidine. If we make the simplifying assumption that in the resting state of the enzyme there is no net interaction between Asp-102 and the other active-site residues and that the transition state has a charge of −1 in the oxyanion hole and +1 on Nε2, then Table

I, row 2, predicts that Asp-102 lowers the activation energy for catalysis by about 4–5 kcal/mol. The results without crystallographic waters predict an effect of about 3 kcal/mol while the results (not shown in the table) using an internal dielectric constant of 2 for the protein predict an effect of about 6–7 kcal/mol. Given the uncertainties in the presence or absence of bound water and in the internal dielectric constant, a conservative interpretation of Table I is that the electrostatic effect of Asp-102 is to lower the activation energy of catalysis by 3–6 kcal/mol. Wild-type trypsin has an activity that is about 25 000 times higher than that of the variant Asn-102 trypsin (Craik et al., 1987), which corresponds to an activation energy difference of 6.1 kcal/mol.

A number of factors complicate a direct comparison of the theoretical and experimental results. The major problem is that the geometry of the active site in the transition state of the variant proteins is not known. In the crystal structure of Asn-102 trypsin at pH 6.0 (where His-57 is protonated as it is in the transition state) the side chain of His-57 is partitioned between the gauche conformation found in native trypsin and a trans conformation in which it is displaced from the active site toward the solvent (Sprang et al., 1987). Perhaps more significantly, the tautomeric form of the side chain in Asn-102 trypsin is such that the Nϵ atom is no longer in a position to accept a proton from the Ser-Oγ. Thus, the histidine must undergo a tautomerization in order to participate in catalysis. A positive free energy change is associated with this step; Warshel et al. (1989) have calculated a value of 2 kcal/mol for the process. If we assume that this number is approximately correct, the electrostatic contribution of Asp-102 is about 4 kcal/mol, quite close to the values reported in Table I.

A final uncertainty concerns the structure and electronic distribution in the transition state. For example, we have assumed in extracting activation energies from Table I that the positive charge on the histidine is localized on Nϵ2 rather than being delocalized throughout the imidazolium ring. Similarly, we have assumed that the negative charge is entirely in the oxyanion hole although it is almost certainly distributed on the serine-Oγ as well. In order to determine the sensitivity of the results to the charge distribution assumed for the transition state, we have calculated the interaction energy of Asp-102 with transition-state charge distribution suggested in the recent studies of Warshel et al. (1989). Somewhat suprisingly, we found that the results obtained for the simple transition state (charge on Nϵ2 and oxyanion hole) were essentially identical with those obtained when the positive and negative charges were delocalized over the entire active site.

We have assumed in the discussion of the catalytic mechanism, in agreement with experimental results (Markley & Ibanez, 1987; Kossiakoff & Spencer, 1981), that Asp-102 is ionized while His-57 is neutral in the resting state of the enzyme. In order to check whether the FDPB calculations are consistent with this result, we have calculated the pK shifts of both of these residues in going from solution to their locations in trypsin. The change in pK in the protein relative to the pKs of the isolated amino acids in solution is given by $\Delta pK = \Delta\Delta G°(\text{elec})/1.4$, where $\Delta\Delta G°(\text{elec})$ is the difference in the total electrostatic energy of the amino acid in the two locations. Total electrostatic energies can be obtained from the FDPB method as has recently been described (Gilson & Honig, 1988). The calculations account both for changes in solvation energy and for interactions of the charged residues with the permanent and partial charges on the protein. The two active-site residues are predicted to be in the proper ionization state for catalysis. In both cases the small pK shifts result from stabilizing interactions in the protein that compensate for the loss of aqueous solvation. Full details of these calculations will be published separately (Yang and Honig, in preparation). Warshel and Russell (1986) have reported similar results with the PDLD method.

It is of interest to compare the results of Table I to those reported by Warshel and co-workers, who used the protein dipole Langevin dipole (PDLD) method to study the effect of Asp-102 on the catalytic mechanism of trypsin. The PDLD method bears some similarities to the FDPB method in that both treat the solvent by assigning polarizabilities (or, equivalently, a dielectric constant) to points on a three-dimensional lattice and, in addition, both methods account for electronic polarization. In the FDPB method electronic polarization is incorporated into the dielectric constant while in the PDLD method it is described in terms of inducible point dipoles at atomic centers. A detailed study of different approaches to the treatment of electronic polarization is currently in progress (Sharp, Yang, and Honig, unpublished results). Our concern in this work is in numerical values obtained from two totally independent methods.

Warshel et al. (1989) calculated that Asp-102 stabilizes the transition state of trypsin relative to the ground state by 5.4 kcal/mol. This number is only slightly larger than those reported in Table I for our FDPB calculations so that the two methods make essentially identical predictions for the effect of Asp-102. It should be pointed out, however, that the results in Table I were obtained for a protein dielectric constant of 4 while the PDLD calculations were carried out on the assumption, in agreement with the crystallographic data (Sprang et al., 1987), that the protein structure did not change when Asp-102 was replaced with Asn-102. [This can be seen from the zero contribution from the permanent dipoles in Table I of Warshel et al. (1989).] Since the structure is kept fixed for the mutation, this corresponds to a case where only electronic polarizability is accounted for and thus a dielectric constant of 2 is appropriate (see above). In order to enable a comparison of the two methods under equivalent conditions, we carried out FDPB calculations with an internal dielectric constant of 2. The predicted effect of Asp-102 is 5.5 kcal/mol, essentially identical with the result obtained from the PDLD calculations. While this near-perfect agreement between two independent methods is partially fortuitous, it should not come as a complete surprise since both methods include the same physical interactions.

Effect of High pH. As pointed out above, rat trypsin maintains its activity in the pH range 9–10 while the activity of cow trypsin is inhibited. We have attempted to determine whether the difference between the two enzymes is electrostatic in origin by calculating the electrostatic potential at the α-amino group of Ile-16 whose deprotonation is believed to shift the equilibrium toward an inactive form of chymotrypsin (Fersht, 1972). Excluding the effects of Asp-194 (which forms a salt bridge with Ile–16 so that those effects should be identical in both enzymes), the electrostatic free energy change $\Delta\Delta G°(\text{el})$ for the binding of a proton at Ile-16 was found to be 2 kcal/mol more negative in the rat protein. The change in pK due to a change in electrostatic energy is given by $\Delta pK = -\Delta\Delta G°(\text{el})/(RT \ln 10)$. This predicts a p$K$ increase for the rat enzyme (relative to cow) of about 1.4 units, which appears sufficient to account for the increased stability of the rat enzyme at high pH. The effect is not due to a single residue but rather to the cumulative effects of the net charge on the surface of the protein.

We have also investigated the possibility that other electrostatic factors are responsible for the inactivation of cow trypsin at high pH. Under the assumption that, other than Ile-16, the only groups in the protein that titrate at pH 9.5–10.5 are lysines and tyrosines, we calculated the interaction energy of each tyrosine and lysine with the oxyanion hole, the Ser-Oγ, the His Nϵ, Asp-102, and the α-amino group of Ile-16. All interactions involving lysines were found to be small in both enzymes while the interactions of the tyrosines were found to be large (particularly for the salt bridge) but, with one exception, similar in both proteins. The only significant interaction that was different in cow and in rat was between Asp-102 and Tyr-94 in cow trypsin. The hydroxyl group of Tyr-94 is about 4 Å from the negatively charged oxygens of Asp-102. Tyr-94 is missing in rat trypsin where it is replaced by a phenylalanine. It is conceivable that the Asp-102···His-57 hydrogen bond is disrupted in bovine trypsin at high pH, leading to an inactivation similar to the one that occurs in the Asp-102 to Asn-102 mutant in rat trypsin. On the other hand, the strong interaction between Asp-102 and Tyr-94 suggests that the tyrosine's pK will be increased due to the interaction, thus arguing against this mechanism for inactivation.

Discussion

The major finding of this work is that charged amino acids that are not part of the active site have no significant effect on the energetics of the catalytic mechanism of cow and rat trypsin. The electrical potentials induced by surface charge are small in absolute magnitude, and moreover, they produce no gradient across the active site that might affect the stability of the transition state. It appears that the low dielectric interior of the protein shields the active site from the effects of surface charge (by diverting the electric field through the high dielectric solvent), thus making it possible for two enzymes that differ by 12.5 units in net charge to have essentially identical active-site potentials. The structural origin of this behavior is the placement of charge on the surface of both proteins. The distribution of charged amino acids near the active site is very similar in each but becomes substantially different at larger distances from the active site. This suggests the possibility that these enzymes have been "designed" by evolution to minimize the effects of surface charge. We plan to consider this issue further in future work where electrical potentials in the entire family of serine proteases will be studied. Preliminary results suggest that the effects of surface charge are also small in chymotrypsin, elastase, and subtilisin despite the different net charges on these proteins.

The results do not imply that surface charge is never a factor in active sites; indeed, the recent studies of Dao-Pin et al. (1989) and Pickersgill et al. (1988) suggest that surface charge may play a role in other enzymes. Moreover, the enhanced pK stability of rat trypsin appears due to the cumulative effects of many small interactions involving surface charge. The calculations suggest that the pK of the α-amino group of Ile-16 in rat trypsin is about 1.5 units higher than in cow trypsin. This is due to the excess negative charge of the rat enzyme which affects the electrostatic potential at Ile-16. It should be pointed out in this regard that pK shifts depend on electrostatic *potentials* while stabilization of the transition state requires a sharp electrostatic *gradient* between His-57 and the oxyanion hole. Clearly, long-range interactions can affect potentials more easily than gradients. Despite the correlation between the effects of surface charge and pH stability, the possibility that the free energy difference between active and inactive forms is larger in rat than in cow trypsin due to nonelectrostatic interactions cannot be excluded. For example, rat trypsin might remain active at high pH simply because hydrophobic effects favor the active form.

The results of this work emphasize the importance of using a correct description of the electrostatic screening effects of both protein and solvent. It is striking that the Coulombic potentials given in Table I yield a large difference in electrostatic potential between cow and rat trypsin. An analysis based on these potentials, and assuming a dielectric constant of 4, would predict a different catalytic rate for the two enzymes, which is clearly not correct. However, charge–charge interactions between groups on the surface of proteins are strongly screened by the solvent (Gilson & Honig, 1988) so that a dielectric constant of 4, though appropriate to the interior of the protein, is a poor guess for the interactions between surface charges and the active site. A dielectric constant of 40 is likely to be a better average value, and its use would reduce the values of the Coulombic interactions in Table I by an order of magnitude. This would yield the correct prediction that surface charge has little effect on the active site. On the other hand, a dielectric constant of 40 would also predict a minor effect for Asp-102, which is at variance with the experimental results. This highlights the fact that the extent of dielectric screening is a sensitive function of position in the protein (Gilson et al., 1985; Gilson & Honig, 1988) and that Coulombic expressions will not, in general, be valid.

A further conclusion from this study is that Asp-102 affords approximately 4 kcal/mol electrostatic stabilization energy to the transition state of serine proteases. The major stabilizing interaction is with His-57 but this is reduced somewhat by repulsive interactions with the negative charge in the oxyanion hole. Our results thus support the idea [see, e.g., Bachovchin (1986) and Warshel et al. (1989)] that, in addition to orienting His-57, Asp-102 plays an important role in transition-state stabilization. The calculated value of about 4 kcal/mol is in close agreement with the results recently reported by Warshel et al. (1989). That two different methods make very similar predictions as to the effects of Asp-102, both of which are quite close to what is observed experimentally, argues that the role of the active-site aspartic acid in the catalytic mechanism of serine proteases is now well established.

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Purification and Characterization of Bovine Heart Phosphoinositide-Specific Phospholipase C: Kinetic Analysis of the Ca^{2+} Requirement and La^{3+} Inhibition†

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ABSTRACT: Bovine heart contains multiple phosphoinositide-specific phospholipase C (PIC) activities separable by ion-exchange chromatography. One PIC activity was purified to apparent homogeneity and migrated as a single band of M_r 85 000 on SDS-PAGE. The purified PIC was characterized with sonicated suspensions of either pure phosphatidylinositol 4,5-bisphosphate (PIP_2) or phosphatidylinositol (PI) as substrates. At pH 7, apparent V_{max} and K_m values were higher for PIP_2 than for PI, but the value of V_{max}/K_m was similar for the two substrates. PIC required Ca^{2+} for the hydrolysis of either PI or PIP_2, and increasing free Ca^{2+} concentrations from 20 to 300 nM saturated PIC activity. The requirement of Ca^{2+} for PIC activity and the sensitivity of PIC to Ca^{2+} concentrations in the physiological range suggested the ion may be a cofactor. The PIC reaction mechanism was determined by two-substrate kinetic analysis; the data fit a model in which PIC contained single sites for Ca^{2+} and phosphoinositide, and utilized a rapid-equilibrium, random-order ternary mechanism for phosphoinositide hydrolysis. The K_{Ca} value for either PI or PIP_2 hydrolysis was approximately 30 nM, suggesting resting intracellular free Ca^{2+} concentrations are sufficient to saturate the Ca^{2+} site of PIC. La^{3+} was used as a calcium analogue to modulate PIC activity. Low concentrations of $LaCl_3$ (0.01-0.3 μM) inhibited PIC activity competitively with respect to calcium, consistent with a Ca^{2+} binding site on the enzyme.

Phosphoinositide-specific phospholipase C (PIC)[1] is the intracellular enzyme that hydrolyzes phosphoinositides upon stimulation of cells with Ca^{2+}-mobilizing agonists (Hirasawa & Nishizuka, 1985). PIC is a family of enzymes; multiple isoforms are present in most tissues (Rhee et al., 1989), including the heart (Low & Weglicki, 1983). Several distinct PIC enzymes have been purified from mammalian tissues, ranging from 60 to 150 kDa (Rhee et al., 1989). Isozymes of 85-kDa PIC (PIC δ and ϵ) have been purified from brain

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[1] Abbreviations: BSA, bovine serum albumin; DG, 1,2-diacylglycerol; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetate; IP_3, inositol trisphosphate; MOPS, 3-(*N*-morpholino)propanesulfonate; PI, phosphatidylinositol; PIC, phosphoinositide-specific phospholipase C; PIP_2, phosphatidylinositol 4,5-bisphosphate; SD, standard deviation; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SEM, standard error of the mean.

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