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Electrostatic fields in the active sites of lysozymes

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ABSTRACT **Considerable experimental evidence is in support of several aspects of the mechanism that has been proposed for the catalytic activity of lysozyme. However, the enzymatically catalyzed hydrolysis of polysaccharides proceeds over 5 orders of magnitude faster than that of model compounds that mimic the configuration of the substrate in the active site of the enzyme. Although several possible explanations for this rate enhancement have been discussed elsewhere, a definitive mechanism has not emerged. Here we report striking results obtained by classical electrodynamics, which suggest that bond breakage and the consequent separation of charge in lysozyme is promoted by a large electrostatic field across the active site cleft, produced in part by a very asymmetric distribution of charged residues on the enzyme surface. Lysozymes unrelated in amino acid sequence have similar distributions of charged residues and electric fields. The results reported here suggest that the electrostatic component of the rate enhancement is >9 kcal·mol^{-1}. Thus, electrostatic interactions may play a more important role in the enzymatic mechanism than has generally been appreciated.**

X-ray structural studies of hen egg white lysozyme (HEWL) (1, 2), human lysozyme (HUL) (3), and bacteriophage T4 lysozyme (T4L) (4, 5) have led to proposals for the catalytic mechanism (refs. 1, 6, and 5; see refs. 7 and 8 for reviews) shown diagrammatically in Fig. 1. Experimental evidence from isotopic substitutions suggests that the rate-limiting step in the reaction is movement of charge from the protonated 1–4 bridge oxygen to the C-1 carbon (10, 11), resulting in the breakage of the C-1–O bond and formation of a charged intermediate. From the very earliest works (1, 6, 12–14), electrostatic interactions of specific active site residues with the substrate were felt to be an important feature of the catalytic mechanism. However, the results reported here suggest that the charge distribution of the enzyme as a whole plays an important role in the enzymatic mechanism. We propose that the electrostatic field in the active sites of lysozymes acts to promote movement of charge as well as to stabilize charged intermediates.

METHODS

Our approach has been to use the classical two-dielectric model of an enzyme–solvent system first discussed for spheres by Tanford and Kirkwood (15) and subsequently generalized by others (16, 17). The enzyme molecule is assumed to have a continuous uniform low dielectric interior with fixed point charges at known locations, while the solvent is a region of uniform high dielectric constant, possibly containing counterions. The differential equation for the electrostatic potential energy $\Phi(x,y,z)$ of the system, the linearized Poisson–Boltzmann equation (reviewed in ref. 18),

$$\nabla\cdot(\varepsilon\nabla\Phi) - \bar{\kappa}^2\Phi = -4\pi\rho,$$

can be solved by standard numerical techniques in which the molecule is placed on a grid. Here, ε (the dielectric constant), $\bar{\kappa}$ (the modified Debye–Huckel parameter), and ρ (the charge density) are all functions of the spatial coordinates. The numerical algorithm of Klapper *et al.* (17) has been implemented. The determination of the molecular boundary is not trivial and the present procedure differs somewhat from that of Klapper *et al.* We chose to expand the van der Waals radii of all atoms that are not solvent accessible by 1.0 Å to fill internal gaps and to set the dielectric constant of grid points covered by any atom to the interior value. All other grid points are assumed to have a dielectric constant equal to the solution value. There is only one variable parameter in the model. This is the dielectric constant ε inside the molecule, which is unknown but widely believed to be in the range of 2–10 (15–17, 19, 20). Electric fields are obtained from the potential by numerical differentiation.

The implementation of the Klapper algorithm was tested for simple cases with known analytical solutions and found to be accurate to 15% except at a charge location (where the analytical solution has a singularity) and within ≈1 Å of the dielectric boundary, where the numerically obtained potential can be too low by as much as 67%.

As a further test of the model, and to study the effects of various values of ε, electrostatically induced shifts in the pK_a of charged groups were calculated for two systems in which experimental values are available. In the first system, the pK_a shift of Glu-35 in HEWL due to ethylation (neutralization) of Asp-52 has been measured by Parsons and Raftery (21). In the second system, the pK_a shifts of His-64 in the active site of *Bacillus amyloliquefaciens* subtilisin due to site-directed mutagenesis of two charged residues have been measured by Russel *et al.* (22). The numerical estimates agree well with the experimental results except at very low ionic strength (Table 1). These results are also in agreement with those calculated by Sternberg *et al.* (19) and Gilson and Honig (20) but differ due to the way molecular surface is determined (in particular the latter authors excluded solvent ions in a layer 2.0 Å from the protein). As can be seen, these results are insensitive to the value of ε, so $\varepsilon = 4$ was chosen for further calculations as this value has been experimentally determined from crystals of amino acids (25). In performing these calculations, we discovered that the inclusion of bound solvent molecules, which are treated by the program as a low dielectric medium, yields unsatisfactory results. Better results were obtained after removal of bound solvent from the crystallographic models, suggesting that the dielectric constant of bound water molecules is close to that of bulk water.

RESULTS

The electrostatic potential for the three lysozymes was calculated by using the Klapper algorithm with the catalytic glutamate uncharged, as this represents the state of this side chain when the molecule is considered to be catalytically

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Abbreviations: HEWL, hen egg white lysozyme; HUL, human lysozyme; T4L, bacteriophage T4 lysozyme.

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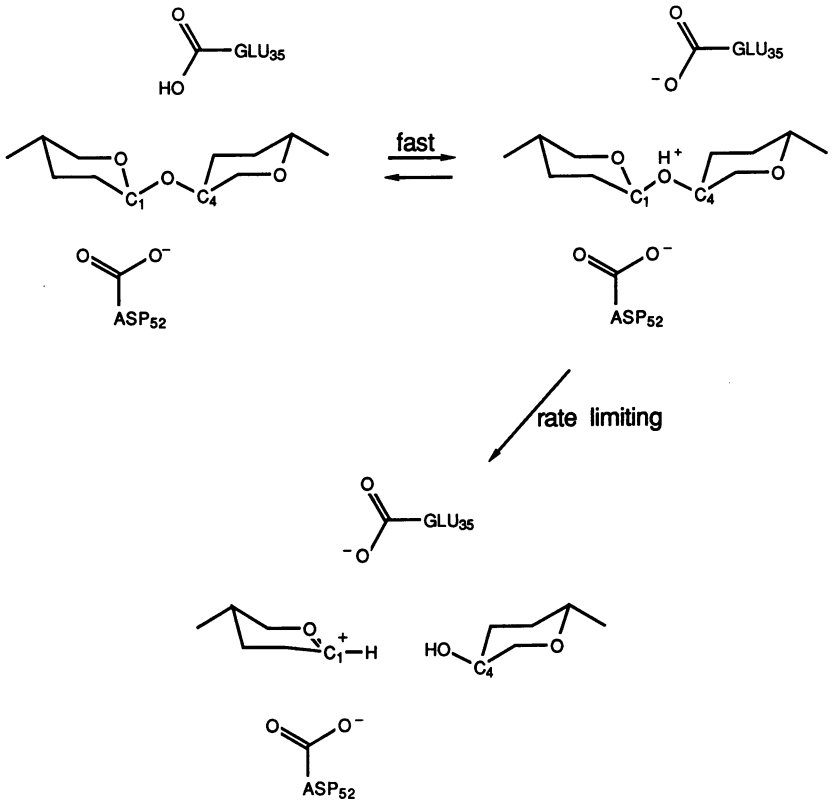

FIG. 1. Scheme of the first half of the proposed (1, 6) reaction catalyzed by lysozyme. The breakage of the C-1–O bond is catalyzed by a neutral glutamic acid (Glu-35 in HEWL and HUL, Glu-11 in T4L), resulting in the breakage of the exocyclic C-1–O bond and the formation of a carboxonium ion intermediate (bottom center). The enzyme is thought to stabilize this intermediate by binding energy, largely by interaction with a negatively charged aspartate, Asp-52 (HEWL), Asp-53 (HUL), or Asp-20 (T4L), until the reaction of a water molecule with C-1 proceeds to form the product in the reverse of the first half-reaction. Post and Karplus (9) have proposed an alternative mechanism that invokes a carboxonium ion intermediate formed by C-1 and the 1–4 bridge oxygen, after breakage of the endocyclic C-1–O bond.

Table 1. Comparison of experimental and calculated pK_a shifts at His-64 of subtilisin (22) and Glu-35 of HEWL (21)

			Change in pK_a				
				Calculated			
				This work			
Model	Perturbed residue	Ionic strength, M	Observed	$\varepsilon = 2.0$	$\varepsilon = 4.0$	$\varepsilon = 8.0$	Ref. 20
Subtilisin	His-64	0.001	0.40*	0.56	0.49	0.43	0.34
Asp-99 → Ser		0.005	0.38	0.46	0.44	0.38	0.31
		0.01	0.42	0.41	0.39	0.35	0.29
		0.025	0.36	0.31	0.30	0.28	0.25
		0.1	0.26	0.13	0.14	0.14	0.18
		0.5	0.09	0.08	0.07	0.08	0.10
Glu-156 → Ser	His-64	0.001	0.39	0.81	0.74	0.61	0.42
		0.005	0.32	0.74	0.68	0.56	0.39
		0.01	0.42	0.67	0.62	0.52	0.37
		0.025	0.41	0.53	0.50	0.43	0.34
		0.1	0.25	0.27	0.26	0.24	0.27
		0.5	NA	0.20	0.18	0.17	0.19
HEWL							
Asp-52 ethylated	Glu-35	0.15	0.9†	0.78	0.71	—§	
HUL							
Asp-53 ethylated	Glu-35	0.15	—‡	0.72	0.51	—	

Potentials calculated by the Klapper algorithm (17) at 298 K on a 65 × 65 × 65 grid. Coordinates [obtained from the Protein Data Bank (23)] were scaled such that no atom was closer than 15 Å from the edge of the grid, resulting in a grid spacing of ≈1.2 Å. Boundary conditions of Klapper *et al.* (17). Iterations were terminated when the maximum change in potential in the final iteration is <0.001 kT/e^+. pK_a shifts were calculated from the change in potential due to the indicated perturbation by the method of Tanford and Roxby (24). No experimental values are available for HUL. Each calculation required between 1 and 2 hr of central processing time on a Digital Equipment Micro-VAX II.

*See ref. 22.

†See ref. 21. Not calculated by Gilson and Honig (20).

‡Has not been measured.

§Not calculated.

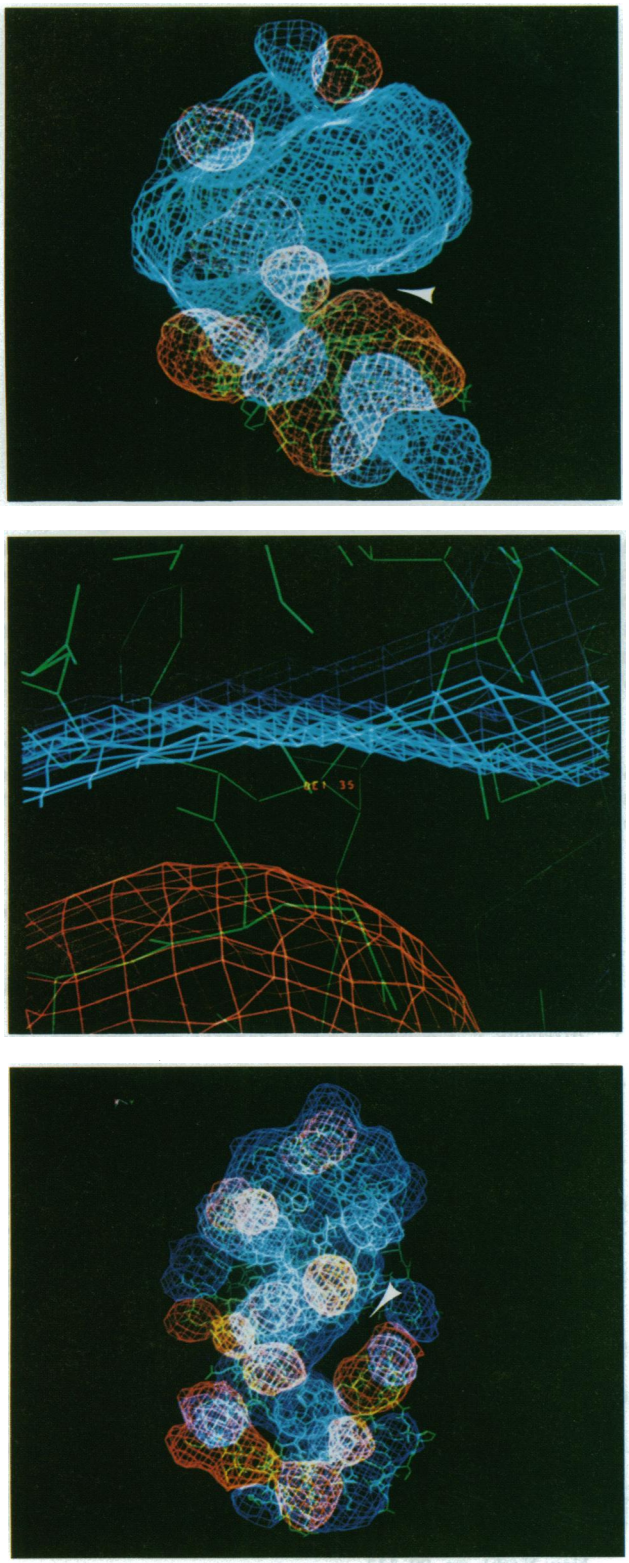

FIG. 2. (*Top*) The electrostatic potential for human lysozyme, contoured at +5 kT (blue) and −5 kT (red), calculated by the Klapper algorithm (17). Conditions are as in Table 1 except ionic strength = 0.15 M and $\varepsilon = 4$. The contours represent equipotential energy surfaces for a hypothetical test charge. Bound solvent molecules were removed from the models and Glu-35 was assumed electrically neutral. All other appropriate side chains, including histidine and the N and C termini, were assumed to be fully charged. The active site cleft is indicated by the arrowhead. (*Middle*) Detail of top near Glu-35, showing the potential gradient across the active site cleft. (*Bottom*) The electrostatic potential for T4 phage lysozyme calculated and contoured as in *Top*.

competent (1, 6). Fig. 2 (*Top* and *Middle*) shows contoured images of the electrostatic potential of HUL calculated for side-chain charges only. There are two striking features in Fig. 2 (*Top*), the first being the obvious gradient between the positive and negative equipotential surfaces that coincides exactly with the active site cleft. The second is the relatively uniform positive potential that coincides with the "upper" lobe of the molecule, while the "lower" lobe is approximately neutral. The proximity of the positive and negative equipotential surfaces (Fig. 2 *Middle*) indicates that there is an electrostatic potential difference of 10 kT (6 kcal·mol^{-1}) across a distance of ≈4 Å in the active site cleft. This corresponds to an electric field of ≈6 × 10^6 V·cm^{-1} in the appropriate direction to promote movement of positive charge from the catalytic glutamate toward the substrate.

Very similar results were obtained for HEWL (Table 2). There is 60% identity between the amino acid sequences of HEWL and HUL, with several changes at charged sites. The results are not sensitive to the differences in the placement of charged groups on these otherwise rather similar enzymes. In a subsequent calculation, coordinates for the amide hydrogens were calculated, and partial charges were assigned to the peptide backbone according to Hol *et al.* (27). The results were qualitatively similar, but the electric field approximately doubles (Table 2). Also, we note that this field is quite large even if the catalytic aspartyl residue is uncharged (Table 2), indicating that these are long-range effects.

Fig. 2 (*Bottom*) shows a contour image of the potential surface obtained from an electrostatic potential calculation for T4L under the same conditions used for Fig. 2 (*Top*). As demonstrated by Fig. 2 these enzymes, entirely dissimilar in amino acid sequence, have a remarkably similar disposition of electrostatic potential. All three enzymes have in common two distinctive features, the first being a clustering of positive charges on the "upper" lobe, the second being a large electric field across the active site cleft from the glutamic acid toward the catalytic aspartate.

The hypothetical fully discharged state of the enzyme could in principle catalyze the reaction of Fig. 1. It is then clear that the electric field due to the other charges actually present in lysozymes will either promote or inhibit the proposed movement of charge from the catalytic glutamate to the C-1 carbon. To investigate whether the observed electric field could be an important component of the reaction rate enhancement, the electrostatic potential was calculated for the complex of HEWL with a tetrasaccharide lactone (ref. 26; coordinates with the D site sugar in the sofa conformation, both Glu-35 and the substrate uncharged). This compound binds to lysozyme in a manner thought to resemble the binding of the carboxonium ion intermediate (Fig. 1). The potential difference between oxygen O$^{\varepsilon 1}$ of Glu-35 and carbon C-1 of the lactone, which is the free energy for movement of positive charge between these locations, was found to be 9.8 kcal·mol^{-1} for an interior dielectric of 4. This component of the reduction of the reaction energy barrier corresponds to a reaction rate enhancement of 1.2 × 10^7 over that of the hypothetical discharged enzyme. However, the result strongly depends on the value of ε used for the calculation. Use of $\varepsilon = 2$ leads to an electrostatic rate enhancement of 18 kcal·mol^{-1}, $\varepsilon = 8$ leads to 5.3 kcal·mol^{-1}, and $\varepsilon = 80$ (a model for the identical reaction proceeding in aqueous solution) leads to 0.6 kcal·mol^{-1}. Clearly, it will be necessary to obtain a better estimate for ε to fully evaluate the importance of these observations. On the other hand, as one would predict (see *Discussion*), the rate enhancement does not depend strongly on ionic strength. At $\varepsilon = 4$, the calculated rate enhancement at 1.0 M ionic strength is only 0.2 kcal·mol^{-1} less than that at 0.05 M.

Table 2. Magnitude of calculated electrostatic field

Model	Notes	Electric field	
		$V \cdot cm^{-1} \times 10^{-6}$	$kcal \cdot mol^{-1} \cdot Å^{-1} \cdot e^{-1}$
HUL	No solvent or partial charges	3.9	0.92
HEWL	No solvent or partial charges	5.4	1.26
T4L	No solvent or partial charges	13.7	3.21
HUL	Asp-53 uncharged	1.9	0.45
HEWL	Asp-52 uncharged	1.6	0.38
HEWL	Bound solvent	6.7	1.56
HEWL	Peptide partial charges included	7.7	1.83
HEWL	TACL complex	13.7	3.19*

Magnitude of calculated electrostatic field, obtained by numerical differentiation of the potential, at Glu-35 $O^{\epsilon 1}$ (HEWL and HUL) or Glu-11 $O^{\epsilon 2}$ (T4L), the oxygen atom closest to substrate. At ionic strength 0.15 M, there was no bound solvent or peptide partial charges except as noted. Other conditions are as in Table 1; interior dielectric $\epsilon = 4$. TACL, HEWL–tetrasaccharide lactone complex (26). For convenience the electric field is given in two different systems of units.
*Electric field at C-1 of the lactone = 9.2 $kcal \cdot mol^{-1} \cdot Å^{-1} \cdot e^{-1}$.

DISCUSSION

There are three sources of error in our calculations. The first is the assumption of a classical two-dielectric model. The second is the numerical approximation used, which is at its worst at the dielectric boundary. Third, use of the linearized Poisson–Boltzmann equation rests on the assumption that the potential of a counterion in the solvent region is less than kT. In the calculations, potentials of several kT make limited excursions into the solvent region, suggesting that the nonlinear Poisson–Boltzmann equation would be more appropriate. Finally, the calculations are rather sensitive to the manner in which the molecular surface is treated (e.g., whether there is an ion exclusion radius or Stern layer), indicating that this aspect of the algorithm can be improved. However, the fact that experimentally obtained pK_a shifts can be calculated with reasonable accuracy suggests that the present simple approach is acceptable. Also, by neglecting partial charges, we appear to have underestimated the electric field and hence the rate enhancement.

These results suggest that in lysozyme, movement of charge is promoted by a large electric field in the active site cleft. This electric field appears to arise from a clustering of positive charge on the "upper" lobe of the molecule, which focuses field lines along the scissile bond in the direction of the catalytic aspartate. Peptide partial charges may also play a prominent role. Interestingly, Table 2 indicates that Asp-52 of HEWL is not essential for catalytic activity and this has recently been borne out experimentally (28).

A striking result of this study is that lysozymes with totally dissimilar amino acid sequences have similar distributions of charged residues, which results in similar electric fields in their active sites. The use of site-directed mutagenesis as recently applied to T4 lysozyme (29) or chemical modification combined with field calculations may help to clarify the roles of individual charged groups in establishing these fields and to more accurately estimate ϵ. It should be noted that the experimentally available pK_a shift measurements were made for perturbations that are relatively close to the group whose pK_a was monitored and this may in part account for the insensitivity of the calculated pK_a shifts to the value used for ϵ. It would be desirable to make changes in charge on the opposite side of the protein from the group being monitored.

One might at first think that the reaction rate would be very dependent on the ionic strength of the solution, but this would not be expected for a neutral substrate as counterions and free solvent molecules are excluded from the vicinity of the substrate when bound to the enzyme. The asymmetric charge distribution implies that lysozymes are macrodipoles and could possibly be oriented by electric fields in solution. These calculations probably cannot distinguish between the mechanisms proposed by Phillips, Vernon, and colleagues (1, 6) and that proposed by Post and Karplus (9). The electrostatic rate enhancement proposed here would be valid for both, as both mechanisms invoke separation of charge. The results shown here are in reasonable agreement with the solvation model discussed by Warshel (12) and the work of Levitt and Warshel (13, 14), although the treatments are very different.

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