ELECTROSTATIC FREE ENERGY OF LYSOZYME

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ABSTRACT The electrostatic free energies of native and acetylated lysozymes were computed by the fixed charge model (Tanford, C., and J. G. Kirkwood, 1957, *J. Am. Chem. Soc.*, 79:5333–5339). For each computation, the charges were transformed into a sphere of fixed radius without changing their depths or the distance between charges. The depths of charges were assumed proportional to one minus accessibility. When the conversion factor was set to 1.62 Å, the computed titration curve fitted best to the experimental data. The calculated electrostatic free energies for native and acetylated lysozymes were consistent with our earlier finding that acetylated lysozyme is less stable than native around neutral pH (Imoto, T., K. Fukuda, and K. Yagishita, 1976, *J. Biochem.* [*Tokyo*], 80:1313–1318; Imoto, T., S. Moriyama, and K. Yagishita, 1976, *J. Biochem.* [*Tokyo*], 80:1319–1325). The contribution of each charge to the stabilization of the protein and the apparent pK's of ionizable groups were computed by this method.

INTRODUCTION

We have shown that protease digestions of lysozyme proceed in an all or none type mechanism that reflects the N ⇌ D transition of the protein (Imoto et al., 1976*a*, *b*). Lysozyme contains ten carboxyl (Imoto et al., 1981*b*), seven amino, and eleven guanidino groups (Canfield, 1963; Jolles et al., 1963). Modification of the amino groups reduces the net charge of lysozyme and it leads to destabilization of the protein's native conformation. To investigate the theoretical basis of this phenomenon, I have computed the electrostatic free energy of lysozyme and its acetylated derivative.

We have compared (Imoto and Ono, 1981) the titration curves of native and a modified lysozyme that has an ester linkage between Glu 35 and oxindolealanie 108 (Imoto and Rupley, 1973; Beddel et al., 1975). The results indicate that the fluctuating native lysozyme molecule (Nakanishi et al., 1973) shows a quite similar titration curve to the modified lysozyme that has a considerably more fixed conformation (Imoto and Rupley, 1973; Imoto et al., 1974, 1976*a*, *b*; Johnson et al., 1978). This finding is compatible with the idea of computing electrostatic free energy employing x-ray crystallographic data.

First, the electrostatic free energies of native and acetylated lysozymes were computed by the smeared charge model (Linderstrøm-Lang, 1924; Tanford, 1961). This model was not successful in explaining the above finding. Second, the energies were computed by the fixed charge model (Tanford and Kirkwood, 1957). In this case, the depth of charge (d) from the surface of the molecule is important. The surface of a protein is usually not smooth and a determination of d is quite difficult. Orttung (1970) and Tanford and Roxby (1972) set all d's at 0 and 0.4 Å, respectively. Shire et al. (1974) and Matthew et al. (1979) corrected obtained electrostatic energy of interaction by the surface accessibility factor of the critical charge. Friend and Gurd (1979) obtained the electrostatic free energy in myoglobin by correcting with the accessibility factors of the pair of charges. Matthew and Richards (1982) employed the average accessibility of the pair of charges to correct the obtained electrostatic energies of pair of charges. However, in their approximations, the depth of the individual charge was not directly included in the computation by the Tanford-Kirkwood method. Considering the importance of the depth of charge i (d_i) and j (d_j) and the distance of the charge (r_{ij}), I have transformed the charges into a sphere of fixed radius (17.5 Å here) without changing d_i, d_j, and r_{ij} on each computation. The depth of charge was assumed proportional to one minus the fraction of accessibility of charge. As the conversion factor of accessibility to depth, I employed the value where the deviation of the calculated titration curve from the experimental one was minimized. The electrostatic free energies computed here by the fixed charge model were in accord with our finding that acetylated lysozyme is less stable than native enzyme around the neutral pH region.

EXPERIMENTAL

Materials

Five times recrystallized hen egg-white lysozyme was employed. Exhaustively acetylated lysozyme (Imoto et al., 1976*a*) and hemoglobin tryptide (Imoto et al., 1981*a*) were prepared as previously reported. Nagarse was donated by Nagase & Co., Ltd. (Osaka, Japan). Pepsin was obtained from Worthington Biochemical Corp. (Freehold, NJ). 2,4,6-trinitrobenzene sulfonic acid was purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan).

Protease Digestion

Protease digestions at various pH's were analyzed by the 2,4,6-trinitrobenzene sulfonic acid method (Adams et al., 1976): (*A*) sample solution containing 5 to 15 mg of substrate and 0.75 mg of nagarse (pH 4–12) or 1.5 mg of pepsin (pH 1–3) in 6 ml of 0.05 M buffer solution

(citrate, pH 1–5; phosphate, pH 6–8; borate, pH 10 and 11; phosphate, pH 11 and 12); the solution was kept at 40 °C; (*B*) 0.5% 2,4,6-trinitrobenzene sulfonic acid solution in water; (*C*) 0.002 M Na HSO_3 solution in 0.1 M Na H_2PO_4; (*D*) 0.5% sodium dodecylsulfate solution in 0.1 M Na $H_2B_4O_7$. After the addition of protease, the solution *A* was pumped through Teflon tubing and was mixed with solutions *B*, *C*, and *D*. The flow rates of *A*, *B*, *C*, and *D* solutions were 12, 30, 123, and 123 ml/h, respectively. The mixed solution was incubated at 40 °C for 10 min in the reaction coil. Absorbance of the reaction mixture at 420 nm was recorded against time using a wavelength tunable effluent monitor (Hitachi Ltd., Tokyo, Japan). The data were analyzed assuming pseudo-first-order kinetics.

Methods of Computation of Electrostatic Free Energy

The radius of native lysozyme (b) and that for ion exclusion volume (a) were set at 17.5 and 20.0 Å, respectively (Tanford and Roxby, 1972). Dielectric constants of water (D) and inside of protein (D_i) were set at 87.5 and 4 (Tanford and Roxby, 1972), respectively. The values of the intrinsic pK's (pK_{int}'s) employed were Asp (4.0), Glu (4.4), α-carboxyl (3.8), α-amino (7.5), His (6.3), ϵ-amino (10.4) (Nozaki and Tanford, 1967), Arg (12.5), and Tyr (10.2) (Shire et al., 1974). The pK_{int} of Glu 35 of native lysozyme was set at 6.1. Temperature and ionic strength were kept at 25 °C and 0.1 OsM, respectively, unless otherwise mentioned. Electrostatic free energy, W_i, for a hypothetical protein devoid of charge i was computed by setting the charge of critical group as zero. That for the protein devoid of more than one charge was also computed in the similar manner. For computation of acetylated lysozyme, all charges of amino groups were set to zero.

Smeared Charge Model. According to Linderstrøm-Lang (1924), the dissociation of protonated groups in proteins can be approximated as Eq. 1

$$\log \frac{\alpha_i}{1-\alpha_i} = \mathrm{pH} - (\mathrm{pK_{int}})_i + 0.868wZ, \qquad (1)$$

where

$$w = \frac{\epsilon^2}{2DkT}\left(\frac{1}{b} - \frac{\kappa}{1+\kappa a}\right) = 0.08351,$$

κ is the Debye-Hückel parameter, and subscript i denotes each ionizable group. At each pH, initial Z was obtained from the fraction of dissociation of ith charge, α_i, computed by setting $Z = 0$ in Eq. 1. The computation of α_i was repeated using the new Z values until all $|\Delta\alpha_i|$'s converged to <0.057, that is, $|\Delta pK_i|$'s < 0.1, where Δ means the deviation from the latest value. The plot of the final Z value against pH can be regarded as the theoretical titration curve. Electrostatic free energy of the protein was calculated by using this Z value and Eq. 2 (Tanford, 1961)

$$W = \frac{Z^2\epsilon^2}{2Db}\left(1 - \frac{\kappa b}{1+\kappa a}\right) = Z^2kTw. \qquad (2)$$

Denatured Protein. Electrostatic free energy of the denatured protein (W_D) was computed according to Eq. 3 (Tanford, 1961)

$$W_D = \frac{9Z^2\epsilon^2}{2\kappa^2 D(R^3 - R_0^3)}\left[\frac{1}{3} + \frac{1+\kappa R}{\kappa^3(R^3 - R_0^3)}\right.$$
$$\left.\times \frac{(1-\alpha\kappa R)\dfrac{1+\alpha\kappa R_1}{1-\alpha\kappa R_1}e^{2\alpha\kappa(R-R_1)} - (1+\alpha\kappa R)}{(1+\alpha)\dfrac{1+\alpha\kappa R_1}{1-\alpha\kappa R_1}e^{2\alpha\kappa(R-R_1)} - (1-\alpha)}\right]. \qquad (3)$$

The net charge of the denatured protein, Z, was calculated from pK_{int} (pK_{int} of Glu 35 was set at that of normal Glu [4.4]). The radius of central core of denatured protein, R_1, was very insensitive to the computation and was set at zero (Tanford, 1961). Then α is expressed as $\sqrt{(R^3 - R_0^3)/R^3}$, and the radius of the native protein, R_0, was set at 17.5 Å, and the radius of denatured protein, R, was set at 27.5 Å (Tanford et al., 1974). As the latter value (R) was for the S—S reduced protein and not for the S—S intact denatured protein considered here, the W_D computed here would be an underestimate. For example, the values of W_D at pH 7 were 1.2 and 3.3 kcal/mol when employing 27.5 and 22.5 Å as R, respectively.

Fixed Charge Model. According to Tanford and Kirkwood (1957), the free energy of charge interaction (W_{ij}) between two sites, i and j, on the protein molecule is given by

$$W_{ij} = \frac{\epsilon^2}{2b}Z_iZ_j(A_{ij} - B_{ij}) - \frac{\epsilon^2}{2a}Z_iZ_jC_{ij} \qquad (4)$$

where $A_{ij} = b/(D_i r_{ij})$,

$$B_{ij} = \frac{1-2\delta}{D_i(1 - 2\rho_{ij}\cos\theta_{ij} + \rho_{ij}^2)^{1/2}} + \frac{1}{D\rho_{ij}}$$
$$\times \ln\left[\frac{(1 - 2\rho_{ij}\cos\theta_{ij} + \rho_{ij}^2)^{1/2} + \rho_{ij} - \cos\theta_{ij}}{1 - \cos\theta_{ij}}\right],$$

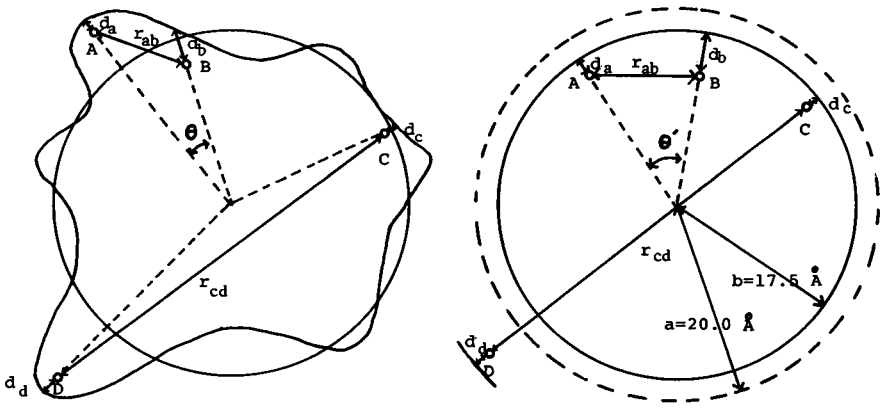

FIGURE 1 Transformation of the coordinates of charges in protein into the sphere with radius of 17.5 Å.

$\delta = D_i/D$, $\rho_{ij} = r_i r_j/b^2$, and C_{ij} is the correction term caused by ionic strength in which the summation index, n, was set to 20 (Orttung, 1970).

It has been shown (Tanford, 1957) that the crucial parameters affecting the values of W_{ij} are the distance between the sites (r_{ij}) and the depths d_i and d_j from the protein surface at which these sites are located. The size of the protein (expressed as a and b) is less important. Thus, it is reasonable to transform charges into the sphere with the radius of 17.5 Å without affecting d_i, d_j, and r_{ij} (see Fig. 1). The transformation was performed with i and j charges on each calculation. A trouble that may arise from the transformation is that $|r_i - r_j| \geq r_{ij}$, that is $\cos\theta'_{ij} \geq 1$. In this case, $\cos\theta'_{ij}$ was set to 0.9999 in the computer program. This occurs when r_{ij} is very small (where the electrostatic interaction is strong). However, in the case of lysozyme, this did not occur because the largest $\cos\theta'_{ij}$ was 0.9844. Another potential problem is that $r_i + r_j < r_{ij}$, that is, $\cos\theta'_{ij} < -1$. In this case, $\cos\theta'_{ij}$ was set to -1 in the computer program, that is, the radius of protein b was increased (Fig. 1). This occurs only when r_{ij} is very large where the electrostatic interaction is weak. In the case of lysozyme, this problem was found in several charges and the interactions tended to give negative $A_{ij} - B_{ij}$ values, and the values were set to zero for computation.

The depth of charge d was estimated assuming that it was proportional to one minus the fraction of the accessible surface area of the polar atoms in protein relative to model peptide (Gly-X-Gly). The accessible surface areas are taken from the paper of Shrake and Rupley (1973). The computed values showed reasonable agreement with those computed

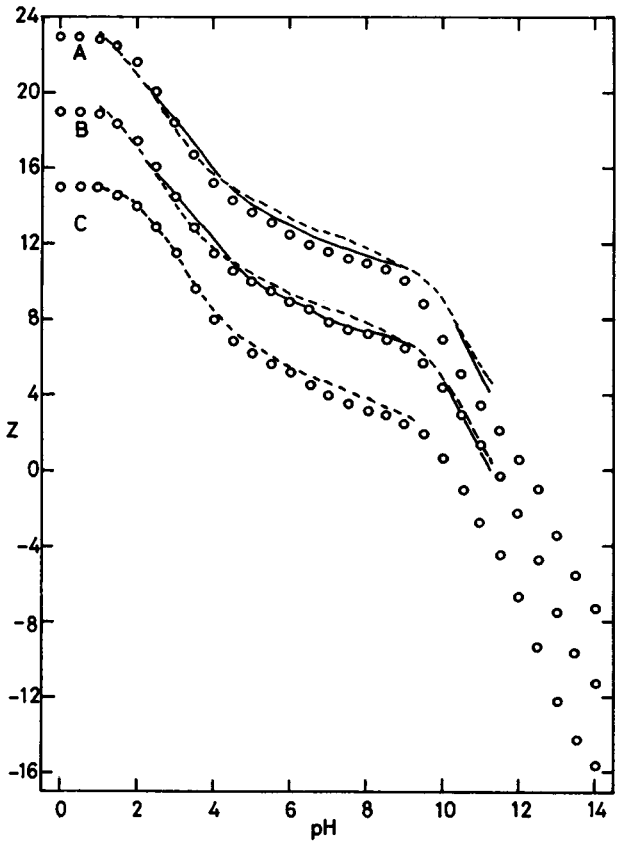

FIGURE 2 Comparison of experimental and computed titration curves. (—), experimental curve by Sakakibara and Hamaguchi (1978) (0.2 M KCl). (---), those by Tanford and Roxby (1972) (0.1 M KCl) and Imoto and Ono (1981) (0.1 M KCl). Each circle indicates the computed value. The ordinates of A and C were shifted by +4 and −4, respectively. A, ionic strength 0.1 OsM, smeared charge model; B, ionic strength 0.1 OsM, fixed charge model; C, ionic strength 1 OsM, fixed charge model and experimental line (---) is from Tanford and Roxby (1972) (1 M KCl).

TABLE I
CHARGES IN LYSOZYME

Group	d	$pK_{1/2}$	$pK^*_{1/2,experimental}$			ΔW_i
		acetylated	native	native		
Carboxyl	Å					*kcal/mol*
α	0.56	3.7	1.9	3.1		−4.6
Asp 18	1.06	3.9	3.3	—		−1.6
Asp 48	1.22	3.4	3.3	4.5		−0.9
Asp 52	1.14	3.9	3.8	3.4		+0.7
Asp 66	1.62	3.2	3.2	1.6		−1.4
Asp 87	0.46	3.2	3.0	(4)		−1.1
Asp 101	0.22	3.8	3.6	4.5		−0.9
Asp 119	0.90	2.1	1.9	—		−5.3
Glu 7	0.96	2.5	2.5	2.6		−4.4
Glu 35	1.30	6.2	6.0	6.1		−0.3
Amino						
α	1.40	—	7.4	—	7.2‡	+0.1
Lys 1	0.72	—	11.8	10.6	10.0‡	−3.2
Lys 13	0.98	—	12.5	10.3	9.8‡	−5.5
Lys 33	0.04	—	10.2	10.4		+0.3
Lys 96	0.80	—	10.7	10.7		+0.1
Lys 97	0.20	—	10.5	10.1		−0.1
Lys 116	0.82	—	10.3	10.2		+0.8
1 and 13	—	—	—	—		−8.7
All	—	—	—	—		−8.8
Others						
His 15	1.30	6.3	5.8	5.8		—
Tyr 20	0.60	10.1	9.6	10.3		—
Tyr 23	0.98	10.1	9.8	9.8	10§	—
Tyr 53	1.38	11.9	11.8	12.1		—

Depth (d) employed for computation and apparent pK ($pK_{1/2}$) are listed. Contribution (ΔW_i) of charge i to the stabilization of lysozyme at pH 7 is also listed. Ionic strength employed was 0.1 OsM.
*Kuramitsu and Hamaguchi (1980) and references therein.
‡Gerken et al. (1982).
§Dobson et al. (1978).

employing the accessible surface areas of Lee and Richards (1971). The conversion factor of 1.62 Å, which gives a closest fit to the results of experimental titrations, was employed for the calculation (see Fig. 2). In this instance, values of d fell between 0 to 1.62 Å, and the d values are shown in Table I. The coordinate that was employed for the computation of accessible surface (Shrake and Rupley, 1973) was kindly supplied by Dr. J. Rupley.

The effect of charge interaction on the pK of the particular group i is given by

$$pK_i = (pK_{int})_i + \sum_{i \neq j} \Delta pK_{ij} \quad (5)$$

and

$$\Delta pK_{ij} = -W_{ij}/2.303 Z_i kT \quad (6)$$

(Tanford and Roxby, 1972). The electrostatic free energy of protein is obtained by considering all interaction of charges as follows

$$W = \frac{\epsilon^2}{2b} \sum_{\substack{i=1 \\ i \neq j}}^{n} \sum_{j=1}^{n} Z_i Z_j (A_{ij} - B_{ij}) - \frac{\epsilon^2}{2a} \sum_{i=1}^{n} \sum_{j=1}^{n} Z_i Z_j C_{ij} \quad (7)$$

(Tanford and Kirkwood, 1957).

RESULTS AND DISCUSSION

The computation based on the smeared charge model (Linderstrøm-Lang, 1924) has been widely employed for the theoretical treatment of protein titration curves. Kuramitsu and Hamaguchi (1980) employed this model to elucidate the titration curve of lysozyme. As shown in Fig. 2 the titration curve computed here was in accord with the experimental curves. The electrostatic free energy for native protein (W_N) computed by this method is shown in Fig. 3 *A*. That for denatured protein (W_D computed by Tanford's method (Tanford, 1961) is shown in Fig. 3 *B*. The difference (ΔW) between W_N and W_D can be considered as the net stabilization energy by electrostatic interactions in native protein over denatured protein (Fig. 3 *C*). These results did not explain our earlier finding that acetylated lysozyme is less stable than native around neutral pH region (Imoto et al., 1976*a*, *b*).

Next, the electrostatic free energy was computed by the fixed charge model (Tanford and Kirkwood, 1957) where one must consider the positions of charged groups. When the conversion factor from the accessibility to the depth was set to 1.62 Å (see Experimental section), the computed Z values fitted best to the experimental titration curves (Fig. 2). The computed Z values at the ionic strength of 1.0 OsM also gave a reasonable fit to experimental curve (Fig. 2).

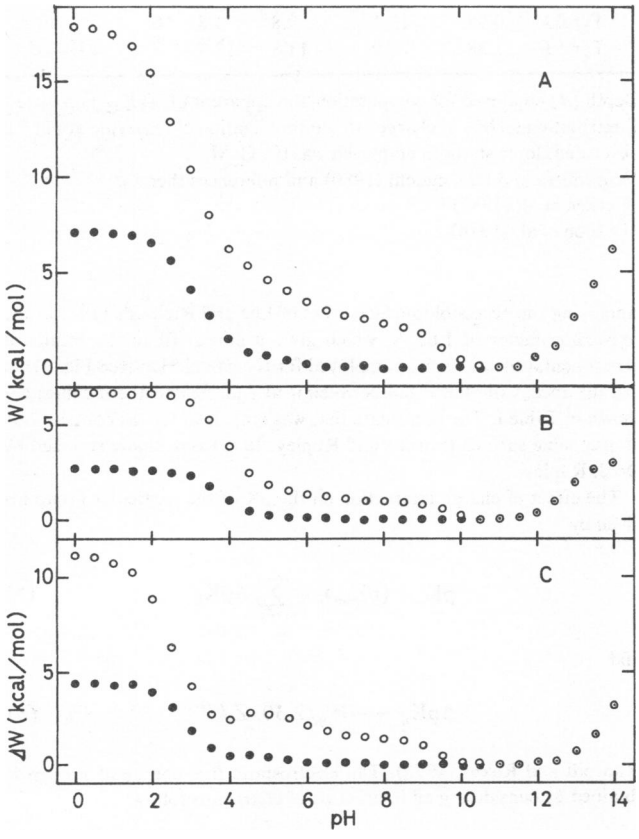

FIGURE 3 *A*, electrostatic free energies (W_N) of native (○) and acetylated (●) lysozymes computed by the smeared charge model. *B*, electrostatic free energies for the denatured proteins (W_D); *C*, electrostatic stabilization energies ($\Delta W = W_N - W_D$). Ionic strength was 0.1 OsM in all cases.

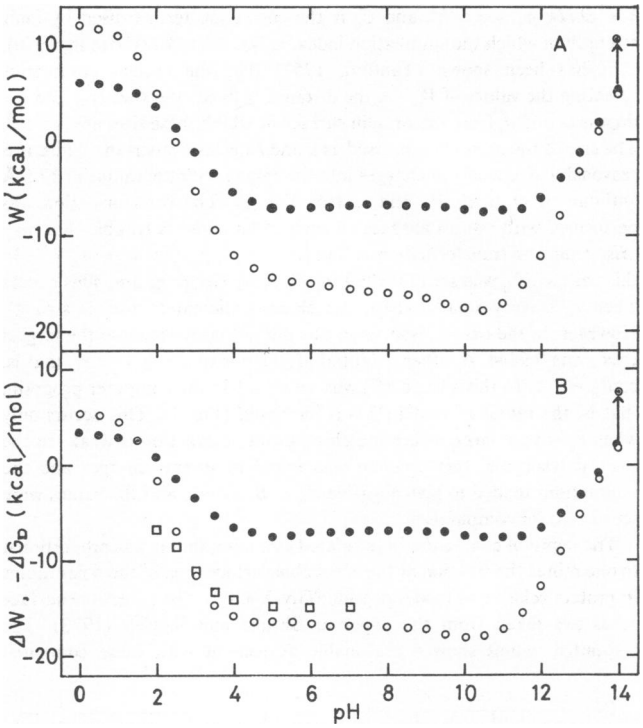

FIGURE 4 Electrostatic free energies (W_N) of native (○) and acetylated (●) lysozymes computed by the fixed charge model. *B*, electrostatic stabilization energies (ΔW) computed by the fixed charge model. Ionic strength were 0.1 OsM in all cases. Apparent Gibbs energy of denaturation (ΔG_D) reported by Pfeil and Privalov (1976) was reproduced (□).

The electrostatic free energies (W_N) for native and acetylated lysozymes were computed similarly (Fig. 4 *A*). The difference in energies (ΔW) between W_N obtained here and W_D obtained before (Fig. 3 *B*) are also shown (Fig. 4 *B*). The latter values were compared with the results obtained by protease digestion of native and acetylated lysozymes (Fig. 5). We have shown that lysozyme is digested only in the unfolded form (Imoto et al., 1974, 1976*a*, *b*) and the ratios of the rate constant of digestion of protein (k) to that of hemoglobin tryptide (k_0) would be roughly proportional to the equilibrium constant of denaturation, K_D (Imoto et al., 1976*a*). As shown in Fig. 5, $\ln(k/k_0)$ showed a similar pH dependence to ΔW computed by the fixed charge model. Thus, it is concluded that acetylated lysozyme is less stable than native around neutral pH region because of the loss of electrostatic interaction between charged groups in native lysozyme.

Pfeil and Privalov (1976) have examined the thermodynamics of lysozyme denaturation. The pH dependence of the apparent Gibbs energy of denaturation (ΔG_D) obtained by them is also shown in Fig. 4. A reasonable coincidence was obtained between ΔG_D and ΔW. These results indicate that the fixed charge model employed here is a proper way to elucidate the electrostatic free energy of a protein.

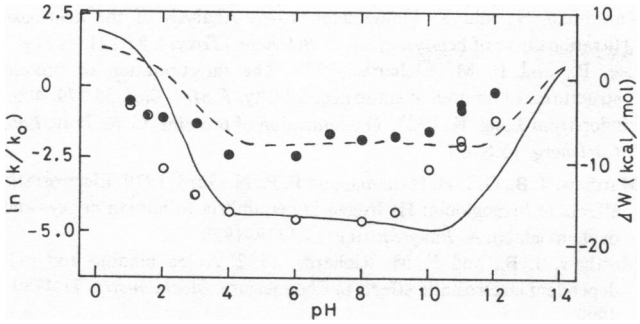

FIGURE 5 Logalism of the ratio of the rates of protease digestions of native (○) and acetylated (●) lysozymes to hemoglobin tryptide was plotted against pH. Lines are ΔW's for native (—) and acetylated (---) lysozymes computed by the fixed charge model.

The electrostatic free energy, W_i, for a hypothetical protein devoid of charge i was computed by setting the charge of critical group as zero, and the difference ($\Delta W'_i$) between W_N and W_i was considered as the contribution of the ith charged group on W. The contributions of more than one group are also computed in a similar manner. The values computed at pH 7 are listed on Table I. ϵ-amino groups of Lys 1 and 13 form salt bridges with carboxyls of Glu 7 and COOH terminus, respectively (Imoto et al., 1972; Shindo et al., 1978; Gerken et al., 1982). Almost all W evoked by amino groups (8.8 kcal/mol) can be explained by the contributions of these two amino groups (8.7 kcal/mol) (Table I). The predominant contributions of Glu 7, Asp 119, and COOH terminal carboxyls are also evident from Table I. A considerable contribution of Asp 119 is also explained by the close proximity of the negative charge of this residue with the positive charge of Arg 125 (3.3 Å). A large portion of the electrostatic stabilization free energy can be explained by a few pairs of proximal positive and negative charge interactions. It is also interesting that the ion pair that is strongly destabilizing the protein conformation was not found among carboxyl and amino groups in lysozyme (see Table I).

Apparent pK's were obtained as follows. By plotting fraction of dissociation, α_i, against pH, the pH at which α_i is 0.5 was considered as apparent pK ($pK_{1/2}$) of charge i. The apparent pK's thus obtained are listed in Table I for native and acetylated lysozymes. The carboxyl of Glu 35 is abnormal and when normal pK_{int} of Glu (4.4) was employed, a rather lower apparent pK of 4.1 was obtained. While the proximal negative charge of Asp 52 increased the pK of Glu 35, positive charges such as Arg 112, 114, Lys 33, and 116 were found to strongly decrease the pK of Glu 35. It was also not successful to obtain high apparent pK of Glu 35 by increasing the depth of the charge of Glu 35. The apparent pK of 6.0 was obtained only when abnormally high pK_{int} of 6.1 was adopted to Glu 35. Tanford and Roxby (1972) also concluded that the abnormality of pK of this group could not be explained only by electrostatic interactions. This residue lies close to Trp 108 and can be easily linked to indole ring of Trp 108 by iodine oxidation (Imoto and Rupley, 1973; Beddel et al., 1975). A possible interaction of carboxyl group of Glu 35 and the indole of Trp 108 was estimated by nuclear magnetic resonance (NMR) spectra (Campbell et al., 1975*a*, *b*; Cassels et al., 1978).

ϵ-amino groups of Lyl 1 and 13 form salt bridges with carboxyls and showed abnormally high pK's of 11.8 and 12.5, respectively, in my computation. In contrast, NMR studies assigned normal pK's for these groups (Bradbury and Brown, 1973; Gerken et al., 1982). For this reason, Gerken et al. (1982) suggested that these residues are in unusual environments. However, we must consider that NMR studies were performed after the methylation of amino groups. In these instances, salt bridges might be partially affected.

Lysozyme was assumed to have a few carboxyl groups with abnormally low pK's (Imoto et al., 1972). Three carboxyls, Glu 7 ($pK_{1/2}$ = 2.5), Asp 119 (1.9), and COOH terminus (1.9) showed abnormally low pK's and these are all close to positive charges of Lys 1 (3.5 Å), Arg 125 (3.3 Å), and Lys 13 (3.0 Å), respectively.

The depth of charge (d) is also an important factor. For example, the $pK_{1/2}$ for Glu 7 was computed to be 2.5 in present method, where the depth of Glu 7 was 0.96 Å and that of Lys 1 (3.5 Å away) was 0.72 Å. When all depths were set to 0 Å as employed by Orttung (1970), $pK_{1/2}$ for Glu 7 was computed to be 3.6. Even when all depths were set to 0.4 Å as employed by Tanford and Roxby (1972), $pK_{1/2}$ for Glu 7 became 3.3 with my way of computation. Thus, I have reconfirmed the importance of the distance and depths of the pair of charges. The present method that emphasizes the distance and depth of charge of ionizable groups is considered adequate for the computation of electrostatic free energy contribution of charged groups in protein.

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