Proton Equilibria of 5-Dimethylamino-1-naphthalenesulfonyl Group Conjugated to Bovine Serum Albumin. II. Neighboring Effects in Urea and Guanidinium Chloride Solutions

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Protonation to the fluorescent probe, 5-dimethylamino-1-naphthalenesulfonyl group, which had been conjugated to bovine serum albumin, was investigated in aqueous solutions of urea and guanidinium chloride. pK values not less than those of the free probe were present even in the positive range of net charge on the protein at high concentration of the denaturants. This was interpreted in terms of the electrostatic and nonpolar effects based on short-range interaction between the probe and its neighboring residues. These effects were substantiated by the reduction of the disulfide bonds in the protein and by the variation of the dielectric constant of the local environment surrounding the probe.

Studies on the acid-base equilibria of optical probe-protein conjugates have been carried out in order to clarify the short-range interaction among the constituent residues.[1-6] A report was given on the acid-base equilibria of the fluorescent probe, 5-dimethylamino-1-naphthalenesulfonyl group (DNS) conjugated to bovine serum albumin (BSA) with various degrees of conjugation.[6] It was found that the apparent dissociation constant, K_a, of the dimethylamino moiety of the DNS–BSA conjugates with less than 1.0 mol of the probe per mol of the protein changes according to the conformational change by acid denaturation. However, the pK_a values of the DNS conjugates at various pH were less than the theoretical values estimated from the Debye-Hückel electrostatic shielding effect on the surface of the protein. The deviation might be ascribed in part to the effect of the nonpolar environment surrounding the probe owing to its hydrophobicity in the basic form.

In order to evaluate the effect of the nonpolar environment, the behavior of the pK_a values of the DNS conjugate was studied at various concentrations of denaturants, such as urea and guanidinium chloride (GuHCl). The denaturants interact strongly with peptide backbone,[7,8] enhancing the solubility of nonpolar groups either by altering the bulk properties of the solvent or through more localized effect.[7,9,10] The former weakens interpeptide hydrogen bonds, the latter reducing hydrophobic interactions. Thus, the denaturation on urea or GuHCl makes the conformational state of the protein less compact and simultaneously, brings about the lowering in hydrophobicity or non-polarity inside the protein. We might expect that the change in the pK_a value is influenced by only the charge of the protein at high concentrations of the denaturants.

The results of the variation of pK_a values at such high concentrations of the denaturants show that the negatively charged groups are present in the neighborhood of the probe in the positive range of the net charge of the protein. The neighboring effect due to the electrostatic interaction is maintained by the disulfide bonds in the protein even in the denaturant solutions.

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Experimental

Materials. The DNS–BSA conjugate used in this experiment was prepared as described previously[6] and contained less than 1.0 mol of the probe per mol of the protein. Such a degree of conjugation has no influence on the conformation of the protein.[6] The reductive cleavage of the disulfide bonds of the DNS–BSA conjugate in 6 M (1 M= 1 mol dm^{-3}). urea or GuHCl solution were accomplished by dithiothreitol (DL-*threo*-1,4-dimercapto-2,3-butanediol) and iodoacetamide according to the procedure of Bewley *et al.*[11] Water was deionized and then distilled. Urea and GuHCl were recrystallized from methanol–water solution. Electrolytes of reagent grade were used without further purification.

Fluorescent Measurements. Solutions of the DNS–BSA conjugate with 1.1×10^{-5} M of the protein were prepared by taking requiste amounts of a stock solution of the DNS–BSA conjugate, solid urea or GuHCl, and water in volumetric flasks. Measurements of the pH and fluorescent spectra of the solutions were carried out as reported[6] with the excitation wavelength taken at 340 nm where only the probe in the basic form absorbs light. In order to obtain the true emission spectra, the measured emission spectra were corrected by using the known emission spectrum of quinine sulfate as a standard.[12]

Potentiometric Titration. Potentiometric titration was carried out in order to determine the number, $\bar{Z}_H$, of proton bound to the protein. The solution of BSA with an appropriate concentration of the denaturants was dialyzed for 48 h against the same concentration of CO_2-free denaturant solution at 25 °C. The protein concentration was in the range 10^{-4} M, about 10 times that for the fluorescent measurements for obtaining the accurate values of $\bar{Z}_H$. Titration of 30 mL (1 L=1 dm^3) of prepared solutions was carried out under nitrogen atmosphere at 25 °C with a Hitachi-Horiba F-7AD pH meter equipped with a Hitachi-Horiba 6326-05C combination electrode. Titrant, 0.5 M HCl, was added with a Mitamura Riken Mechanical Buret (syringe type; a minimum volume of readings, 2.5 μL). The solvent containing the corresponding denaturant was titrated in the same way. No difference in titration curves between the unconjugated and conjugated proteins could be detected in the case of 0.01 M NaCl, 6 M GuHCl, and 8 M urea; the unconjugated protein was employed in the other cases.

Results and Discussion

Fluorescence of DNS Conjugate. The fluorescence

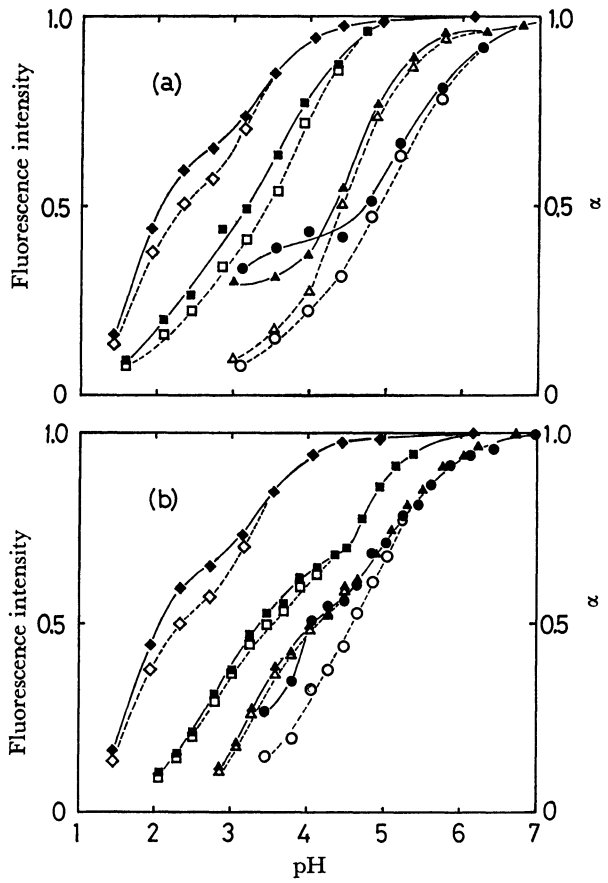

Fig. 1. Variation of fluorescence intensity (open symbols) and fraction in the basic form (closed symbols) of the DNS–BSA conjugate as a function of pH in denaturant solutions. (a) Concentration of urea: 8 M (○, ●), 4 M (△, ▲), 2 M (□, ■), and 0 M (◇, ◆). (b) Concentration of CuHCl: 6 M (○, ●), 4 M (△, ▲), 2 M (□, ■), and 0 M (◇, ◆). Fluorescence intensity is defined by taking a maximum in the intensities measured at each concentration of the denaturants as 1.0. α is calculated with Eq. 2.

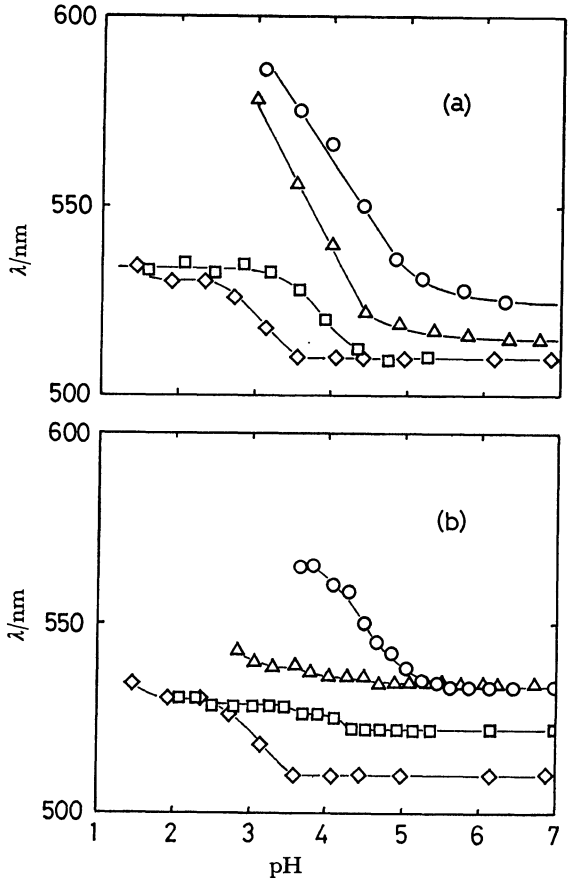

Fig. 2. Plots of peak wavelength of the fluorescence of the DNS–BSA conjugate against pH of the denaturant solutions. (a) Concentration of urea: 8 M (○), 4 M (△), 2 M (□), 0 M (◇). (b) Concentration of GuHCl: 6 M (○), 4 M (△), 2 M (□), 0 M (◇).

intensities of the DNS–BSA conjugate obtained at different concentrations of urea and GuHCl are shown as a function of pH in Figs. 1(a) and 1(b), respectively. In the absence of denaturants the 0.01 M NaCl solution was employed. A maximum in all the fluorescence intensities measured at each denaturant concentration is arbitrarily assigned the numerical value of 1.0 on the ordinates in the figures. Each sigmoid curves can be ascribed in part to the acid-base equilibrium of the conjugated probe since the protonation of the probe quenches the fluorescence, only the probe in the basic form giving the fluorescence.[16]

The peak wavelengths corresponding to the fluorescence in the solutions of urea and GuHCl are also shown in Figs. 2(a) and 2(b), respectively. The peak shifts progressively to higher wavelengths both with an increase of the denaturant concentration and with a decrease of the pH value, reaching 590 nm for the most acidic solution of 8 M urea and 6 M GuHCl. The shift is closely dependent on the change of the polarity of the environment surrounding the probe. Similar shifts in peak wavelength was observed in the fluorescence of DNS–amino acid conjugates in various organic solvents.[13–15] This type of change in the fluorescence with change of solvent polarity has been explained theoretically in terms of the differences in solvent interaction energies of the ground and excited states arising from differences in the polarities or dielectric constants of the states.[13,14] Thus, with increase in the dielectric constant of the organic solvent the peak shifts to higher wavelength, the quantum yield of the fluorescence decreasing. Variation of the peak wavelength and quantum yield with the dielectric constant of solvent was reported by Abe *et al.* for the fluorescence of three DNS–amino acid conjugate, *viz.* DNS–L-alanine, DNS–L-leucine, and DNS–L-phenylalanine, in various organic solvents.[15]

pK_a *Values in Denaturant Solutions.* The relative fluorescence intensities (Fig. 1) change not only with quenching by the protonation of the probe but also with the change in quantum yield of the probe in the basic form.[16] The relative fluorescence intensity, F, should be corrected in order to calculate the true fraction, α, of the probe in the basic form.

Since the fluorescence observed arises from the basic form,[16] F can be related to the quantum yield, Φ, and the concentration, C, of the basic form:

$$F \propto I_0 \varepsilon C \Phi,$$

where I_0 is the strength of the incident beam, and ε the molar absorption coefficient of the basic form at 340 nm. Factors ε and I_0 are constant, while C is dependent upon the degree of the protonation to the probe, Φ changing with the peak wavelength, λ, of the fluorescence measured at each pH value of a denaturant solution. When the fluorescence at different pH values of the solution give the peak wavelengths, λ_1 and λ_2, the relation between the relative fluorescence intensities, $F(\lambda_1)$ and $F(\lambda_2)$, can be written as

$$\frac{F(\lambda_1)}{F(\lambda_2)} = \frac{C_1\Phi(\lambda_1)}{C_2\Phi(\lambda_2)}, \quad (1)$$

where $\Phi(\lambda_1)$ and $\Phi(\lambda_2)$ are the quantum yields giving the corresponding wavelengths, and C_1 and C_2 the respective molarities of the probe in the basic form existing in the solution with the different pH values. If $F(\lambda_2)$ is taken as the fluorescence intensity in the case where all the probe becomes the basic form, α is equal to C_1/C_2, so that

$$\alpha = \frac{F(\lambda_1)\Phi(\lambda_2)}{F(\lambda_2)\Phi(\lambda_1)}. \quad (2)$$

When λ_1 is equal to λ_2, the α value is equal to the ratio $F(\lambda_1)/F(\lambda_2)$. Then, the relative intensity of $F(\lambda_1)$ is identical with the α value if $F(\lambda_2)$ is taken as 1.0 (Fig. 1).

Since the peak wavelength was shifted, $\Phi(\lambda_1)/\Phi(\lambda_2)$ was evaluated from the results obtained by Abe *et al.*[15] The α values calculated by Eq. 2 are shown by closed symbols in Figs. 1(a) and 1(b). The maximum of all the measured fluorescence intensities is taken as the α value of 1.0. The apparent dissociation can be calculated from the α and pH values by the following Henderson-Hasselbalch equation:

$$pK_a = pH - \log\frac{\alpha}{1-\alpha}. \quad (3)$$

The pK_a values in urea and GuHCl solutions are shown as a function of pH in Figs. 3(a) and 3(b), respectively.

Neighboring Effect on pK_a. The pK_a values determined at each pH value increased with an increase in concentrations of the denaturants, suggesting that the probe inside the protein is exposed to the solvent according to the concentration of the denaturants which weaken the hydrophobic interaction between the probe and the nonpolar residues. In the absence of the hydrophobic interaction, the shift of pK_a from the logarithm of the reciprocal of dissociation constant, pK_0, of the free probe can be attributed to the electrostatic interaction between the probe and the charged residues. Taking only the long-range electrostatic effect of the protein based on the Debye-Hückel theory into account,[17] the pK_a values in the range of positive net charge of the protein might be less than the pK_0 values.

The pK_0 values were determined for 1.0×10^{-5} M of DNS-glycine conjugate as a model of the free probe in the corresponding solution: 3.94 in 0.01 M NaCl; 3.93, 4.00, and 4.17 in 2, 4, and 8 M urea; 3.99, 4.00, and 3.99 in 2, 4, and 6 M GuHCl, respectively. The results show that the dissociation constant gives about 4.0 in pH units, either in the presence or in the absence of the denaturants. The value of 4.0 is shown by a dashed line in Fig. 3.

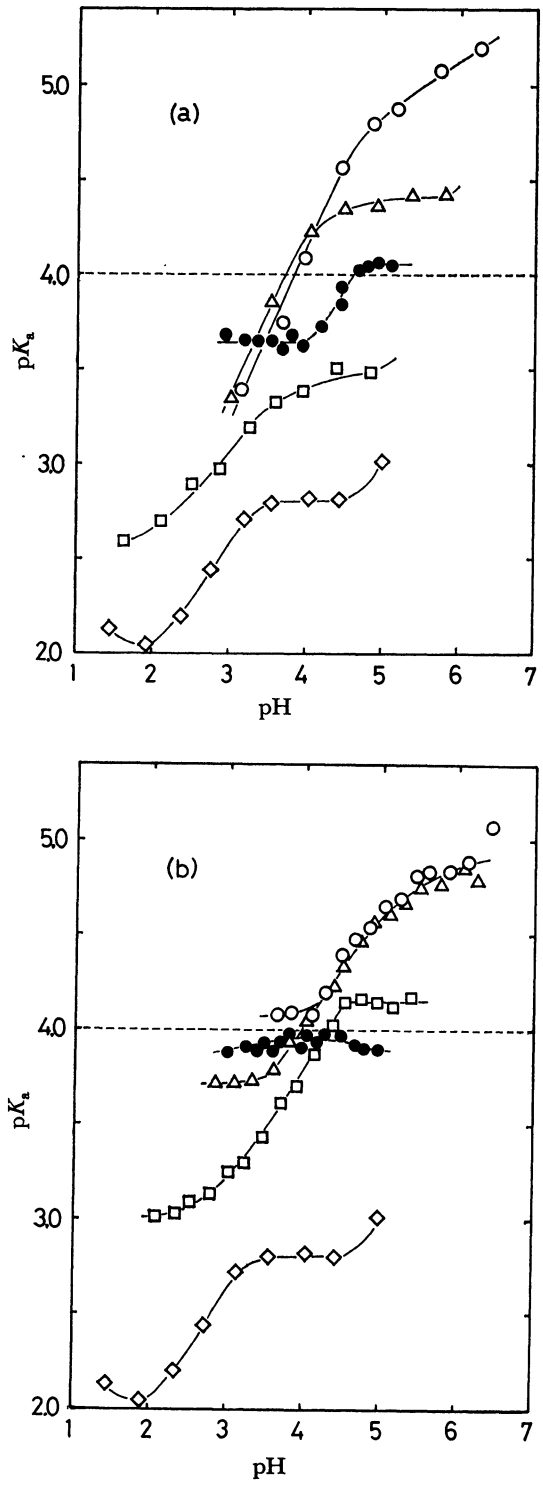

Fig. 3. Apparent pK_a values as a function of pH in the solution of urea (a) and GuHCl (b). Open symbols are obtained for the DNS–BSA conjugate with disulfide bonds at the same concentration of the denaturants as those in Fig. 2. Closed circles are obtained for the DNS–BSA conjugate with disulfide bonds ruptured by the reduction at 6 M of urea (a) and of GuHCl (b).

The pK_a values are not less than the pK_0 values under high concentration of the denaturants. This can be at least anticipated to involve a contribution of the electrostatic effect of negatively charged residues

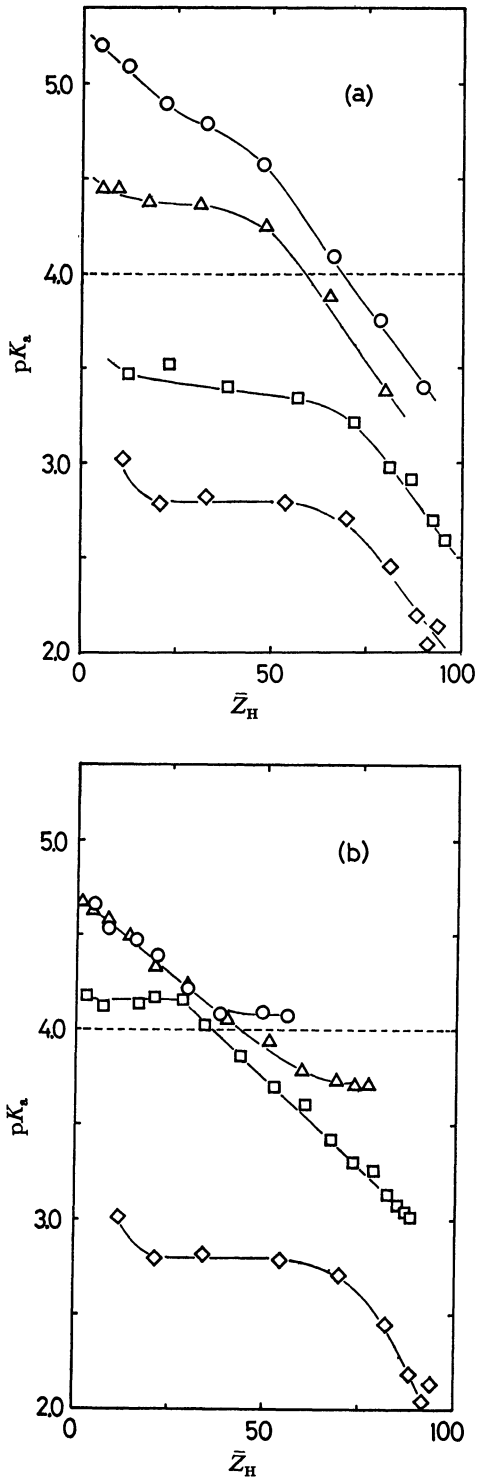

Fig. 4. Apparent pK_a values as a function of $\bar{Z}_H$ in the solutions of urea (a) and GuHCl (b). Symbols are the same as in Fig. 2.

on the acid-base equilibrium of the probe, since the positively charged residues apparently weaken the basic properties of the probe. If we assume that the electrostatic effect is attributable to only the long-range electrostatic interaction, the phenomenon should appear in the range of negative net charge of the protein. The net charge, $\bar{Z}_H$, from the isoelectric point, pI, of the protein was determined at various pH in each denaturant solution. The pI values were 5.3 in 0.01 M NaCl; 6.4, 6.6, and 7.0 in 2, 4, and 8 M urea; 5.3, 5.3, and 5.6 in 2, 4, and 6 M GuHCl. The pK_a values are plotted in Fig. 4 as a function of $\bar{Z}_H$ instead of pH. The pK_a values not less than the pK_0 values are observed even in the range of positive $\bar{Z}_H$ values. This makes it impossible to account for the acid-base equilibrium at the high concentration of the denaturants in terms of the long-range electrostatic interaction. A possible explanation for the observed behavior may be the contribution of the short-range electrostatic interaction between the probe and the negatively charged residues situated in the neighborhood of the probe.

Variation of pK_a *in Reduction of Disulfide Bonds.* Proteins denatured by urea and GuHCl exist as random coils, but the transition to the denatured state is often incomplete even at the highest denaturant concentrations, especially when the native conformation is stabilized by disulfide bonds.[18] It is possible that the local structures surrounding the disulfide bonds maintain the structure of the native protein even in the concentrated denaturant solution. Since a molecule of BSA has seventeen of cystine with a disulfide group as seen from the complete data for the primary structure of BSA reported by Brown,[19] the fixed structures around such disulfide bonds might contribute largely to the neighboring effect on the acid-base equilibrium of the probe.

For the sake of confirmation, the disulfide bonds in the DNS–BSA conjugate were subjected to reductive cleavage by dithiothreitol in 6 M of urea or GuHCl solutions. The pK_a values for the DNS–BSA conjugate with the reduced disulfide bonds were determined in these denaturant solutions (Fig. 3). The values do not exceed the pK_0 values in the denaturant solutions, *i.e.*, 4.17 in 8 M urea and 3.99 in 6 M GuHCl in the range of pH measured. The results showing that the neighboring effect disappears with the reduction of the protein suggest that the disulfide bonds play a significant role for fixing the relative situation of the probe and the ionized residues even in the denaturant solutions.

The change of pK_a against pH in the case of the reduced protein was not so remarkable as that in the case of unreduced one. $\bar{Z}_H$ values for the reduced protein were not dentermined, the pK_a values for the reduced DNS–BSA conjugate being less than those for the unreduced one in lower pH range. This suggests that the acid-base equilibrium of the probe is affected remarkably by the positively charged residues, as well as negatively charged residues, according to the dissociation of the ionizable group surrounding the probe.

Dielectric Constant of the Environment Surrounding the Probe. Appearance of the electrostatic neighboring effect is manifested by the lowering of the hydrophobicity around the probe. However, the hydrophobicity should also affect the shift of pK_a as the nonpolar neighboring effect. Since the denaturants weaken the hydrophobic interaction between the nonpolar residues, the local environment surrounding probe might have a solvent-like polarity with a high dielectric constant.

The change of such a local environment can be recognized as that of the medium in which the probe

exist. The nonpolar neighboring effect might be related to the medium effect in organic-water solvents. If a neutral species in the basic form, B^0, which gives a pK_0 value in water exists in a medium with a dielectric constant, D, the pK_a value in the medium can be written as

$$pK_a - pK_0 = \frac{e^2}{2.3rkT}\left(\frac{1}{D_w}-\frac{1}{D}\right), \quad (4)$$

where e, k, and D_w are proton charge, Boltzman constant, and dielectric constant of water, respectively, and r is the mean radius of the ions which forms HB^+ and X^- in the protonation of B^0 by an acid, HX, if these ions are regarded as rigid spheres. The relation is derived from Born's model to account for the medium effect theoretically.[20] Equation 4 shows that the shift of pK_a from pK_0 is proportional to $(1/D_w - 1/D)$.

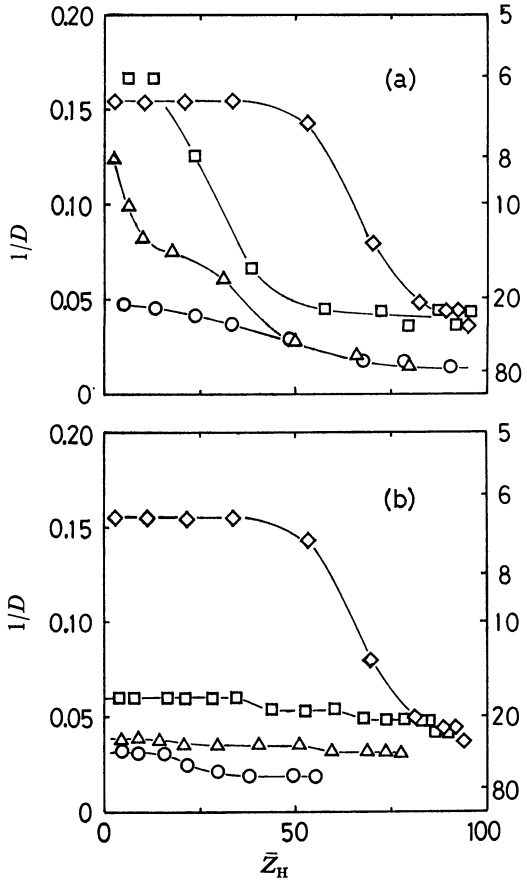

Fig. 5. Plots of the reciprocal of dielectric constant surrounding DNS as a function of $\bar{Z}_H$ in the solutions of urea (a) and GuHCl (b). Symbols are the same as in Fig. 2.

The dielectric constant of the local environment surrounding the probe was estimated by the peak wavelength of the fluorescence of the probe from the data for the fluorescence of DNS–amino acid conjugates in organic solutions.[15] The $1/D$ values estimated are shown as a function of $\bar{Z}_H$ in Fig. 5. In urea solution the $1/D$ values in the lower $\bar{Z}_H$ range decrease remarkably with an increase of the concentration of urea (Fig. 5(a)). This suggests that the nonpolar or medium effects appear in the absence of urea, especially in the lower $\bar{Z}_H$ range. The fact that the pK_a values in 0.01 M NaCl are almost constant in such a range of $\bar{Z}_H$ can be attributed to the nonpolar effect rather than the electrostatic effect. The nonpolar environment is unlikely to give rise to the presence of the electrostatic neighboring effect.

On the other hand, for higher $\bar{Z}_H$ values, the $1/D$ values approach the reciprocal of the dielectric constant of water (=0.0125), either in the presence or in the absence of urea. Thus, the nonpolar effect on pK_a can be ignored at the highest $\bar{Z}_H$ value. The shift of pK_a at such a $\bar{Z}_H$ value might be attributed to the long-range or short-range electrostatic effects. The pK_a values in aqueous solutions in the absence of the denaturants were less than those estimated from the Linderstrøm-Lang equation, especially when the protein formed an expanded state by the protonation.[6] The result can also be interpreted in terms of the short-range electrostatic interaction between the probe and the positively charged residues. The interaction might be caused by the disulfide bonds, similar to the electrostatic interaction between the probe and negatively charged residues in the presence of the denaturants.

In GuHCl solution (Fig. 5(b)), the $1/D$ values behave like those in urea solutions but give a low constant value even in 2 M of GuHCl solution. This is ascribed to the difference of the two denaturants; urea is a neutral molecule and GuHCl is a salt species. In a GuHCl solution with a high ionic strength, it is likely that the neighboring effect is due to the electrostatic interaction rather than the nonpolar interaction as compared with urea solutions.

The following conclusion is made. (1) The shift of pK_a from pK_0 is mainly attributed to the neighboring effects between the probe and its surrounding residues in the case of the unreduced protein. (2) The neighboring effects are attributed to the electrostatic and nonpolar interactions: The former effect is due chiefly to the exposure of the probe to the solvent-like environment by the protonation on the protein or by the denaturants employed, the latter to the probe being buried in the nonpolar environment.

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References

1) G. Weber, *Biochem. J.*, **51**, 155 (1952).
2) I. M. Klotz and J. Ayers, *J. Am. Chem. Soc.*, **79**, 4078 (1957).
3) I. M. Klotz and H. A. Fiess, *Biochim. Biophys. Acta*, **38**, 57 (1960).
4) P. A. Frey, F. C. Kokesh, and F. H. Westheimer, *J. Am. Chem. Soc.*, **93**, 7266 (1971).
5) U. P. Strauss and G. Vesnaver, *J. Phys. Chem.*, **79**, 1558 (1975).
6) N. Ikuta, J. Koga, and N. Kuroki, *Bull. Chem. Soc. Jpn.*, **54**, 228 (1981).
7) Y. Nozaki and C. Tanford, *J. Biol. Chem.*, **238**, 4074 (1963).
8) D. R. Robinson and W. P. Jencks, *J. Am. Chem. Soc.*, **87**, 2462 (1965).

9) W. Bruning and A. Holtzer, *J. Am. Chem. Soc.*, **83**, 4865 (1961).
10) D. B. Wetlaufer, S. K. Malik, L. Stoller, and R. L. Coffin, *J. Am. Chem. Soc.*, **86**, 508 (1964).
11) T. A. Bewley, J. S. Dixon, and C. H. Li, *Biochim. Biophys. Acta*, **154**, 420 (1968).
12) R. J. Argauer and C. E. White, *Anal. Chem.*, **36**, 368 (1964).
13) R. F. Chen, *Arch. Biochem. Biophys.*, **120**, 609 (1967).
14) E. Lippert, *Z. Eletrochem.*, **61**, 962 (1967).
15) I. Abe, J. Koga, and N. Kuroki, *Nippon Kagaku Kaishi*, **1975**, 879.
16) Since the probe in the acidic form absorbs no light at excited wavelength of 340 nm,[6] the fluorescent quantum yield of the probe in the acidic form can be taken as zero.
17) C. Tanford, *Adv. Protein Chem.*, **17**, 69 (1963).
18) C. Tanford, *Adv. Protein Chem.*, **23**, 120 (1969).
19) J. R. Brown, *Fed. Proc.*, **35**, 2141 (1976).
20) E. J. King, "Acid-Base Equilibria," in "The international Encyclopedia of Physical Chemistry and Chemical Physics," 1st ed, ed by E. A. Guggenheim *et al.*, Pergamon Press, Oxford (1965), Vol. 4, Chap. 11, pp. 248—279.