Proton Equilibria of a 5-Dimethylamino-1-naphthalenesulfonyl Group Conjugated to Bovine Serum Albumin. I. Effects of The Conjugation on Structural Alternation

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The proton equilibria of an optical probe, the 5-dimethylamino-1-naphthalenesulfonyl group, conjugated to bovine serum albumin with various molar ratios to the protein in an acidic solution, were investigated by means of the change in the fluorescence intensity as well as that in the absorbance. Below the molar ratio of 1.7 the apparent pK values for the dimethylamino moiety of the optical probe decreased remarkably with a decrease in the pH of the solution, accompanying conformational changes of the protein caused by the proton binding to the protein. For molar ratios of more than 2.0, the alternation of the higher structure of the protein induced by the conjugation of the probe was detected by ORD measurements, while the pK values of those conjugated probes were lowered at pH's higher than 3.0. For comparison, the probe conjugated with glycine as its free state gave the pK value of 3.99 in an aqueous solution. The shifts of the apparent pK values of the conjugated probe in the protonated protein compared with those for the free probe were, particularly, too large to arise from the Debye-Hückel electrostatic shielding effect. These results are, therefore, interpreted in terms of a change in the short-range interaction in the neighboring residues surrounding the probe.

The interpretation of the ionization of acid-base groups on a protein molecule is complicated in part by the fact that the measurements reflect the composite behavior of a large number of groups. Even in an acidic solution the groups participating in the proton equilibria of acid-base groups on a protein include not only a large number of carboxyl groups, but also imidazole or tyrosine residues in an appreciable number. Their proton equilibria overlap one another, so that a sharp separation cannot be made by means of the potentiometric titration technique.

In order to avoid obscure titration curves, the spectral titration curves are often obtained for tyrosine residues.[1] A useful method for the same purpose is the attachment of an optical probe which has a desirable acidity constant to proteins by means of a covalent linkage.[2-5] The ionization of the probe is usually accompanied by a significant change in both the absorption and emission spectra. It is thus very easy to obtain the titration curve for this group alone. An optical probe of the kind which has been successively employed in protein studies is the 5-dimethylamino-1-naphthalenesulfonyl (DNS) group. Klotz and Fiess[3] reported the proton equilibria of DNS attached chiefly to amino groups on bovine serum albumin (BSA) by means of absorption measurements. Their equilibria indicated a significant difference from those of DNS-amino acid conjugates and were independent of the pH values of the solution in which BSA undergoes a conformational change by ionization. The DNS group conjugated to BSA (DNS-BSA) prepared by them contained 4 to 11 mol of DNS to 1 mol of the protein. There is, therefore, a fear of the alternation of the secondary and tertiary structures of the conjugated protein in their results.

The present investigation was undertaken to see if the proton equilibria of DNS-BSA followed the conformational change on the acid denaturation of the conjugated BSA, the fine structure of which was not grossly altered by the limited degree of conjugation. The degree of protonation of the DNS conjugate was determined from the fluorescent spectra, which have a better sensitivity, in order to measure the quenching by the protonation of the DNS group at a low degree of conjugation.

Experimental

Materials. BSA (crystallized and lyophilized) from the Sigma Chemical Co. (Lot. 17C-8145) and 5-dimethylamino-1-naphthalenesulfonyl chloride (DNS-Cl) from the Tokyo Chemical Co. (Tokyo) were used without further treatment. DNS-glycine conjugate (DNS-Gly) was purchased from the Sigma Chemical Co. (Lot. D-0875). The DNS-L-phenylalanine conjugate (DNS-Phe) was prepared from DNS-Cl and L-phenylalanine according to the method of Gray and Hartley.[6] The stock solution of these conjugates to amino acids were prepared by dissolving the conjugates in ethanol. All the acids, bases, and salts were reagent-grade.

Preparation of DNS-BSA. A solution of BSA was prepared containing 320 mg of protein in 80 ml of 0.1 M Na_2HPO_4 (1 M = 1 mol dm^{-3}). To this cooled solution was added 4 ml of a cold acetone solution containing 0.5—8 mg of DNS-Cl. The mixture was kept in a refrigerator and shaken sometimes for 24 h. The reaction by-products was then removed by dialysis in the cold for 24 h against three successive portions of 0.01 M acetic acid and thereafter for several days against distilled water. The dialysates were monitored for their gradual disappearance. The fluorescence intensity of the dialysate in the final dialysis could not be detected at the maximum sensitivity of the monitoring fluorescence spectrofluorometer. The protein solution was diluted in a volumetric flask and stored in a refrigerator. The stock solution was not used after one month from the time of preparation. The moles of the DNS conjugates to the protein were calculated from the measurement of the absorption at 340 nm using an extinction coefficient of 3.36×10^6 M^{-1} cm^{-1}.[7]

Fluorescence Titration of DNS Conjugate. In order to prevent concentration quenching, a portion of the stock solution of conjugates to BSA or amino acids was diluted with distilled water to give a solution with an optical density of less than 0.1 at 340 nm. To this diluted solution a measured quantity of hydrochloric acid was then added from a micropipet, after which the pH value and the fluorescence spectrum of the solution were measured. The procedure

was repeated with further samples of the solution and different quantities of the acid. Suitable corrections were made for the dilution of the solution by the added acid or base. The pH was measured at 25.0 °C with a Hitachi-Horiba F-7AD pH meter and a 6326-05C combination electrode. The fluorescence emission spectra were measured at the same temperature with a Hitachi Perkin-Elmer MPF-2A fluorescence spectrophotometer. The excitation wavelength (λ_{exc}) was taken at 340 nm. Both the excitation and emission slits were to cover wavelength ranges of 10 nm. Solutions of quinine sulfate in 0.1 M H_2SO_4 were used as the reference in the range of concentration of 10^{-7} to 10^{-5} M.

The protonation of the dimethylamino moiety of the DNS group gave rise to a quenching of the fluorescence of DNS at $\lambda_{exc}=340$ nm, so that the fraction, α, of the DNS group in the basic form was calculated by means of the following equation:

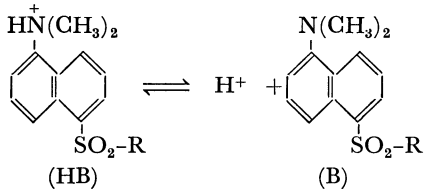
$$\alpha = \frac{\text{(maximum intensity of fluorescence at the pH value of the soln.)}}{\text{(maximum intensity of fluorescence in the basic soln.)}} \quad (1)$$

The emission spectra described in the results were corrected for variations in the detector system by using the known emission spectra of quinine sulfate. In the present experiments, the α values calculated from uncorrected spectra were equal to those from corrected spectra.

Results

Proton Equilibria of Free Probe. The acidity constant of a probe obtained from fluorometric analysis may be evaluated by means of the equilibria containing the components an acid and/or base existing in the excited state. In order to investigate the proton equilibria of the probe conjugates in the ground state, it is necessary to take into account the contribution of the excited probe molecules to the acid dissociation in the ground state. The absorption method is usually employed to determine the proton equilibrium constant in the ground state.

The proton equilibria of DNS conjugated in the ground state may be represented by the following equation:



(2)

When R is replaced by a small molecule such as a glycine residue, $-NHCH_2COOH$, this dissociation can be described quantitatively by the familiar Henderson-Hasselbalch expression:

$$pH = pK_a + \log\frac{\beta}{1-\beta} \quad (3)$$

where pK_a is the apparent acidity constant of the dimethylamino moiety in the DNS group, and β, the fraction of the DNS group in the unprotonated ground state. The absorption spectra of 1.00×10^{-4} M DNS-Gly, as a model of free probes obtained at various pH values, are shown in Fig. 1, where the absorbance is given as a function of the wavelength, λ. The results exhibited isosbestic points at 268 and 304 nm which were identical with those reported for the DNS group conjugated to diaminoethane.[5] The curves corresponding to the highest and lowest pH values in Fig. 1 represent the spectra of the unprotonated (B) and protonated (HB) forms respectively; hence, the fraction of the probe in the protonated ground state, β, is commonly calculated from the amount of the absorption change at each pH value. According to Eq. 3, the plots of the pH values in solution *versus* $\log[\beta/(1-\beta)]$ give a slope of one and a pK_a value which is equal to a pH value at $\log[\beta/(1-\beta)]=0$. For DNS-Gly and DNS-Phe in water, the pK_a values were given as 3.99[8] and 4.00 respectively.

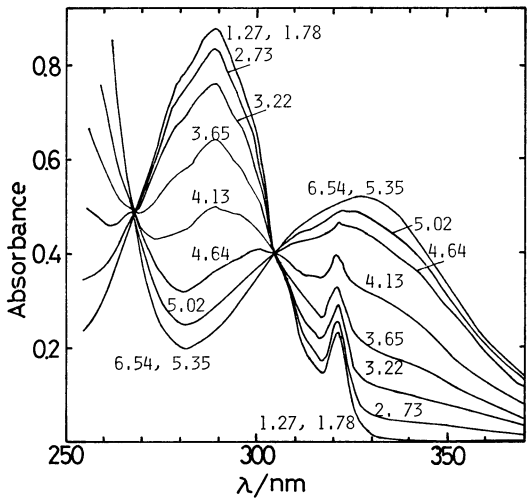


Fig. 1. Absorption spectra of 1.00×10^{-4} M DNS-Gly in water at various pH. Curves are labeled with appropriate pH values. Cell length 1 cm.

The emission spectra of 1.68×10^{-5} M DNS-Gly excited at 340 nm were measured at various pH in water, as is shown in Fig. 2. The spectra were characterized by one band near 590 nm at which the maximum intensity decreased with a decrease in the pH. As is shown in Fig. 1, the absorption at 340 nm was absent in the solution of a lower pH, where all of the dimethylamino moiety of DNS group was protonated. This fact indicates that the DNS molecules are excited only in the unprotonated form when exposed to the light of the 340 nm wavelength. The emission band, therefore, corresponds to the fluorescence from the excited state of the DNS group with the dimethylamino moiety in its unprotonated ground state.

The unprotonated excited molecules, denoted by B*, are treated by the insertion of an additional component in Eq. 2 to give:[9]

$$HB \rightleftharpoons B \rightleftharpoons B^*. \quad (4)$$

The fraction of the unprotonated DNS group in both the excited and ground states make it difficult to estimate from the fluorescence in the above successive equilibrium. The intensity of the emission peak was,

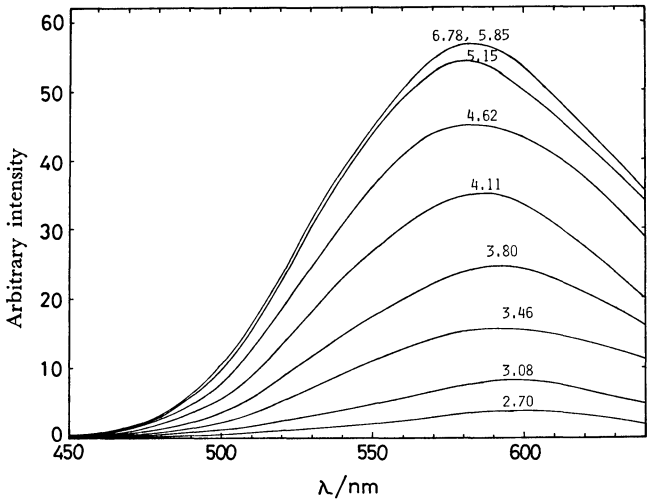


Fig. 2. Corrected emission spectra of 1.68×10^{-5} M DNS-Gly in water at various pH. Curves are labeled with appropriate pH values.

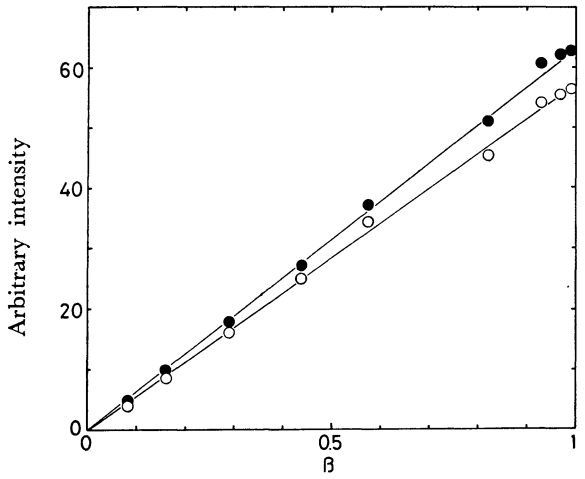


Fig. 3. Maximum intensity of corrected and uncorrected fluorescence spectra of DNS-Gly as a function of the fraction in unprotonated ground state (β). Ordinate scale, I_{max}, is arbitrary intensity of peaks in the corrected (○) and uncorrected (●) spectra.

however, proportional to the fraction of the DNS molecules in the unprotonated ground state (β), as calculated from absorption, as is shown in Fig. 3. The linear relationship implies that the equilibrium between B and HB is scarcely shifted by the processes of excitation and emission between B and B*, which are much faster than those of protonation and unprotonation. Consequently, the fraction of the unprotonated form in the ground state can be calculated from the fluorescence intensity of the DNS group in the same way as from the absorption titration. From the pH value in solution and the α obtained by fluorescence measurement, the pK_a values were obtained as 3.99 and 4.00 for DNS-Gly and DNS-Phe respectively. These values were, as expected, identical with those obtained by the absorption method. Hence, the fraction of the DNS group in the unprotonated form obtained from fluorescence measurements, α, is equal to the β obtained by the absorption method and Eq. 3 can be rewritten as follows:

$$pH = pK_a + \log\frac{\alpha}{1-\alpha}. \quad (5)$$

Proton Equilibria of DNS-BSA Conjugates. The corrected emission spectra of DNS-BSA conjugates with the molar ratio of 1.7 mol of the probe to 1 mol of protein obtained in water as a function of the pH are shown in Fig. 4. The spectra are different from those described before for the free probe. The 580 nm peak of the free probe is remarkably blue-shifted when the DNS group is conjugated to BSA, to 515 nm at high pH values and to 530 nm at low pH values. The large shift is attributable to the hydrophobic environment of the protein at which DNS group is attached. The small red-shift from a high to a low pH corresponds to an energy change of about 0.55×10^3 cm^{-1}, which is slightly larger than that of about 0.48×10^3 cm^{-1} in the case of the free probe. This finding exhibits the effect of the structure change on the environment of the DNS group. The lowering of the quantum yield of the DNS conjugate in unprotonated form with the red-shift may be neglected compared with the quenching by the protonation of the probe. Thus, the proportionality between the fraction of the probe in the unprotonated form and the fluorescence intensity is maintained in DNS-BSA as well as in the free probe, as has been described before. The fraction of DNS conjugates in the unprotonated form can, therefore, be calculated by the same method as in the free probe.

When the DNS group was conjugated to BSA with the molar ratios of 0.9, 2.0, and 2.7 mol of the probe to 1 mol of protein, Eq. 5 was no longer adequate to represent the dissociation over the pH range measured. As is shown in Fig. 5, Henderson-Hasselbalch plots of the DNS-BSA conjugates in aqueous solutions gave three successive straight lines, indicating the three dissociation characteristics, denoted by I, II, and III,

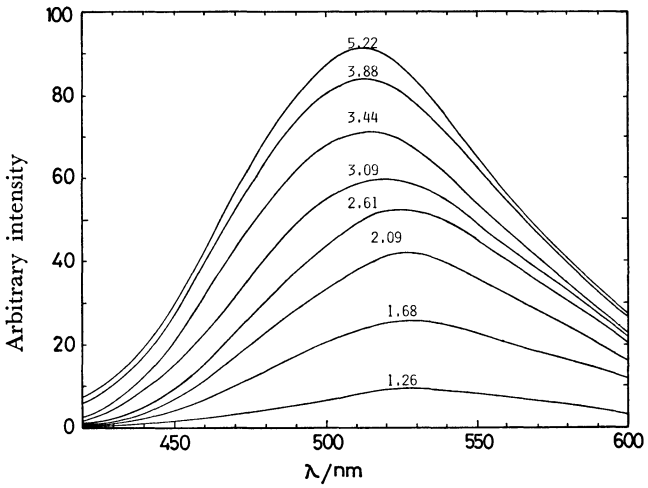


Fig. 4. Corrected emission spectra of 1.08×10^{-5} M solutions of DNS-BSA conjugates with the molar ratio of 1.2 mol probe to 1 mol protein in water at several pH. Curves are labeled with appropriate pH values.

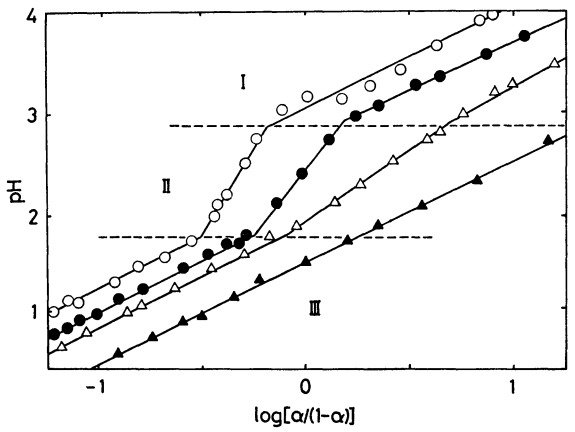


Fig. 5. Henderson-Hasselbalch plots of DNS-BSA conjugates with the molar ratio of 0.9 (○), 2.0 (●), 2.7 (△), and 7.2 (▲) mol probe to 1 mol protein. Two dashed lines divide the plots into three regions denoted by I, II, and III.

in the order of decreasing pH. The slopes were nearly one in I and III, but more than one in II: hence, the apparent pK_a values in both the I and III regions, as calculated from Eq. 5, were almost constant, but those in the II region were not. In the II region it is adjustable to a generalized Henderson-Hasselbalch expression with a parameter, denoted by n, of interaction among dissociation groups in a polyelectrolyte as follows:[10]

$$pH = pK_a + n \log \frac{\alpha}{1-\alpha}. \quad (6)$$

In this study, n may be attributed to the interaction between the DNS group and the other residues in the protein. We should note that the apparent pK_a in the II region obtained from Eq. 6 makes it difficult to discuss quantitatively the proton equilibria of DNS group conjugated to BSA, because the parameter, n, is physicochemically ambiguous in the process of the extension of the simple Maxwell-Portington equation,[11] as described by Katchalsky and Spitnik.[10] The apparent pK_a value in the II region is useless for comparison with the pK_a values in other regions; hence, the value has not been determined. In the case of the DNS-BSA conjugate with the molar ratio of 7.2, Henderson-Hasselbalch plots gave one straight line, from which the constant pK_a and a slope of 1.0 were calculated over the pH range measured. The results of the pK_a values and slopes in each region, summarized in Table 1, show the two following features. Firstly, the pK_a values in the I region are greater than those in the III region except in the case of the molar ratio of 7.4. Secondly, the pK_a values in the I region come close to those in the III region and the slope in the II region decreases to one with an increase in the molar ratio of the conjugates. The former may be attributed to the conformational change on acid denaturation, and the latter to the alternation of the protein by the conjugation of the probe, as was pointed out by Takagi *et al.*[12,13]

The α-helix contents of the DNS-BSA conjugates

Table 1. Apparent pK_a and slope n of DNS-BSA conjugates in water

Molar ratio[a]	Region I		Region II	Region III	
	n	pK_a	n	n	pK_a
0.9	1.0	3.05	3.5	1.0	2.30
2.0	1.0	2.70	2.6	1.0	2.05
2.7	1.0	2.18	1.5	1.0	1.90
7.4[b]	$n=1.0$, $pK_a=1.55$				

a) Moles of dansyl group conjugated to 1 mol of BSA.
b) The regions were indistinguishable in Fig. 5.

were calculated from the ORD measurement with the Moffitt-Yang equation in order to elucidate the alternation of the protein by the conjugation of the probes and the protonation of the ionizable groups in the protein. The α-helix contents of the conjugated proteins with the molar ratios of 0.7 and 5.2 were 46 and 28% in pH 5.0, and 28 and 23% in pH 2.0, respectively. In the unconjugated protein, the α-helix contents were 46% in pH 5.0 and 27% in pH 2.0. It can be seen from the ORD data that both the conjugation of the probe and the binding of the proton to the protein cause a lowering of the α-helix content of the native protein in a neutral solution. Because of the similar α-helix content of the conjugated protein even with the molar ratio of 5.2 in pH 2.0 and 5.0, the constant pK_a obtained for the conjugated protein with the molar ratio of 7.4 should be attributable to the impaired protein, which maintains its structure regardless of the protonation of the protein.

In the case of DNS-BSA, with an average molar ratio below 1.7, it can be seen that the proton equilibria of the DNS group present the same behavior, as is shown in Fig. 6. This finding implies that the BSA conjugated with DNS group with a small molar ratio consists of a conformation which is similar to that of unconjugated BSA, judging from the agreement in α-helix content between the unconjugated BSA and the conjugated BSA with the molar ratio of 0.7.

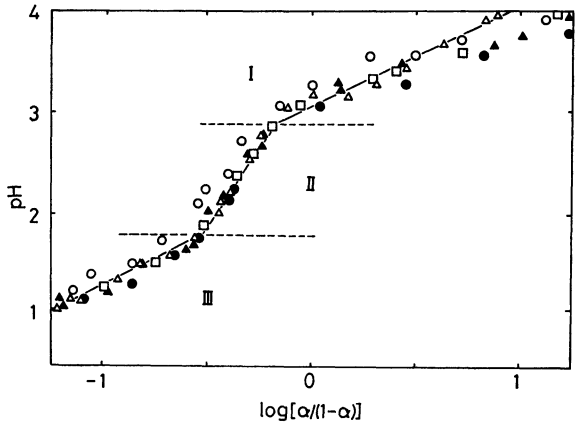


Fig. 6. Henderson-Hasselbalch plots of DNS-BSA conjugates with the molar ratio of 0.3 (○), 0.5 (●), 0.9 (△), 1.6 (▲), and 1.7 (□). Two dashed lines are drawn at the same position of pH values in Fig. 5.

Discussion

BSA undergoes a marked structure change in the region near pH 4, termed "N-F transition," from the normal state (N) to a form (F) having a faster rate of migration in electrophoresis.[14] Futhermore, the protein structure of the F-form is changed into a structure with an expanded form (E) in the region below pH 3.5.[15]

As is shown in Fig. 6, the pH value of the boundary between the I and II regions is 3.0, in agreement with the pH value beginning to occur upon the unfolding by means of the acid expansion. A possible explanation for the agreement may be that, in the I and III regions, the conjugated DNS group exists in two different environments, in the protein of the N- or F-form and in that of the E-form respectively, and that in the II region a transfer of the DNS group proceeds along with the expansion of the protein structure. As has been described in the Results section, the protein conjugated with the molar ratio below 1.7 showed the same behavior on acid denaturation as the unconjugated protein, so the variation in pK_a values against the pH depends only on the effect on the conformational change upon acid denaturation.

The fact that all of the pK_a values obtained for DNS-BSA are smaller than that for DNS-Gly, denoted by pK_o, shows that the protein environment has a profound effect on the ionizing abilities of the dimethylamino moiety. Such an effect can be expressed thermodynamically from the difference between pK_a and pK_o values in the following equation:

$$pK_a = pK_o - 0.434\frac{\Delta G_p}{RT}, \qquad (7)$$

where ΔG_p is the free-energy change resulting from the transfer of the probe from water to a inside of the protein in the solution of a pH value, and where pK_o is estimated for the free probe in the solution which consists of the same solute as in the determination for DNS-BSA; pK_o=3.99 in the present case. The left-hand side in Eq. 7, pK_a, can be calculated from the pH value of a solution and the fraction, α, in Eq. 5; hence, ΔG_p may be estimated. In Fig. 7 the experimental pK_a values are plotted against the pH values in the case of those of DNS-BSA in Fig. 6; the ΔG_p scale at 25.0 °C is indicated on the right-hand side.

If ΔG_p depends only upon the Debye-Hückel electrostatic shielding effect, Eq. 7 can be expressed by the following equation containing Linderstrøm-Lang's parameter:[18]

$$pK_a = pK_o - 0.434\frac{\Delta G_{el}}{RT}\bar{Z}_p, \qquad (8)$$

where ΔG_{el} is the electrical free-energy change for charging an assumed-spherical protein molecule uniformly with a total unit charge and where $\bar{Z}_p$ represents the mean net charge of the protein molecule. Although the values of $\Delta G_{el}/RT$, usually represented by 2ω, may be computed on the basis of the electrostatic theory,[19,20] we shall obtain ω as an experimental parameter and adopt the numerical values for it which have been found by Tanford, Swanson, and Shore to fit the potentiometric titration data.[19] We must also choose values for $\bar{Z}_p$ which are primarily due to a bound or dissociated proton, but which are also influenced by bound salt. In a rough approximation, however, it may be assumed that $\bar{Z}_p$ is equal to the number of protons bound by BSA the values of which can be computed from the data of Tanford, Swanson, and Shore.[19]

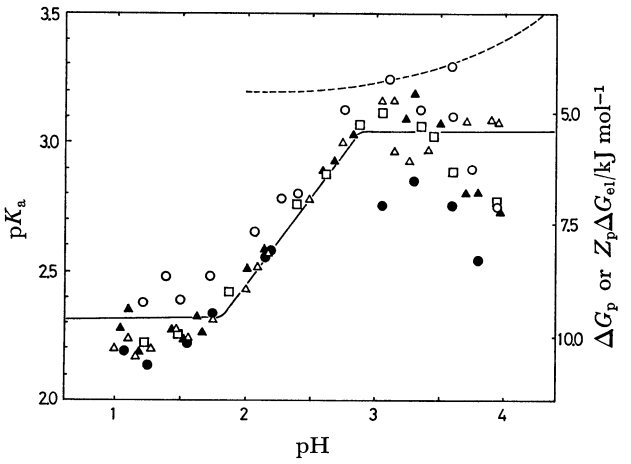


Fig. 7. Apparent pK_a values of DNS-BSA conjugates as a function of pH in aqueous solutions. The pK_a values in symbols and solid line were calculated from the data in Fig. 6 with Eq. 5. BSA conjugates have the molar ratio of below 1.7 mol probe to 1 mol protein. Symbols as in Fig. 6. Dashed line was estimated with Eq. 8 from data of Tanford, Swanson, and Shore.[19]

The calculated results are also illustrated in Fig. 7 for a solution with an ionic strength of 0.03. The experimental curves are shifted greatly from the curves computed on the basis of electrostatic considerations, especially in the lower-pH range. This feature suggests that ΔG_p is constituted not only of ΔG_{el} depending on long-range electrostatic force, but also of the free-energy changes proceeding from several short-range interactions, such as the hydrophobic effect, the hydrogen-bonding, and the alternation of the solvent structure by the intensive electrical field around the protein. Such interactions, resulting from the displacement between the experimental and computed curves, must be greatly changed with the conformational transition accompanying the acid expansion. As Klotz and Fiess have pointed out,[3] the dimethylamino group contributes its pair of electrons to a resonance with the aromatic ring; hence, it cannot be a good acceptor of a hydrogen-bonding. Thus, the difference between ΔG_p and ΔG_{el} is unlikely to involve the contribution of any hydrogen-bonding, and the short-range interactions must arise from some other causes.

Klotz and Fiess,[3] in their study of DNS-BSA similar to this paper, have found that the pK of the dimethylamino group is 1.67 when attached to BSA and 3.99 when attached to glycine. The DNS-BSA conjugates prepared by them, however, have the molar ratio

of 5.9 at which the conjugation causes an alternation of the protein structure because of our results that, at molar ratios of more than 1.7, the normal protein structure no longer maintains its structure, even in a solution near the isoionic point in the I region. They have preferred to interpret their results in terms of a postulated ice-like character of the hydration water in the vicinity of the nonpolar portions of the protein, including the DNS group. Such an environment would tend to stabilize the uncharged form of the dimethylamino group, as the creation of a charged group in place of the uncharged one would require some breakdown of the ice lattice. Thus, the factor of ice lattice is unlikely to contribute to the shift of ΔG_p.

Strauss and Vesnaver,[5] studying the acid-base equilibria of the DNS group conjugated to a copolymer of maleic acid and butyl vinyl ether, have found that the pK values of the dicarboxylate groups and of the dimethylamino group of the probe pass through a maximum as the macromolecule undergoes a transition from a compact to a random-coil conformation. These results have been interpreted in terms of changes in the local solvent surrounding the probe, taking into account both the electrostatic forces due to the negative carboxylate groups and the influence of the nonpolar or hydrophobic butyl groups. The former effect is acid-weakening, and the latter, acid-strengthening, with regard to the $(CH_3)_2NH^+$ group.

In the case of DNS-BSA, BSA can be regarded as a polycation instead of the polyanion studied by Strauss and Vesnaver,[5] so the electrostatic and nonpolar environment effects would reinforce one another, both acting to increase the acid strength of the probe. The integrity of the albumin internal structure may be essential for both the effects, possibly as a consequence of the environment of the DNS group in nonpolar or hydrophobic regions of the intact protein. Such a hydrophobicity would favor the neutral basic form of the dimethylamino group over the ionic acidic form. The cationic charges in $\bar{Z}_p$ neighboring the hydrophobic environment have an intensive electric shielding effect in the inert protein involving the probe compared with that in bulk water.

On account of the hydrophobic residues in the protein, the charges formed by proton binding on the protein are considered to have interesting effects on the pK_a values. The effects of the charges should be investigated further in the proton equilibria of DNS-BSA in aqueous solutions of electrolytes and/or denaturants.

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8) The value was identical with the pK_a value reported for DNS-Gly by Klotz *et al.*[3]
9) The protonated form of the DNS group in an excited state should not be included because the energy level of the unprotonated form in an excited state is lower than that of the protonated form in an excited state, judging from the observation of the absorption and emission spectra. This makes the energy transfer from the unprotonated to the protonated form in the excited state very unlikely.
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