IONIC EQUILIBRIA IN A PROTEIN CONJUGATE OF A SULFONAMIDE TYPE

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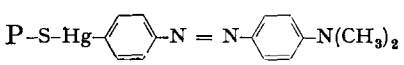
SUMMARY

The acidity constants of the $(CH_3)_2N$-group of 5-dimethylamino-1-naphthalene sulfonyl conjugates of glycine and of bovine serum albumin have been compared. In the native protein environment, pK_a is shifted by over 2.3 pH units. Denaturation of the protein by 8 *M* urea reduced the shift in pK_a to 0.9 unit. These and related observations are interpreted in terms of changes in the nature of the hydration lattice of the protein molecule.

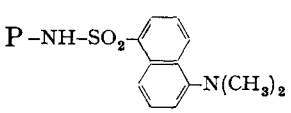
INTRODUCTION

It has been shown recently[1,2] that some interesting features of the behavior of protein molecules may be followed in studies of the ionization equilibria of artificially conjugated complexes. The conjugate described previously was prepared from an azomercurial which combined with the protein through mercaptan side chains (I):



(I)

The use of a mercaptide linkage, however, confines one to combinations with sulfhydryl-containing proteins, a limitation which would rule out studies of many interesting systems. To extend the range of the approach, therefore, we have examined some conjugates depending on an $-SO_2-$ linkage (II), which allows us to form a bond with a lysine (or perhaps serine) residue:



(II)

The specific compound used, 5-dimethylamino-1-naphthalene sulfonyl chloride, was suggested by the work of WEBER[3] and of others[4,5], who showed that this substance combines readily with several proteins under very mild conditions. The ionic equilibria of the $-N(CH_3)_2$-group in the conjugate so produced, (II), can be followed readily through changes in the absorption or the fluorescence of radiation. With conjugate (II), as with (I), acid-base equilibria have been found to change strikingly in the environment of a protein molecule.

EXPERIMENTAL

Preparation of conjugates

A solution of bovine serum albumin was prepared containing 80 mg of protein in 20 ml of 0.1 *M* Na_2HPO_4. To this cooled solution was added 1 ml of a cold acetone solution containing 2 mg of 5-dimethylaminonaphthalene-1-sulfonyl chloride. The mixture was kept in a refrigerator for 24 h. It was then placed in cellophane tubing and dialyzed in the cold first, for 18 h with shaking, against 0.1 *M* NaCl, secondly for 31 h against three successive portions of 0.01 *M* acetic acid and finally for several days against distilled water. At each stage the fluorescence intensity of the solution outside the dialysis bag was measured. It declined to a negligible value in the final dialysis, the amount coming off in the last stage representing less than 0.3 % of the dye originally added.

The moles of dye bound to the protein were computed from absorbance measurements at 340 mμ, plus the assumption that the extinction coefficient of $3.36 \cdot 10^6$ cm^2/mole found by HARTLEY AND MASSEY[6] for the chymotrypsin-dye conjugate is also applicable to albumin-dye conjugates. Preparations containing 4–11 moles of dye/mole of protein were obtained.

The glycine-dye conjugate was prepared by Mr. E. C. STELLWAGEN following the procedure of HARTLEY AND MASSEY[6] with minor alterations. The coupling was carried out in 0.5 *M* Na_2HPO_4 instead of 0.5 *M* NaOH. The twice recrystallized product had a m.p. of 158–9°, a yellow fluorescence, an absorption peak at 325 mμ at pH 9.1, and an R_F value of 0.81 in the butanol–formic acid–water solvent system of HARTLEY AND MASSEY[6]. All of these properties, except the absorption peak, agree with those reported[6]. The small discrepancy in position of the absorption peak is probably due to a typographical error.

Titration procedure

A portion of the solution of protein conjugate was diluted with distilled water to give a solution with an O.D. at 340 mμ of 0.3–0.4. The pH was then measured, and the absorption spectrum obtained over the region of 280–400 mμ. A portion of this solution was weighed to within 0.01 g, and then a measured quantity of hydrochloric acid was added from a microburet. The pH and spectrum of the solution were obtained. The procedure was repeated with further samples of solution and different quantities of acid (or base) until enough data had been collected to provide a smooth spectrophotometric titration curve. Suitable corrections were made for dilution of the solution by added acid or base.

The fraction, α, of the conjugate dye in the acid form was calculated by means of the equation[1]

$$\alpha = \frac{\text{O.D. soln. } a \text{ — O.D. basic soln.}}{\text{O.D. very acid soln.—O.D. basic soln.}} \qquad (1)$$

The pH's were measured with a Beckman meter model G.

Materials

Bovine serum albumin was a crystallized sample purchased from Armour and Co. The 5-dimethylaminonaphthalene-1-sulfonyl chloride was a purified grade obtained from the California Foundation for Biochemical Research. Glycine was C.P. grade of

H.M. Chemical Co. All acids, bases and salts were reagent grade. Acetone was Eastman Kodak spectroscopic grade.

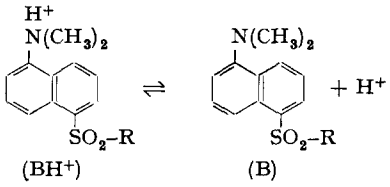
Optical measurements

The absorption of light was measured with the Beckman spectrophotometer, model DU, at about 25°. Fluorescence intensities were obtained with a fluorometric attachment to the Beckman spectrophotometer as described by FLETCHER, WHITE AND SHEFTEL[6a]. A solution of 2 mg of quinine sulfate in 100 ml of water was used as the reference.

RESULTS AND DISCUSSION

Computation of acidity constants

The equilibrium being studied may be represented by the equation



(2)

If R represents a small molecule, such as a glycine residue, $-NHCH_2COO^-$, then this dissociation can be described quantitatively by the familiar simple mass-law expression in logarithmic form,

$$pH = pK + \log \frac{(B)}{(BH^+)} \tag{3}$$

A more convenient graphical representation of this equilibrium is a plot of α, the fraction of conjugate in the acid form BH^+, *vs.* the pH. A graph for the glycine conjugate is illustrated in Fig. 1. At the midpoint, $\alpha = 0.5$, $(BH^+) = (B)$, and hence the pK of the dimethylamino group is readily determined as 3.99 for the naphthalenesulfonylglycine conjugate.

When the dye is attached to a protein, eqn. (3) is no longer adequate to represent the ionization. Since the protein molecule carries a charge which varies with pH, the uptake of protons by the $(CH_3)_2N$-group will be affected electrostatically[7–9]. This electrostatic influence is generally treated by insertion of an additional term in eqn. (3) to give

$$pH = pK + \log \frac{(B)}{(BH^+)} - 0.868 \frac{\Delta F_{el.}}{RT} \overline{Z}_P \tag{4}$$

where ΔF_{el} is the electrical free energy change (per mole) for charging an assumed-spherical protein molecule uniformly with a total of unit charge and $\overline{Z}_P$ represents the mean net charge of the protein molecule.

Values of $\Delta F_{el}/RT$, usually represented by w, may be computed from electrostatic theory[7–9]. However, since it has been clearly shown that such values cannot be used (without additional *ad hoc* assumptions) to describe the titration behavior of bovine serum albumin in acid solutions, we shall merely consider w as an experimental parameter and adopt numerical values for it which have been found by TANFORD, SWANSON AND SHORE[7] to fit the electrometric proton titration data.

To apply eqn. (4) to our spectrophotometric titrations of the albumin-dye conjugate, we must also choose values for $\overline{Z}_P$. To a first approximation we might

assume that $\bar{Z}_P$ in solutions acid to the isoionic point is equal to r_H, the number of protons bound by serum albumin as we proceed from the isoionic point to the given pH. Values of r_H can be computed from the data of TANFORD, SWANSON AND SHORE[7]. Thereafter, if we adopt the assumption that the *intrinsic* affinity of the $(CH_3)_2N$-group

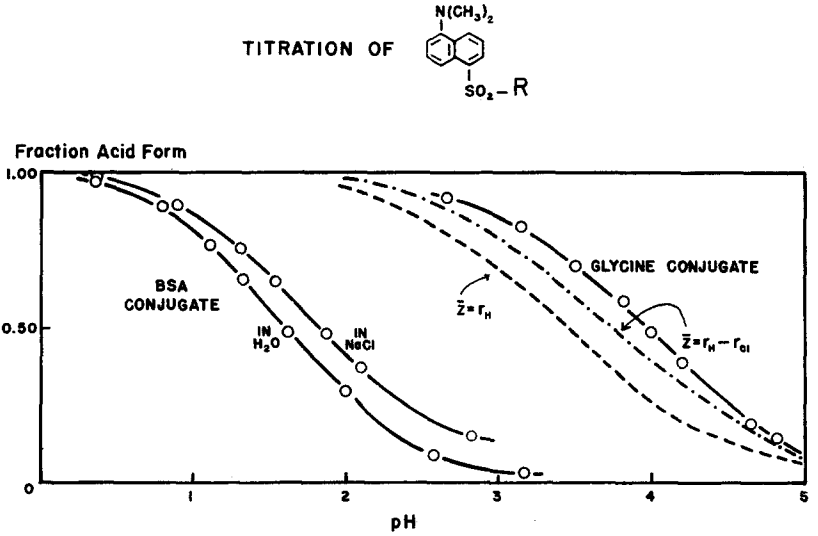


Fig. 1. Optical titrations of 5-dimethylaminonaphthalenesulfonyl conjugates of bovine serum albumin and glycine, respectively. Circles represent experimental points; ——— represents curve computed theoretically for the $-N(CH_3)_2$ group on serum albumin whose net charge $\bar{Z}$ equals r_H, the number of protons bound from the isoionic point to the given pH; — — — — represents theoretical curve correcting $\bar{Z}$ for the moles of anion, r_{Cl}, bound by the protein at the given pH. The theoretical curves are for protein in solutions of 0.1 ionic strength.

is the same whether the dye is attached to protein or to a small molecule such as glycine, we can compute readily (B)/(BH$^+$) and hence a. Fig. 1 shows a titration curve calculated in this way for solutions with an ionic strength $\mu = 0.1$.

An alternative computation might also be made which takes cognizance of the fact that serum albumin binds anions, such as chloride, which are present during the titration. Thus we should write $\bar{Z}_P = r_H - r_{Cl}$, where r_{Cl} is the number of moles of chloride bound/mole of protein at the pH being considered. Some rough estimates of r_{Cl} may be made from the experiments of SCATCHARD, COLEMAN AND SHEN[8]. Again a titration curve for the albumin-dye conjugate may be calculated; this result also is illustrated in Fig. 1, again for solutions with $\mu = 0.1$.

Experimentally observed titration curves for a dye-albumin conjugate (containing a 5.9:1 dye to protein ratio) are also shown in Fig. 1. A small difference* is observed between the curve in the presence of 0.1 M NaCl and that in the absence of salt. Nevertheless both curves are displaced by a striking amount from the curves computed from electrostatic considerations.

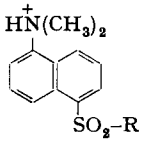
For purposes of comparison we may define[1] as pK_a for the protein conjugate that

* The shift in pK_a of the protein conjugate in salt as compared to water is in the correct direction and of about the proper magnitude if the experimental values of TANFORD, SWANSON AND SHORE[7] are used for w and if the intrinsic pK_a of the $-N(CH_3)_2$-group is assumed to be the same in both solutions. However, this intrinsic pK_a is far lower than that for the glycine conjugate of the dye.

pH at which (B) = (BH^+), that is the midway point in the graph of α *vs.* pH (Fig. 1). Values of pK_a computed in this way are listed in Table I.

A few experiments were also run with conjugates containing different ratios of bound dye to protein. These are summarized in Table II. There is a slight upward trend in pK_a's in water with increasing mole ratio of dye: protein, but the differences are too small to be considered significant.

TABLE I

ACIDITY CONSTANTS OF  AT 25°

R	*Solution*	pK_a
$-NHCH_2COOH$	Water	3.99
	8 *M* urea	4.18
–OH	Water	4.27
–Bovine serum albumin (5.9/1)*	Water	1.67
	0.1 *M* NaCl	1.86
	0.1 *M* NaSCN	2.05
	8 *M* urea	3.27

* The numbers within the parentheses refer to the (average) number of dye molecules attached to each protein molecule.

TABLE II

VARIATION OF ACIDITY CONSTANTS WITH MOLE RATIO OF DYE: SERUM ALBUMIN IN CONJUGATE

Dye: protein	pK_a
4.1	1.60
5.7	1.64
5.9	1.67
10.9	1.70

Nature of influence of protein environment on pK_a

The fact that the pK_a of the $(CH_3)_2NH^+$- group is 1.67 when it is attached to the protein and 3.99 when it is on a small molecule in water shows that the protein environment has a very profound effect on the ionizing abilities of this group. The analysis outlined above indicates strongly that electrostatic effects are not responsible for this behavior. For several reasons, it seems unlikely, furthermore, that hydrogen-bonding between $(CH_3)_2\ddot{N}$- and a hydrogen donor group causes the shift in pK_a. The $(CH_3)_2\ddot{N}$- substituent of an aromatic ring contributes its pair of electrons to resonance with the ring and hence is not a good receptor of an H-bond. From the hydrogen-bonding viewpoint one would expect a much greater shift in the pK of $-COO^-$ groups of a protein since these are much stronger H-bond acceptors. In addition, in the conjugate of type (I), spectroscopic behavior is inconsistent with that observed[1] for related systems where hydrogen-bonding is present. Also as has been pointed out previously, it is difficult to see from a hydrogen-bonding viewpoint, why the addition of a long-chain anion should lower the pK_a of (I) even further.

As has been suggested earlier, the displacement of the titration curve of conjugate II (Fig. 1) toward lower pH's, *i.e.* toward greater H^+-ion concentrations, may be thought of advantageously as a "masking" effect. Thus a denaturing agent, such as urea, which can reveal naturally-occurring masked groups, also removes most of the unusual behavior of the artificially-produced masked groups. As is evident from Table I, the addition of urea to conjugate II raises pK_a to 3.27, a very substantial return toward the value of the dissociation constant of the $(CH_3)_2NH^+$-group attached to a small molecule. The effect of urea on conjugate II thus is similar to that on I.

Since a H^+–ion can rapidly reach the $(CH_3)_2N$- group on the protein, we have assumed that an aqueous passage is available to the proton, but since the basicity of the dimethylamino group is so different when it is attached to the protein, we have reason to assign some special properties to the water in the immediate neighborhood of the macromolecule. In addition to much evidence cited previously[1,2] for the assumption of a special ice-like character of regions of the hydrate water of proteins, we might also point to the isolation and structure determination of many crystalline hydrates of non-polar molecules[10,11], such as those of CH_4, C_3H_8, $(CH_3)_2O$, $(CH_3)_3CH$, C_6H_6, C_3H_7Br, CH_3SH, etc. It is apparent that a wide variety of non-polar molecules are capable of stabilizing water in crystalline structures containing large cavities (5–7 Å in diameter). In the absence of the non-polar molecules these structures would be unstable with respect to normal ice.

Such a direct demonstration of "icebergs" around non-polar molecules is not possible for dissolved solutes, but further indirect evidence is available. Among recent papers one might cite two dealing with thermal measurements. It has been shown by FRANK AND WEN[12], for example, that the apparent molal heat capacity, φC_{p_2}, of tetrabutylammonium ion in water solution is about 150 cal $mole^{-1}$ deg^{-1} higher than one might expect from additivity rules. Similarly GODDARD, HOEVE AND BENSON[13] have found that the heat capacity of octanoate ion is some 70 cal $mole^{-1}$ deg^{-1} higher in aqueous solution than one would expect from the behavior of the hydrocarbon chain in the liquid state. In both cases, the excess heat capacity has been attributed to the effect of the organizing of a solvent structure around the non-polar solute molecule. With a rise in temperature, some heat must be absorbed in the progressive thermal disorganization of the hydrate ice; it is this heat which contributes to the unexpectedly large $\bar{C}_{p_2}$ of these non-polar solutes.

In a protein molecule many non-polar side-chains exist in juxtaposition. Thus there could be a "coupling" of the ice lattices formed around each residue with a consequent strengthening of the resultant hydrate structure. It is of interest in this connection to recall the crystalline double-gas hydrates described by v. STACKELBERG AND MÜLLER[10] in which two non-polar molecules act cooperatively in stabilizing the aqueous crystal to a degree substantially greater than that possible with one type of solute molecule. The structure of the lattice formed by coupling of hydration water of protein side-chains may not be the same as that of the gas hydrates but the possibilities for cooperative interaction in the macromolecule should be even greater since the residues are even initially relatively fixed in position by the framework of the protein molecule.

The presence of patches or ribbons of ice-like coverings around various regions of a protein molecule would have both kinetic and equilibrium consequences.

Rates of diffusion of essentially all substances to a reactive site under an ice-layer

would be greatly reduced. For ions the relative rates of diffusion in the presence and absence of the hydrate might be estimated from the values found by EIGEN AND DE MAEYER[14] for Li^+ in ordinary ice and water, $< 10^{-8}$ *vs.* $4 \cdot 10^{-4}$ cm^2 $volt^{-1}$ sec^{-1}. On this basis one can readily understand why Ag^+ titrates very slowly with –SH-residues in many proteins. Judging from the observation that mercaptans form crystalline hydrates[10], we might reasonably conclude that –SH-groups in proteins could participate cooperatively with neighboring side-chains in the formation of an ice patch. Diffusion of Ag^+ through this ice patch would be greatly hindered as compared to the movement of this ion in ordinary water. Consequently the protein mercaptan acts as if it were masked.

Hydrogen and hydroxyl ions, in contrast to all other substances, encounter no difficulties in traversing ice[14] because of the unique mechanism by which they can be transported. In the present studies of reaction (2), however, it is the equilibrium and not the rate* of ionization which is affected when the $(CH_3)_2N$-group is attached to protein. The shift in pK_a toward lower values means in essence that the environment is more favorable to the $(CH_3)_2N$ rather than to the $(CH_3)_2NH^+$-group. Such a bias seems very reasonable, for the creation of a charged group in place of the uncharged one would require some breakdown of the ice lattice of the protein. It is this requisite disorientation of the hydration lattice which obstructs the formation of the acid form of the amine.

Thus the behavior of the sulfonamide conjugate (II) parallels that of the mercaptide conjugate (I) studied previously[1,2]. Furthermore, in both systems, the ionization equilibria can be understood in terms of interactions of the protein, serum albumin, with solvent molecules. With this broadening of the range of available conjugates it should now be possible to compare the behavior of a series of proteins of different sizes and shapes.

ACKNOWLEDGEMENT

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REFERENCES

1 I. M. KLOTZ AND J. AYERS, *J. Am. Chem. Soc.*, 79 (1957) 4078.
2 I. M. KLOTZ, *Science*, 128 (1958) 815.
3 G. WEBER, *Biochem. J.*, 51 (1952) 155.
4 R. M. CLAYTON, *Nature*, 174 (1954) 1059.
5 V. MASSEY, W. F. HARRINGTON AND B. S. HARTLEY, *Discussions Faraday Soc.*, 20 (1955) 24.
6a M. H. FLETCHER, C. E. WHITE AND M. S. SHEFTEL, *Ind. Ang. Chem., Anal. Ad.*, 18 (1946) 204.
6 B. S. HARTLEY AND V. MASSEY, *Biochim. Biophys. Acta*, 21 (1956) 58.
7 C. L. TANFORD, S. A. SWANSON AND W. S. SHORE, *J. Am. Chem. Soc.*, 77 (1955) 6414.
8 G. SCATCHARD, J. S. COLEMAN AND A. L. SHEN, *J. Am. Chem. Soc.*, 79 (1957) 16.
9 J. T. EDSALL AND J. WYMAN, *Biophysical Chemistry*, Academic Press, Inc., New York, N.Y., 1958, Vol. I, pp. 512–518.
10 M. V. STACKELBERG AND H. R. MÜLLER, *Z. Elektrochem.*, 58 (1954) 25.
11 M. V. STACKELBERG AND B. MEUTHEN, *Z. Elektrochem.*, 62 (1958) 130.
12 H. S. FRANK AND W.-Y. WEN, *Discussions Faraday Soc.*, 24 (1957) 133.
13 E. D. GODDARD, C. A. J. HOEVE AND G. C. BENSON, *J. Phys. Chem.*, 61 (1957) 593.
14 M. EIGEN AND L. DE MAEYER, *Proc. Roy. Soc. (London) A*, 247 (1958) 505.

* No special effort was made to examine the rate of proton uptake by fast-reaction techniques Within the few minutes required for mixing the solutions and taking an O.D. reading, equilibrium was attained.