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Excited-State Proton Transfer in Hydroxynaphthaldehydes Covalently Bound to Proteins

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The kinetics and equilibrium of excited-state proton transfer (ESPT) in 2-hydroxynaphthaldehyde-1 (HNA-2.1) bound to proteins (bovine serum albumin, cytochrome *c*, and lysozyme) by an alkylamino bond was studied by means of fluorimetric steady-state and time-resolved methods. The results were compared to analogous data for 1-hydroxy naphthaldehyde-4 (HNA-1.4) bound to proteins and for other 2-naphthol derivatives bound to proteins by a sulfonamide bond. Conclusions concerning the influence on ESPT of the mode of binding and of intramolecular hydrogen bonds occurring in the case of HNA-2.1 were drawn. An intramolecular hydrogen bond enhances the rate of ESPT but the molecular environment in the protein leads to an opposite effect by increasing reorganization energy during proton transfer. The results obtained prove that the mode of binding and the kind of group linking fluorophores to proteins influence considerably the rate and mechanism of ESPT. In naphthol groups bound to proteins by an alkylamino bond, proton dissociation depends strongly on the molecular environment in the macromolecule. This is due to the short length of the alkylamino bridge and its small interaction with electronic orbitals of the aromatic system. Fluorophores bound to proteins by a sulfonamide bond show a higher rate of ESPT, which is due partly to the electron withdrawing effect of the linking arm. The efficiency of ESPT for naphthol groups bound to proteins by a sulfonamide bond is, in most cases, sufficient for acidification of the medium and influence of the proton gradient in biological membranes.

KEY WORDS: Excited-state proton transfer; hydroxynaphthaldehydes; proteins.

INTRODUCTION

Excited-state proton transfer (ESPT) in fluorophores absorbed on the protein surface or bound chemically to macromolecules has been studied with several spectrofluorimetric techniques [1–5]. Compounds undergoing ESPT have been used for rapid local acidification of biological samples [3,4]. For 2-naphthol derivatives bound to proteins by a sulfonamide bond, it has been shown that illumination of a sample in a neutral water solution with a light beam of a suitable wavelength range causes efficient acidification of the medium due to ESPT reaction [6–9]. It is known that proton gradients on cell membranes and proton transfer play a very essential role in many biological processes, especially in biological energy transformation systems [10]. Therefore it can be supposed that a fluorophore undergoing ESPT—if it was bound selectively to a suitable site on the energy transforming membrane—can exert a strong biological effect which would be controlled by the incident light intensity. Essential for proper application of this phenomenon is suitable localization of the fluorophore and efficiency of the ESPT reaction.

The scope of the present work is to recognize the optimal conditions for modification of proteins by 2-naphthol (2-Np) derivatives and for ESPT reaction in groups bound to proteins. ESPT in 2-Np groups bound

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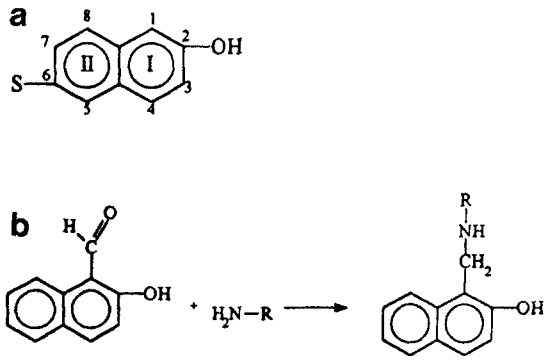
Table I. Calculated Changes in Electronic Charge Density During Proton Transfer in 1- and 2-Naphthol Analogues with Electron Withdrawing Substituents in the Ground (G) and Excited (E) States

Substituents in naphthalene	DQ I		DQ II		DQ O		DQ S	
	G	E	G	E	G	E	G	E
2-OH	−0.210	+0.006	−0.303	−0.540	−0.326	−0.147		
6-F, 2-OH	−0.212	+0.015	−0.276	−0.510	−0.319	−0.150	−0.033	−0.037
6-Cl, 2-OH	−0.208	+0.021	−0.252	−0.489	−0.318	−0.146	−0.063	−0.072
6-Cl, 2-OH (d)[a]	−0.186	+0.149	−0.208	+0.185	−0.301	−0.252	−0.145	−0.818
6-SO_3H, 2-OH	−0.194	+0.054	−0.218	−0.385	−0.340	−0.184	+0.095	−0.106
1-OH	−0.234	+0.043	−0.228	−0.827	−0.313	−0.191		
4-OH_3, 1-OH	−0.153	−0.466	−0.183	−0.581	−0.305	+0.112	−0.070	−0.076

[a] Taking into account 3*d* orbitals.



Scheme I. (a) 2-Naphthol derivatives for protein modification. S, substituent. (b) Modification of proteins by formation of an alkylamino bond.

to proteins by an alkylamino bond is compared to an analogous process in 2-Np bound by a sulfonamide bond. It can be predicted that the sulfonamide group—because of its electron withdrawing power—would favor ESPT. This supposition is quantitatively verified by our calculation results presented in Table I.

QUANTUM CHEMICAL CALCULATIONS

Quantum chemical calculations for 1-naphthol and 4-methyl 1-naphthol, as analogues of naphthol groups bound to proteins by an alkylamino bond, and 2-naphthol, 6-chloro 2-naphthol, 6-fluoro 2-naphthol, and 2-naftolo-6-sulfonic acid as analogues of 2-naphthol groups bound to proteins by a sulfonamide bond were performed as described in Ref. 11. Unfortunately no calculations for the sulfonamide group as a substituent in napthalene could be performed.

The electronic charge density in a given atom in naphthalene derivatives in the protonated (ROH) form was substracted from that in the dissociated (RO^-) form. These difference values were summed up for atoms in ring I (DQ I; Scheme Ia) in ring II (DQ II) in substituent (DQ S) and in phenolic oxygen (DQ O). These results are shown in Table I.

It follows from these data that in 2-naphthol derivatives with electron withdrawing substituents (e.g., Cl), the ESPT reaction is accompanied by migration of the electronic charge from ring I to a substituent. It must be noted here that the Cl (with 3*d* orbitals) atom is a better analogue of the sulfonamide group simulating its electron withdrawing properties than the charged SO_3H^- group. No such electronic charge migration takes place in the case of 4-methyl 1-naphthol as an analogue of 1-naphthol bound to proteins by an alkylamino bond in position 4. Such electronic rearrangements can influence considerably the proton transfer in the case of 2-Np groups bound to proteins by a sulfonamide bond. This effect may enhance the rate of proton transfer and the dependence of proton transfer on the polarity of the environment and induce another variation in the mechanism of this reaction. Therefore a comparison of excited-state proton transfer in 2-Np groups bound to proteins by a sulfonamide bond and by an alkylamino bond, where no such electronic migration during proton transfer takes place, is necessary for understanding the influence of a linking arm on the ESPT reaction in 2-Np groups bound to proteins.

MATERIALS AND METHODS

Syntheses. Hydroxynaphthaldehyde 2.1 (HNA-2.1) was synthethized as described in Ref. 12. The product crystallized from methanol had a m.p. = 77–78°C and was identified by mass spectroscopy and IR. 1-Hydroxynaphthaldehyde-4 (HNA-1.4) was synthethized accord-

ing to Ref. 13. The product was identified by its absorption spectrum in aqueous methanol (UV 212, 236, 242, 337 nm [13]).

Labeling of Proteins. Proteins used for modification were commercial preparates—bovine serum albumin (BSA; molecular weight, 68,000) and cytochrome *c* (CytC; MW 12,380; from horse heart type 01)—supplied by Sigma (USA). Lysozyme (Lyso; MW, 14,300) was from C. Roth (Germany).

Five milligrams of HNA-2.1 or HNA-1.4 was dissolved in 0.3 ml of dimethylformamide (DMF) and added in small portions, alternately with solid $NaBH_4$, to a stirred and cooled solution of BSA (1.5 μmol) in 3 ml of 0.066 *M* phosphate buffer, pH 7.5 [14]. In the case of labeling of CytC and lysozyme, HNA-2.1 was dissolved in ethanol or tetrahydrofurane and some diethyl ether was added to prevent foam formation. During the modification of lysozyme, some tendency to precipitation of the protein was noticed. In such a case the addition of aldehyde was stopped. This procedure yielded a preparate of modified lysozyme (Lyso-HNA-2.1 preparate A) of a very low modification degree. Therefore in another case the addition of HNA was continued, which caused the precipitation of a large part of the material, but some part (about 40%) remaining in solution showed a much higher degree of modification (Lyso-HNA-2.1 preparate B). After addition of the reagent the reaction mixture protected from light was stirred at room temperature during 12 h. The solution was then applied to a Sephadex G-25 (30 × 800 mm) column equilibrated with 0.01 *M* ammonium carbonate (pH 8.7). The elution was monitored spectrophotometrically. For comparison sake, lysozyme was also modified by a succinimidyl ester of 6-hydroxynaphthalene 2-sulfonamide of α-L-phenylalanine (NPSE) during 20 min. The detailed procedure for lysozyme modification by succinimidyl ester was described elsewhere [8]. In this preparate (Lyso-SE) the 2-Np group is bound to the protein by a sulfonamide bond. Lyso-SE was characterized by a higher degree of modification than Lyso-HNA(B), but in obtaining Lyso-SE no marked precipitation of protein was observed, though at a 0.9 molar ratio of NPSE to protein, some turbidity of the solution was noted and the addition of the reagent was stopped. The labeled proteins were lyophilized and stored at 5°C.

Spectroscopic Measurements. Spectrophotometric and spectrofluorimetric (steady-state) measurements were performed as described elsewhere [6–8]. Lifetime determinations were performed by means of an SLM Aminco 48000S phase and modulation instrument using a 450-W xenon lamp as a light source and a frequency range of 1–250 MHz. The efficiency of modification, equilibrium constants in the ground state (K_a), rate constant for ESPT (k_{PT}), and accessibility (f_1) of the fluorophore groups to the solvent were determined as described elsewhere [6,7]. Equilibrium constants in the excited state (K_2^*) were determined according to the Foerster cycle [15] by the equation

$$pK_a^* = pK_a - (\nu_{00}^{ROH} - \nu_{00}^{RO^-})\, Nhc/RT = pK_a - (\nu_{00}^{ROH} - \nu_{00}^{RO^-})\, 0.625/T \quad (1)$$

where the frequency of transition between zero oscillation levels (V_{00}) of the ground and excited state for ROH and RO^-, forms of HNA groups were determined as the arithmetic mean of absorption and emission maxima of a given form, *N*, *h*, and *c* are universal constants, and *T* is the absolute temperature.

Mass spectroscopic measurements were performed by means of a Finnigan TSQ 700 instrument with an electrospray ionization source.

RESULTS

Characteristics of Modified Proteins

In the absorption spectra of all protein–HNA conjugates, a band in the range of 310–360 nm appears which must be ascribed to HNA bound to macromolecules. The degree of modification, negative logarithms of dissociation constant in the ground (pK_a) and excited (pK_a^*) states, accessibility of the fluorophore to solvent, and fluorescence quantum yields characterising HNA chromophores bound to proteins are given in Table II.

The efficiency of modification for all preparates studied by HNA derivatives was lower than 1. In the mass spectra of Lyso-HNA-2.1 (preparate B) (Fig. 1a) and CytC-HNA-2.1 (not shown), the band corresponding to the molar mass of the protein plus one HNA molecule bound by an alkylamino bond (156; error, <0.05%) was found. Mass spectra of BSA-HNA-2.1 and BSA-HNA-1.4 were not subjected to mass spectroscopic measurements. Comparing the intensity of the band of protein modified by one HNA group to that of the mother ion of the protein, one obtains modification degrees very near those calculated from absorption data (Table II).

This verifies our assumption of the mode of binding of HNA to proteins by an alkylamino bond. It also follows from the mass spectra that the number of protein molecules labeled by more HNA groups (two, three, etc.) is much lower than that with one marker molecule.

In the mass spectra of Lyso-HNA-2.1 (A, B) and CytC-HNA-2.1 but not of Lyso-SE (Fig. 1b), an addi-

Table II. Characteristics of Protein HNA Conjugates

Modified protein	pK_a	pK_a^*	Accessibility f_i	Fluorescence quantum yield Φ_0	Modification degree, $C_{HNA}/C_{protein}$
BSA-HNA-2.11	pK_{a1} = 10.0 pK_{a2} = 5.2	3.4	0.7	0.12	0.2
BSA-HNA-1.4	9.9	1.5	0.6	0.11	0.15
CytC-HNA-2.1	11.7	2.9	0.4	0.07	0.15
Lyso-HNA-2.1 (A)	9.5	3.0	0.7	0.1	0.05
Lyso-HNA-2.1 (B)	9.3	3.6	0.500	0.2	0.3
Lyso-SE	9.25	−0.4	0.4	0.25	1.3

tional band is present, the position of which depends on the kind of solvent used in the modification reaction. These bands are probably to be attributed to side reactions of protein with solvent during modification. It is concluded that for efficient modification, a very large excess of HNA would be necessary; however, it cannot be attained because in all cases further addition of the reagent caused precipitation of a protein.

Absorption Spectra

Absorption spectra of BSA-HNA-2.1 and Lyso-HNA-2.1 (B) are represented in Figs. 2 and 3. The spectra of other preparates studied are analogous.

The absorption spectrum of BSA-HNA-2.1 at pH $< pK_a$ (pH 6.5; Fig. 2) in the range 310–360 nm, where total absorbance must be attributed to the HNA group, is similar to that of 2-naphthol. In the spectrum of Lyso-HNA-2.1 (B) at pH 7 (Fig. 3), besides the absorption band of the protonated (ROH) form of HNA at 320–360 nm, some weak absorption at 360–370 nm is also observed, which must be ascribed to the dissociated (RO^-) form of HNA. Such an effect is not visible in the absorption spectra of other preparates studied and is probably due to the existence, in the ground state of the fluorophore, of some phenolate (RO^-) species of HNA bound to lysozyme with proton transferred along the hydrogen bond to an acceptor. The occurrence of such forms of intramolecularly hydrogen bonded substances at pH $< pK_a$ in strongly polar media is not exceptional [16,17]. In the case of HNA-2.1 bound to a protein by an alkylamino bond, several forms with an intramolecular hydrogen bond (Scheme II) are possible.

Spectrophotometric titration curves for CytC-HNA-2.1 and for BSA-HNA-1.4 (not shown) have an approximately normal appearance, with one titration threshold corresponding to the equation $pH = pK_a + \log(C_{ROH}/C_{RO}^-)$. For BSA-HNA-2.1 two ionization steps can be detected (Fig. 4), one at pH 4–5 and the other at pH 6–12, but the shape of the titration curve does not allow us to exclude another titration step at pH 7. It follows from these data that in BSA-HNA-2.1 (in contrast to BSA-HNA-1.4), at least two HNA forms differing in acidity are present. This effect may be due to the influence of the environment and/or of intramolecular hydrogen bonding. For Lyso-HNA-2.1 (Fig. 5), the spectrophotometric titration suggests that more than two HNA forms with overlapping pK_a values in the range 3–12 are present in the ground state of the chromophore. Apparent pK_a values for Lyso-HNA-2.1 obtained graphically from spectrophotometric titration and pK_a^* values using Eq. (1) are to be treated as mean values for naphthol groups bound to lysozyme in preparation B.

Negative logarithms of dissociation constants of the phenolic group of HNA-2.1 and HNA-1.4 in the ground and excited states (pK_a, pK_a^*) are close to those of 2-naphthol and 1-naphthol, respectively (see Table II and Ref. 17). This is in contrast to 2-naphthol groups bound to proteins by a sulfonamide bond [6–8], where pK_a^* values are considerably decreased compared to analogous data for 2-naphthol

Fluorescence Spectra

Fluorescence spectra of CytC-HNA-2.1 and Lyso-HNA-2.1 (A) are shown in Figs. 6 and 7.

The spectra of Lyso-HNA-2.1 (B) (not shown) are similar to those of preparation A of the modified lysozyme. In the fluorescence spectra of all studied preparation of proteins modified by HNA, two emission bands are visible. By analogy with HNA-2.1 in a methanol–water (1:1) mixture, one of these bands, positioned at about 360 nm, can be ascribed to the protonated (ROH*) form and the other (about 420 nm) to the deprotonated (RO^{*-}) species arising during the excited-state lifetime by proton dissociation (ESPT). The ratio of intensities of these bands $F^*_{ROH}/F^*_{RO^-}$) in a neutral water solution of pH 6.5 is highest for CytC-HNA-2.1 and lowest for

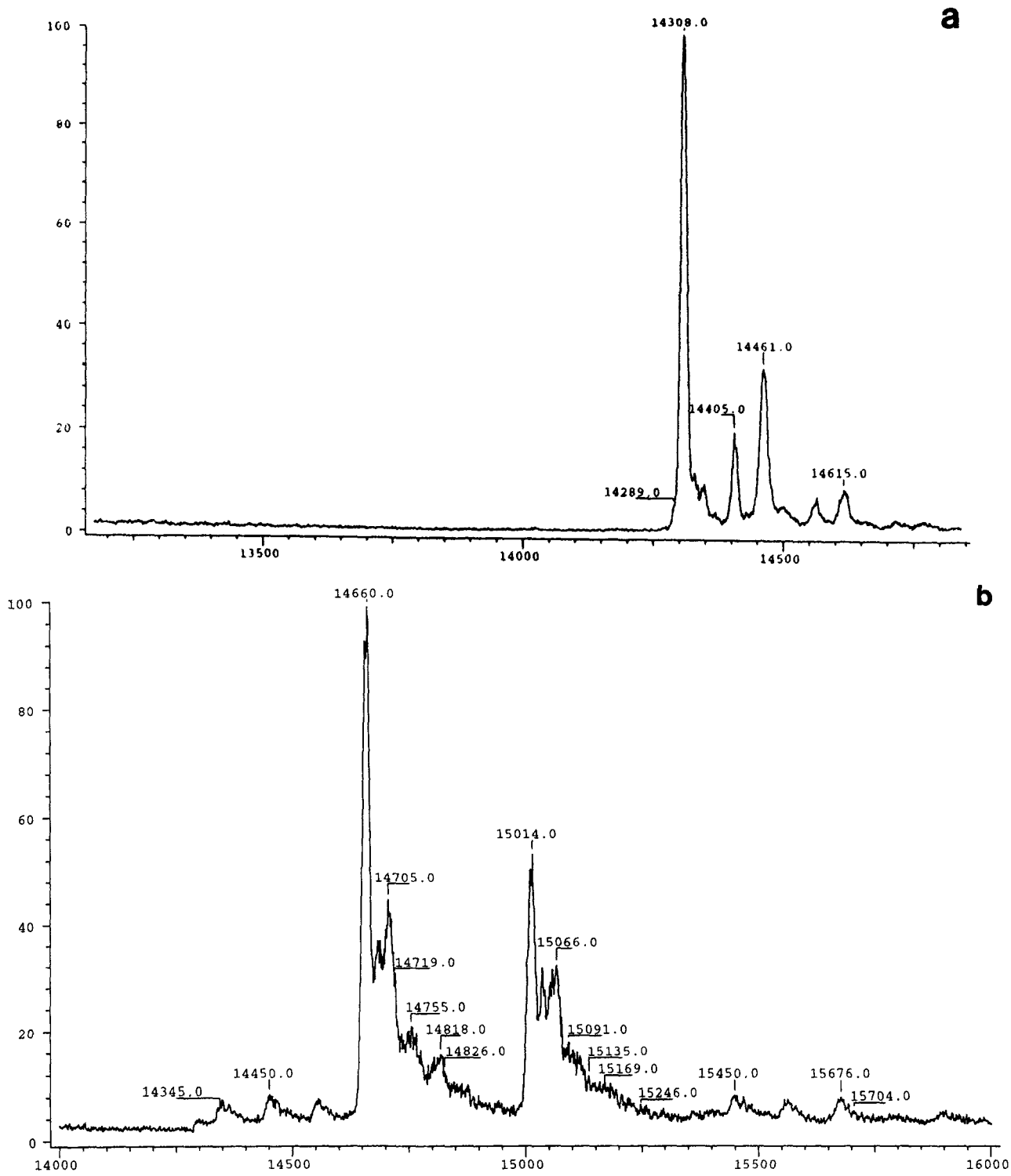


Fig. 1. (a) Mass spectrum of lysozyme modified by HNA-2.1 [Lyso-HNA-2.1 (B)]. The peak at 14,308 is due to unmodified protein. The peak at 14,461 can be ascribed to lysozyme modified by one molecule of HNA. It should occur at 14,465 (14,308 + 157). The error of 4 daltons may be caused by the presence of impurities. (b) Mass spectrum of lysozyme modified by 6-hydroxynaphthalene 2-sulfonamide of α,L-phenylalanine (Lyso-SE). The peak at 14,660 is due to singly modified lysozyme. It should appear at 14,661 (14,308 + 353). The shift of 1 dalton is within the limits of experimental error. No signal of unmodified lysozyme is visible for this preparation.

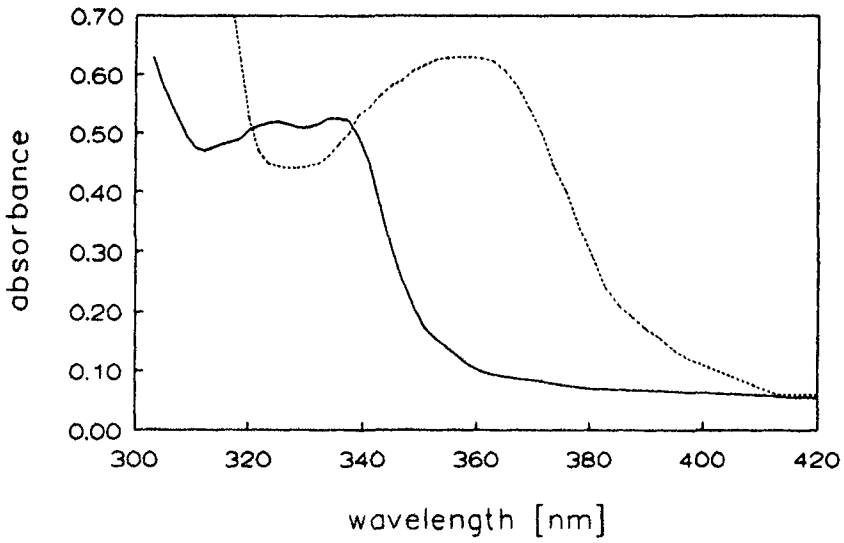


Fig. 2. BSA modified by HNA-2.1. Absorption spectrum in water. c = 10 mg/ml. (——) pH 6.5; (-------) pH 12.

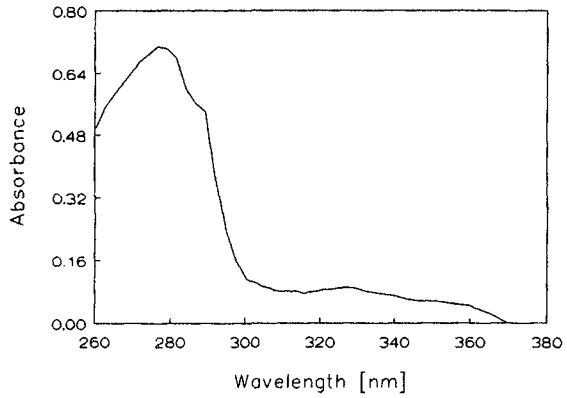


Fig. 3. Lysozyme-HNA-2.1 (B). Absorption spectrum in water (pH 6.5).

BSA-HNA-2.1 (Fig. 8), while both preparation of Lyso-HNA-2.1 have an intermediate position with respect to the ratio $F^*_{ROH}/F^*_{RO^-}$).

The fluorescence spectrum of BSA-HNA-1.4 (not shown) is similar to that of BSA-HNA-2.1 but shows more marked ROH* emission.

Spectrofluorimetric titration curves of BSA-HNA-2.1 and Lyso-HNA-2.1 (B) are shown in Figs. 9 and 10. Data for CytC-HNA-2.1 and Lyso-HNA-2.1 (A) (not shown) are analogous.

Spectrofluorimetric titration of compounds undergoing ESPT such as naphthols and many others including 2-naphthol derivatives bound to proteins by a sulfonamide bond show a relative fluorescence quantum yield (ϕ/ϕ_0) near 1 at pH $\ll$ pK_a*, a decrease in ϕ/ϕ_0 at a pH near pK_a*, a region where ϕ/ϕ_0 is independent of pH (plateau) near neutral pH values, and a decrease in (ϕ/ϕ_0) to 0 at a pH near pK_a where no protonated molecules in the ground state are present in the solution [6,7].

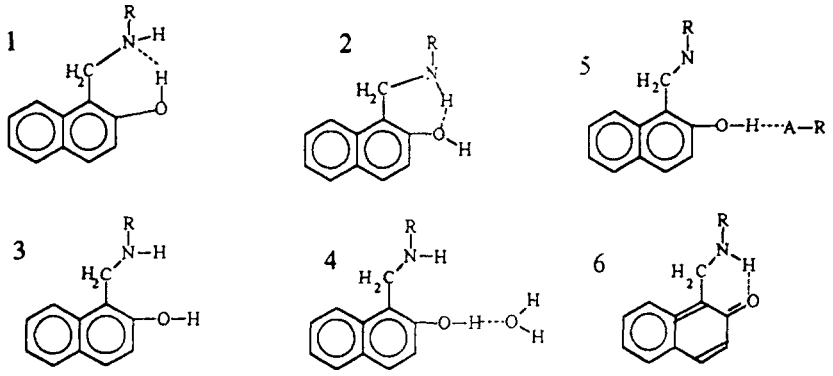
The results for BSA-HNA-1.4 (Fig. 11) resemble the picture for naphthols and their derivatives but the curves of the remaining protein HNA conjugates (Figs. 9–10) and differ considerably.

In Figs. 9 and 10 additional, unexpected transitions are visible which can be explained in accordance with the spectrophotometric titration data discussed above—by the presence of various HNA forms in HNA-2.1 protein conjugates (Scheme II). Forms of the chromophore differing in pK_2 and pK_a* values can be intramolecularly hydrogen-bonded and non-hydrogen-bonded species of the HNA group bound to proteins by an alkylamino bond. However, it must be noted that spectrophotometric and spectrofluorimetric titration curves for a given preparation are not parallel to one another, which means that the equilibrium of ESPT for at least some forms of HNA bound to proteins is not established during the excited-state lifetime and the rate of ESPT is comparable to the rate of emission of fluorescence.

Fluorescence Decay

The results of time-resolved measurements of modified proteins are presented in Table III.

In all these results at least two decay components are visible: one with a lifetime in the nanosecond range



Scheme II. Forms of HNA-2.1 bound to proteins by an alkylamino bond. A (in 5), proton acceptor groups from protein (e.g., His, Lys, Asp, Glu); R, protein.

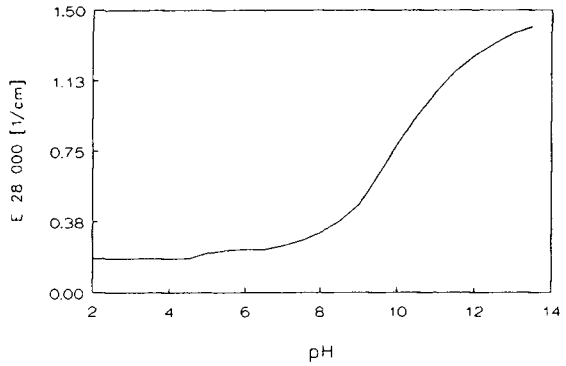


Fig. 4. BSA Modified by HNa-2.1. Spectrophotometric titration.

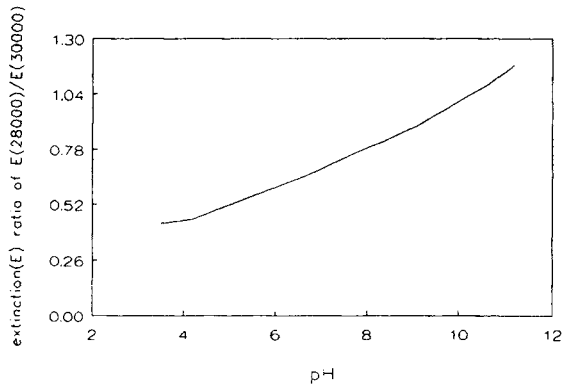


Fig. 5. Lysozyme modified by HNA-2.1. Spectrophotometric titration.

and the other in the picosecond range. The results in Tables III and IV indicate that the rate of ESPT [$k_{PT} = (1/\tau_2) - (1/\tau_1)$][4] in the preparations studied is very high (about 10^{10} s^{-1}). In such a case no fluorescence band of the primary (ROH*) form should be visible in the steady-state fluorescence spectra in water ($\phi/\phi_0 = 1/(1 + k_{PT}\,\tau_2) < 10^{-3}$ [6]) as it is in analogous compounds (low molecular weight Mannich bases [18]). This conclusion, however, is in apparent contradiction to the experimental spectra presented in Figs. 6 and 7, where the fluorescence band with a maximum at about 360 nm is undoubtedly connected with the ROH* form of HNA. The value of k_{PT} calculated from lifetime data [$k_{PT\,(TR)} = (1/\tau_2) - (1/\tau_1)$] is inconsistent with that obtained from steady-state measurements [$k_{PT\,(ss)} = ((\phi_0/\phi) - 1)/\tau_2$] for most of the preparations studied. Concordant values of $k_{PT\,(TR)}$ and $k_{PT\,(SS)}$ were obtained only for BSA-HNA-1.4,

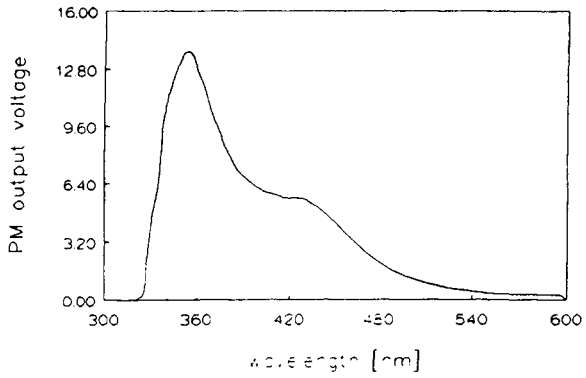


Fig. 6. Cytochrome *c* modified by HNA-2.1. Corrected fluorescence spectrum in water. $\lambda_{excit} = 320$ nm.

[4] $\tau_1 = 1/(k_f + k_q)$ should be measurable at pH $\ll pK_a^*$ and $\tau_2 = 1/(k_f + k_q + k_{PT})$ should be detected at neutral pH values. In our case no such clear picture could be obtained (Table III), but it can be noted that the amplitude of τ_1 decrerases at a pH near 7 and the amplitude of τ_2 goes to zero at a pH near 0.5. It should be noted that the fluorescence quantum yields of ROH* and RO^{*-} forms and therefore the rates of fluorescence (k_f) and radiationless inactivation (k_q) are comparable, which is required for validity of this equation.

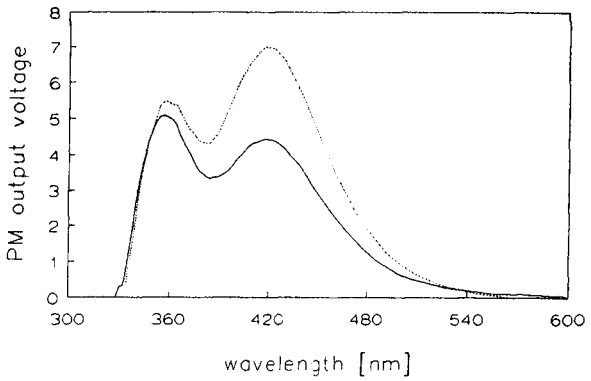


Fig. 7. Lysozyme modified by HNA-2.1. Corrected fluorescence spectra at pH 7.8 and 6.5. λ_{excit} = 320 nm. (——) pH 6.5; (------) pH 7.8.

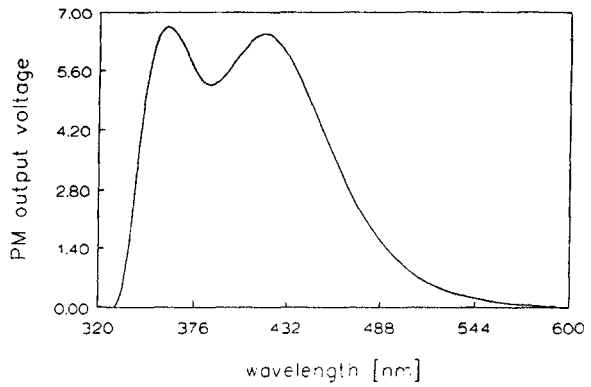


Fig. 8. BSA modified by HNA-2.1 (water solution, pH 6). Corrected fluorescence spectrum. λ_{excit} = 320 nm.

which is explained by the fact that in this case no intramolecular hydrogen bond of the fluorophore is possible.

The appearance of a ROH* fluorescence band in the presence of a very rapid decay component (Table III) could be due to the existence of various hydrogen-bonded and non-hydrogen-bonded forms of HNA bound to proteins in all preparation studied—with the exception of BSA-HNA-1.4. The occurrence of such forms was postulated above on the grounds of spectrophotometric and spectrofluorimetric titration data. Some of these forms may be responsible for the marked ROH* fluorescence band and others for fast proton transfer, causing a picosecond decay component of the fluorescence of ROH* with a considerable amplitude. It is assumed that fast decay components—in the picosecond range—are due to fluorophore fractions with an intramolecular hydrogen bond whose ROH* fluorescence is invisible in the steady-state spectrum. The lifetime of the fluorophore fraction with strong ROH fluorescence—which must be in the nanosecond range—probably is not visible at a neutral pH because of insufficient resolution of the apparatus, which is unable to fit the data to multiexponential decay. The lifetime values (τ_1) and quantum efficiencies of proton transfer (ϕ_{PT}) for these fractions can be calculated from formulas (2) and (3).

$$1/\tau_1 = k_{PT(SS)} + (1/\tau_2) \quad (2)$$

$$\Phi_{PT} = k_{PT(SS)} * \tau_1 \quad (3)$$

The results for τ_1 calculated from (2) and ϕ_{PT} obtained from (3) using calculated τ_1 values are presented in Table IV.

The procedure for calculation of τ_1 from formula (2) may seem arbitrary but we believe that the values of ϕ_{PT} obtained from (2) and (3) are good approximations for comparison of our preparation of proteins modified by HNA with respect to the efficiency of the ESPT reaction. It must be noted, however, that this procedure neglects the probability of geminal recombination in the ESPT reaction in our samples [1], which may be another reason for the inconsistency of steady-state and time-resolved fluorescence measurements.

DISCUSSION

A comparison of the fluorescence spectra of naphthol groups bound to proteins by an alkylamino bond and by a sulfonamide bond shows that the nature of the arm linking a fluorophore to a protein influences the ESPT reaction considerably. This influence is due to the electron withdrawing power of the sulfonamide group, the effect being responsible for the large decrease in pK_a^* values of 2-Np groups bound to proteins by a sulfonamide bond compared to unsubstituted 2-naphthol [7,8]. Such an effect is not observed for 2-Np groups bound to proteins by an alkylamino bond (Table II), for which even some increase in pK_a^* (decrease in excited-state acidity) compared to 2-naphthol (pK_a^* = 2.8) is found. An enhancement of excited-state acidity in 2-Np derivatives bound to proteins by a sulfonamide bond causes an increase in the rate of ESPT reaction compared to 2-naphthol and 2-Np groups bound to proteins by an alkylamino bond in which no intramolecular hydrogen bond is present (e.g., BSA-HNA-1.4). It is known that for analogous compounds differing in pK_a values, the rate of proton transfer should be proportional to the acidity of the group undergoing proton dissociation (Bronsted–Marcus relation) [5,8]. Taking into account the pK_a^* value for BSA-HNA-1.4 (Table II), the rate constant of ESPT (k_{PT}; Table IV) for this preparation is

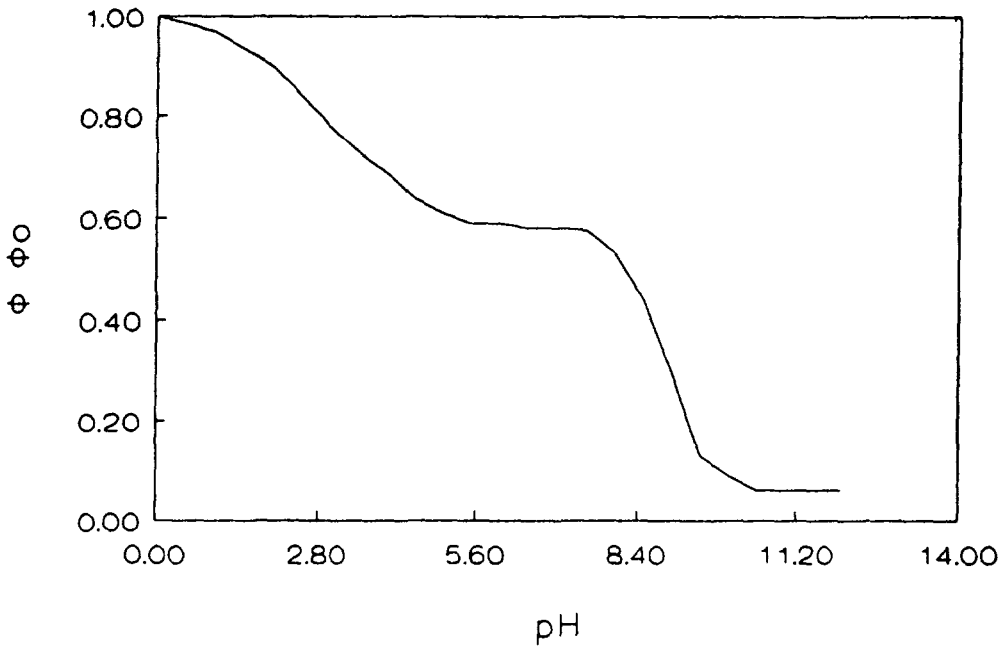


Fig. 9. BSA modified by HNA-2.1. Spectrofluorimetric litration.

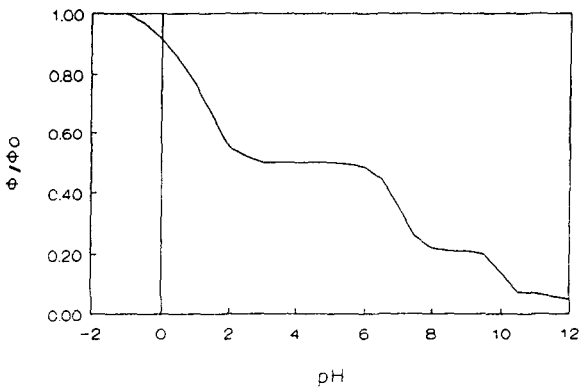


Fig. 10. Lysozyme modified by HNA-2.1 (preparation B). Spectrofluorimetric titration.

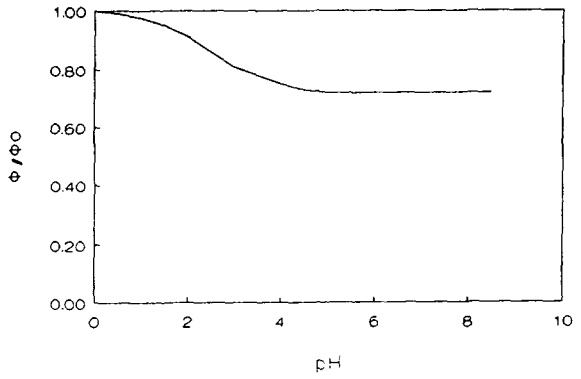


Fig. 11. BSA modified by HNA-1.4. Spectrofluorimetric titration.

much lower than it should be according to the Bronsted relation for naphthol derivatives. The value of k_{PT} for Lyso-SE is much higher (Fig. 12) than predicted on the basis of the Bronsted–Marcus relation. The values of k_{PT} for 2-naphthol groups bound to BSA by a sulfonamide bond are higher than that for BSA-HNA-1.4. The lowering of the rate of ESPT for BSA-HNA-1.4 compared to low molecular weight naphthol derivatives is probably caused by the immobilization of water molecules near the protein surface and increasing energy of medium reorganization during proton transfer [19]. This effect is more significant in the case of naphthol groups bound by an alkylamino bond than by a sulfonamide bond because in the former case the arm linking the fluorophore to the protein is shorter (cf. Scheme II and Ref. 5) and the naphthol group is more susceptible to the influence of the protein microenvironment.

In cases in which an intramolecular hydrogen bond between the phenolic oxygen atom and the amino nitrogen of a protein is formed, much higher ESPT rates can be anticipated. However, the fraction of fluorophores with an intramolecular hydrogen bond varied in the preparation studied. It is suggested by our results that the rate of proton transfer along the intramolecular hy-

Table III. Fluorescence Decay of HNA Bound to Proteins[a]

Preparation	Lifetimes		
	pH 0.5	pH 6.5	pH 10.5
Lysozyme-(HNA-2.1)	5.7 ns, $\alpha_1 = 0.9$	$\tau_1 = 5.7$ ns, $\alpha_1 = 0.23$	$\tau_1 = 9.8$ ns, $\alpha_1 = 0.61$
	$\tau_2 = 4.2$ ns, $\alpha_2 = 0.1$	$\tau_2 = 3.3$ ps, $\alpha_2 = 0.71$	$\tau_2 = 502$ ps, $\alpha_2 = 0.14$
		$\tau_3 = 750$ ps, $\alpha_3 = 0.06$	$\tau_3 = 1.8$ ps, $\alpha_3 = 0.24$
BSA-(HNA-2.1)	$\tau_1 = 10.6$ ps, $\alpha_1 = 0.36$	$\tau_1 = 1$ ps, $\alpha_1 = 0.74$	
	$\tau_2 = 3.6$ ns, $\alpha_2 = 0.33$	$\tau_2 = 4.5$ ps, $\alpha_2 = 0.20$	
	$\tau_3 = 107$ ps, $\alpha_3 = 0.32$	$\tau_3 = 490$ ps, $\alpha_3 = 0.06$	
BSA-(HNA-1.4)		$\tau_1 = 780$ ps, $\alpha_1 = 0.60$	
		$\tau_2 = 1.3$ ns, $\alpha_2 = 0.40$	
Cytochrome *C*-(HNA-2.1)		$\tau_1 = 2$ ps, $\alpha_1 = 0.87$	
		$\tau_2 = 5.2$ ns, $\alpha_2 = 0.13$	
Lyso-SE		$\tau_1 = 119$ ps, $\alpha_1 = 0.16$	
		$\tau_2 = 9.7$ ns, $\alpha_2 = 0.84$	

[a] τ_1, τ_2, etc., are lifetimes and α_1, α_2, etc., amplitudes in the fluorescence decay equation: $I_{(t)} = I_0 \exp(\Sigma\alpha_i\tau_i)$.

Table IV. Comparison of Proton Transfer Efficiency in the Samples Studied

Preparation	τ_2 (ns)	τ_1 calculated (ns)	Φ_{PT}	$k_{PT(SS)}$ (s^{-1})
BSA-HNA-2.1	3.6	2.2	0.399	1.85×10^8
BSA-HNA-1.4	1.3	0.93	0.278	2.9×10^8
Lyso-HNA-2.1 (B)	5.7	Fr. 1,[a] 2.8	0.478	1.74×10^8
		Fr. 2,[a] 1.1	0.798	7.0×10^8
CytC-HNA-2.1	5.2	4.42	0.15	3.4×10^7
Lyso-SE	9.7	0.25	0.91	19×10^8

[a] The values for fraction 1 (fr. 1) are calculated from $\Phi/\Phi_0 = 0.502$, which is the first plateau value visible in Fig. 10, and that for fraction 2 (fr. 2) from $\Phi/\Phi_0 = 0.20$, another plateau in Fig. 10. For BSA-HNA-2.1 (Fig. 9) only one plateau value can be discerned, though at least one other fraction of fluorophores is present.

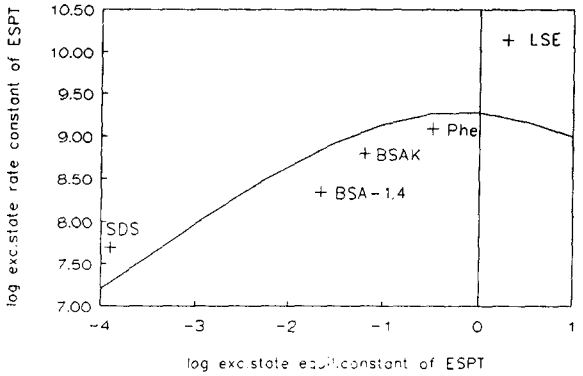


Fig. 12. Bronsted relation for naphthols. Solid curve, Bronsted relation for 2-naphthol derivatives. (+) Data for 2-naphthol groups bound to macromolecules: LSE, Lyso-SE; Phe, 6-hydroxynaphthaleno-2-sulfonamide of α, L-phenylalanine; BSA-1.4, BSA-HNA-1.4; BSAK, preparation of BSA with 2-naphthol groups bound by a sulfonamide bond described in Ref. 8; SDS, sodium dodecyl sulfate with 2-naphthol groups bound by a sulfonamide bond [7].

drogen bond in naphthol groups bound to proteins is slower than that for analogous low molecular weight compounds with an intramolecular hydrogen bond (Mannich bases) [18].

These effects, due to immobilization of water near the interphase boundary [19], are also responsible for a decrease in ϕ_{PT} for naphthol groups bound to proteins by an alkylamino bond. Taking into account the ϕ_{PT} values for the preparation studied, the degree of modification, and the dimensions of the protein molecules labeled, one can estimate the amount of proton released per unit volume. In this way we calculated that in Lyso-HNA-2.1 (B), 1 mol of photons releases 7×10^{-3} mol of protons per dm^3, and in Lyso-SE, 8.5×10^{-3} mol protons per dm^3. In one sample of BSA modified with 2-naphtholo-6-sulfonyl chloride [1,2], by very high degree of modification (39 mol of marker per mol of protein and ϕ_{PT} 0.75) 1 mol of photons releases 0.35 mol of protons per dm^3. This means that light of 320-nm wavelength and 370 W causes local acidification from about pH 6 to pH 0.47 in 1 s. This effect would be sufficient to influence the proton gradient in biological energy transformation systems such as tylakoids, mitochondria, and bacterial cell walls [10]. It is clear that the method of binding naphthol groups to proteins by an alkylamino bond is much less advantageous with respect to proton transfer efficiency and homogeneity of modification compared to that consisting in the formation of a sulfonamide bond. However, in some cases an alkylamino bond may be preferable because of its shorter length and the lesser influence of ESPT on electronic density.

Naphthols (Np) as fluorescent probes for proteins undergoing ESPT are, from some points of view, preferable to probes used by other authors [3,4] such as carboxyfluorescein and hydroxypyrene trisulfonate. Np's affect the intrinsic internal forces of a protein less and thus the results obtained are more representative for understanding proton transfer in unmodified proteins. Fluorescence bands of ROH* and RO* forms of Np's are relatively well resolved, which makes the analysis of steady-state and time-resolved fluorescence data more reliable.

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