Biochemistry. In the article “A common ancestry for multiple catalytic antibodies generated against a single transition-state analog” by Hideaki Miyashita, Tomoko Hara, Ryuji Tanimura, Fujie Tanaka, Masakazu Kikuchi, and Ikuo Fuji, which appeared in number 13, June 1994, of Proc. Natl.

Acad. Sci. USA (91, 6045–6049), the following correction should be noted. Due to a printer’s error, Figs. 2 and 3 on p. 6047 were transposed; the correct figures and their legends are shown below.

Fig. 2. Deduced amino acid sequence comparison of V_{1} cDNA generated from 11 anti-CP monocular antibodies. Dashes denote identity to antibody 6D9 and dots, used to align sequences, represent no amino acid residue at a position. CDR designations and amino acid residue numbering are as described by Kabat et al. (14).

Biophysics. In the article “Excited-state intramolecular proton transfer as a fluorescence probe for protein binding-site static polarity” by Alexander Strychn and Michael Kash, which appeared in number 18, August 1994, of Proc. Natl. Acad. Sci. USA (91, 8627–8630), the following acknowledgement was inadvertently omitted by the authors: “This research was supported under Contract DE-FG05-87ER60517 between the Division of Biomedical and Environmental Research and the Florida State University.”

Cell Biology. In the article “An essential gene of Saccharomyces cerevisiae coding for an actin-related protein” by Masahiko Harada, Anneliese Karwan, and Ulrike Wintersberger, which appeared in number 17, August 16, 1994, of Proc. Natl. Acad. Sci. USA (91, 8258–8262), the authors request that the following change be noted. On page 8258, in line 17 of the Abstract, “hydrophilic” should be replaced by “hydrophobic.”
Excited-state intramolecular proton transfer as a fluorescence probe for protein binding-site static polarity

(proton-transfer fluorescence/protein conformation/protein site polarity)

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ABSTRACT A fluorescence probe is introduced for protein conformation and binding-site monitoring as the proton-transfer (PT) tautomer fluorescence by using 4-hydroxy-5-azaphenanthrene (HAP) as a prototype. A typical grossly-wavelength-shifted PT fluorescence for HAP is observed in the 600-nm spectral region for this UV-absorbing molecule (absorption onset, 400 nm), for which case PT occurs even in protic solvents. It is shown that PT fluorescence of HAP can serve as a protein-binding-site static-polarity calibrator, shifting from a λ_max of 612 nm in cyclohexane to 585 nm in ethanol at 298 K, contrary to the usual dispersion red shift. A small mechanical solvent-cage effect is noted in ethanol at 77 K, but solvent dielectric relaxation is not apparent from the fluorescence spectrum. Thus, HAP serves to distinguish static solvent-cage polarity from dynamical solvent dielectric relaxation and other solvent-cage effects (mechanical restriction of molecular conformation). HAP as a PT-fluorescence probe is applied to human serum albumin (HSA) and beaver apomyoglobin.

Fluorescent-probe spectroscopy of proteins as a methodology yielding structural and dynamical information concerning the chromophore (probe) environment has a long history of the application of excited-state molecular interaction between a probe and its environment (1-4). This interaction is effective for the description of both dipole-reorientational dynamics of molecules surrounding the chromophore and their dielectric properties. Nevertheless, when one is interested in structural changes in the protein as well as the intrinsic structure, such a duality can complicate the interpretation of experimental fluorescence data. Both the instantaneous electronic response of the static environment and the relaxation dynamics of the probe environment can induce analogous spectral shifts of fluorescence.

In principle, several static and dynamical aspects of protein binding site and protein conformation could be considered. The polarity of a binding site and its dimensional and conformational features can be considered as static aspects. On the other hand, dynamical aspects could include dielectric relaxation—e.g., solvent-cage response to dipolar changes in magnitude and reorientation upon electronic excitation of a fluorescence probe—and response to conformational changes of the probe itself. The effect of the protein binding site as a "solvent cage" can then serve to separate dielectric effects from mechanical conformational changes, as deduced from electronic spectral changes.

During the last decade molecular spectroscopy made great advances in the understanding of different modes of molecular excitation, such as fluorescence arising from (i) an electron-transfer excitation, (ii) proton transfer (PT), and (iii) twisted-intramolecular charge transfer. In electron transfer (ET) spectroscopy, the generation of a charge-separated polar state provokes a profound response of the environment of the fluorescence probe. In excited-state intramolecular PT (ESIPT) spectroscopy, a gross frequency shift (up to 13,000 cm⁻¹) from the lowest absorption band permits easy observation without spectral interferences (5). The ESIPT (or PT) phenomenon involves the intramolecular transfer of a proton with femtosecond rise times (6-9) and offers diverse excited-state environmental responses. Another phenomenon, twisted-intramolecular charge transfer (TICT) involves both charge-separation and drastic conformational changes in the excited molecule, offering yet another and more complex fluorescence probe (10, 11).

Excited-state intramolecular ET in bianthranyl (12) has already been applied to the study of dynamical properties of the ligand-binding site of human serum albumin (HSA) (13). The present research explores the advantages of using the proton-transfer (PT) fluorescence probe for protein binding-site studies. For the specific prototype probe selected for PT fluorescence, 4-hydroxy-5-azaphenanthrene (HAP), it will be shown that it can serve as a static polarity calibrator for the binding sites with HSA and apomyoglobin (apo-Mb), separated from dynamical dielectric relaxation effects.

MATERIALS AND METHODS

Spectroscopic Measurements. Absorption spectra were measured with a Shimadzu UV-2100 spectrophotometer. The fluorescence spectra (uncorrected for the detector sensitivity) were recorded with a Baird-Atomic model SFR-100 (Baird-Atomic, Bedford, MA) spectrofluorometer.

Emission of HAP-protein complexes was studied with quartz optical cells of 1 × 1 cm. The temperature (5–65°C) was regulated by an RTE-4 cryothermostat (Neslab Instruments, Portsmouth, NH). All solvents were spectrograde and were checked for fluorescence impurities. Spectroscopic experiments with HAP in ethanol (77 and 298 K) were performed in a quartz Dewar flask.

Preparation of HAP-Protein Complexes. Essentially fatty-acid-free HSA was a product of Sigma. Beaver apo-Mb was obtained from the Biochemistry Department in the L'viv State University, Ukraine. HAP was a gift from Pi-Tai Chou (Department of Chemistry, University of South Carolina). The commercial product (TCI America, Portland, OR) was twice recrystallized from spectrograde methycyclohexane and then vacuum-sublimed. The probe was dissolved in acetone and evaporated with the formation of the film on the inside surface of the vessel; then protein solutions were added in a 0.05 M Hepes buffer (pH 7.0). After being incubated for 1 hr at room temperature and stored overnight in a refrigerator, the solutions were centrifuged for 30 min at 23,000 × g the following day.

Abbreviations: HAP, 4-hydroxy-5-azaphenanthrene; HSA, human serum albumin; ET, electron transfer; PT, proton transfer; ESIPT, excited-state intramolecular PT.
RESULTS AND DISCUSSION

ESIPT in HAP. The excitation sequence for chromophores undergoing ESIPT could be represented by the following steps: $S_0 \rightarrow S_1$, normal molecule absorption (usually in the UV region); then $S_1 \rightarrow S_1^*$ excited-state radiationless PT; followed by the $S_1^* \rightarrow S_0$ fluorescence; and then $S_0 \rightarrow S_0$ reverse PT to the normal tautomer ground state, the primes designating the electronic states of the PT tautomer. Fig. 1 depicts the "normal" tautomer and the PT "tautomer" structures of HAP. This probe has a PT fluorescence in the orange-red region with a $\lambda_{max}$ of 612 nm (in cyclohexane at 298 K) (14). A six-membered-ring strong intramolecular hydrogen bond between the O--H proton and the N atom in HAP makes ESIPT in this molecule free from hydrogen-bonding solvent interference. For example, HAP dissolved in ethanol shows only PT fluorescence and no normal fluorescence (Fig. 2).

Solvent-Cage Effects in the Fluorescence of HAP. A solvent-cage effect can result from a mechanical restriction on intramolecular conformational changes in a fluorescence probe upon its electronic excitation. In such a case, a band origin may shift, and the vibrational or torsional distribution pattern (Franck-Condon vibronic band shape or contour) may change (15-18). This appears to apply to the case for HAP in ethanol at 77 K or 298 K, as evidenced in the spectra of Fig. 2.

The PT fluorescence band at 298 K in liquid ethanol and at 77 K in ethanol glass (Fig. 2) has a maximum at the same position ($\lambda_{max}$, 585 nm). However, this coincidence is illusory when analyzed spectroscopically. The width at half maximum of the fluorescence band ("FWHM") is greatly reduced, from 2333 cm$^{-1}$ to 1650 cm$^{-1}$ when comparing the spectrum at 298 with that at 77 K; moreover, the electronic band origin (measured as onset of fluorescence) is measurably red-shifted ($\Delta \nu = 1300$ cm$^{-1}$). Thus, the change in the fluorescence band shape or Franck-Condon envelope is compensated by the observed red shift in band origins, yielding what appears to be a negligible band shift. In this case of HAP, therefore, we conclude that a mechanical solvent-cage effect is manifest, as well as a dispersion red-shift in the rigid glass medium (77 K) compared with liquid ethanol at 298 K.

Fig. 2. Absorption (curve 1) and fluorescence (curves 2 and 3) spectra of HAP in ethanol at 298 K (curves 1 and 2) and 77 K (curve 3), showing a small mechanical solvent-cage effect on the fluorescence spectrum.

Another effect of a solvent cage would be its restriction of dielectric relaxation, as noted in the case of indole fluorescence spectroscopy (19, 20). In the case of indole, the dipole moment orientational change for the $S_1$ state compared with that for the $S_0$ ground state conditions a corresponding dipolar reorientation in a surrounding polar medium, observed as a large (time-dependent) red shift. This red shift is blocked by the solvent cage when the medium is a rigid glass solution. The contrasting behavior of HAP in ethanol liquid vs. ethanol glass solution indicates that such a dynamical dielectric relaxation and its blockage are absent for this case.

Spectral Blue Shift in the Four-Level PT System. The PT tautomer fluorescence of HAP proves to be quite sensitive to solvent polarity with solvent variation (Fig. 3). This fluorescence shifts markedly to shorter wavelengths with increase of solvent polarity [Reichardt (21) $E^\pi$ index], contrary to a normal-dispersion red shift (Fig. 4). The PT tautomer has a fluorescence maximum at 612 nm (16,340 cm$^{-1}$) in cyclohexane and 585 nm (17,090 cm$^{-1}$) in ethanol (298 K; $\Delta \nu$ change + 750 cm$^{-1}$). The UV absorption band $S_0 \rightarrow S_1$ of HAP shifts toward shorter wavelengths with increasing solvent polarity, the band maximum in cyclohexane occurring at 381 nm (26,250 cm$^{-1}$) and in ethanol at 372 nm (26,880 cm$^{-1}$) (298 K; $\Delta \nu$ change + 630 cm$^{-1}$).

A dispersion blue shift implies a decrease in dipole moment upon electronic excitation so that the ground state in a polar solvent will lie lower in energy than the corresponding excited state. This is illustrated in Fig. 5, for both the normal tautomer (left) and the PT tautomer states (right). The fact that both the $S_0 \rightarrow S_1$ absorption and $S_1 \rightarrow S_0$ fluorescence show a comparable blue shift requires analogous greater
lowering of ground states in going from hydrocarbon solvent to the ethanol polar environment, compared with that for the excited states $S_1$ and $S_i$. The dispersion-force interaction is an instantaneous response of the environmental dielectric field to electronic excitation of the molecular fluorescence probe. Thus, it can be considered a static field effect as far as the probe molecule is concerned, contrasted with the dynamical relaxation effect of dipolar solvent reorientation. In the case of HAP, we would conclude that there occurs a small diminution of electric dipole moment of the excited states $S_1$ and $S_i$ vs. the ground states $S_0$ and $S_i$ and not a gross reorientation of the dipole moment direction, as in the case of indole.

Because excitation of ESIPT is faster than solvent relaxation, even a possible change of dipole-moment orientation of HAP in the $S_i$ state in comparison with that for the $S_0$ state would not change its interaction with the solvent dipoles for the $S_i$ state, if the dipole moment of the solute in the latter state were parallel to that at the $S_0$ state. As a consequence, the $S_i$ state of HAP would be undisturbed by solvent relaxation. Similar conclusions have been made for the 3-hydroxyflavone molecule and for its derivatives, fisetin and $4'$-diethylamino-3-hydroxyflavone (A. S., D. A. Gormin, and M. K., unpublished data). Moreover, the C. B. Harris group (8), studying the femtosecond resolved transient absorption of 3-hydroxyflavone, found that the transition dipole of the $S_i$ state is approximately parallel to that of the $S_0$ state. This parallels the findings of B. J. Schwartz (see ref. 8), on the tautomer emission transition-dipole orientation of 2-(2'-hydroxyphenyl)benzothiazole. A generalization of the $S_0$ and the $S_i$ dipole-moment parallelism is premature for all ESIPT cases, but at least for HAP it seems to be a valid premise.

The fact that HAP PT fluorescence depends on polarity of environment and is independent of its dynamics (mobility) makes this molecule an ideal probe for the prediction of the polarity properties of protein binding sites and their conformational stability. For example, HAP could be very useful in the study of the cell denaturation of proteins.

**Interaction of HAP with HSA.** HSA, as a protein of the physiological circulation system, binds and carries various ligands in the blood stream. The HSA molecule is created by three structurally homologous domains, denoted I, II, and III (22). Each domain is formed by two smaller subdomains, A and B. The principal binding sites of HSA are located in subdomains II A and III A. Since HAP does not dissolve in

**Fig. 5.** Electronic state diagram for HAP, comparing polar solvent shifts in PT spectroscopy (primed states) for the absorption blue shift and PT fluorescence blue shift with increase in solvent polarity. The absorption blue shift is $\Delta \tilde{E} = \Delta S_0 - \Delta S_1$ and analogously for PT fluorescence $\Delta \tilde{E}$.

**Fig. 6.** Absorption (curves 1) and fluorescence (curves 2) spectra of HAP-protein complexes at 298 K in 0.05 M Hapes buffer (pH 7.0). (Upper) HAP-HSA complex. (Lower) HAP-apo-Mb complex.

**Fig. 7.** Fluorescence excitation spectra of HAP at 298 K. Curves: 1, HAP-HSA complex; 2, HAP in ethanol. Excitation spectra were normalized at their maxima, monitored at the PT fluorescence wavelength of 580 nm.
water, HAP–HSA interaction was studied by using thin probe films (see Materials and Methods). The binding pattern was resolved by a Scatchard plot; the HSA molecule was found to bind one molecule of HAP with a $K_d = 11 \mu M$.

Fig. 6 (Upper) shows the absorption and fluorescence spectra of the HAP–HSA complex. Fluorescence emission of this complex has a maximum at 576 nm. Such a short-wavelength position indicates the high polarity of the HAP environment in the HSA ligand-binding site. The single tryptophan residue of HSA is situated in the II A subdomain. Because the fluorescence spectrum of the tryptophan of HSA (donor) overlaps with the absorption spectrum of HAP (acceptor), binding of this probe in the II A subdomain could be followed by the nonradiative energy transfer from Trp-214 to HAP. To check on this possibility, the excitation spectra of the HAP–HSA complex and HAP in ethanol monitored at the fluorescence wavelength of 580 nm were compared (Fig. 7). Both spectra were almost identical at 280 nm. If Trp-214 had been transferring its excitation energy to HAP efficiently, this band at 280 nm for the protein–probe complex would have been much stronger. This result rules out a significant Förster-type energy transfer in HAP–HSA complexes and leads to the presumption of probe binding in the tryptophan-free III A subdomain of HSA.

HAP PT tautomer fluorescence was applied for the investigation of the conformational stability of HSA (Fig. 8). No change in the position of HAP fluorescence in the temperature interval of 12–37°C was found. An increase of temperature results in a red shift of PT tautomer emission and could be attributed to the decrease of polarity of the HAP environment. Temperature-lowering shifts probe fluorescence to the blue. This effect could be due to the increase of polarity of the HAP environment in the protein matrix. Such sensitivity of the HSA molecule to the temperature changes could have a physiological meaning. It is possible that the temperature increase above 37°C changes the role of this protein in metabolism. The same temperature range of HSA conformation stability was observed by using another probe, bianthryl (13), but in that case the interpretation of spectral results was complicated by the sensitivity of bianthryl to both dynamical and conformational changes of the ligand-binding site.

**HAP-apo-Mb Complex.** Mb is the oxygen-carrying protein of muscle. Its structural and dynamical properties are well described (23, 24). If the apo-Mb heme is removed (25), HAP replaces it with high affinity. In apo-Mb the polarity of the HAP-binding site is evidently greater than in HSA, as the probe fluorescence has a maximum at 571 nm (Fig. 6 Lower). In HSA the binding site is probably more flexible to accommodate ligands of different size than in apo-Mb. The heme molecule is larger than HAP, and for the HAP–apo-Mb complex, the free space in the binding site could be substituted by water molecules.

**SUMMARY**

Summing up the results of the ESIPT in the HAP molecule surrounded by protein matrix, one can postulate the following advantages of this probe: (i) fluorescence in the orange-red region is clear of the UV excitation and interfering fluorences; (ii) excited-state tautomer fluorescence of HAP is free from hydrogen-bonding solvent interference (unusual for PT emission) (26); and (iii) the wavelength of the HAP tautomer fluorescence blue shift correlates with the polarity of the environment and is free from the influence of solvent relaxation.

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