LUMINESCENCE PROPERTIES OF PYRON RED
A NEW MOLECULAR PROBE FOR PROTEIN RESEARCH

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The dependence of the luminescence of the new anionic dye Pyron Red (PR) on the polarity of the medium is investigated. Upon passage from an aqueous phase to a nonpolar phase, PR shows a shortwave shift of the fluorescence emission maximum from 675 to 650 nm and an increase in the fluorescence quantum yield from 0.03 to 0.54-0.70. When complexed with human serum albumin, PR shows fluorescence excitation and emission maxima at 525 and 625 nm and a fluorescence quantum yield of 0.8. In a comparison of the luminescence properties of PR with those of the well-known probes ANS and K35 in water and a complex with albumin, PR is shown to have the maximum absolute sensitivity but a lower fluorescence enhancement upon binding with a protein compared to ANS. A convenient criterion of the probe sensitivity toward binding with a protein that is defined as the ratio of the fluorescence intensities of the protein-bound and the free probe $A_F = F_b/F_f$ is proposed. The value of $A_F$ (35) for the PR probe ranks between those for the K35 probe with a low $A_F$ (18) and ANS with a high $A_F$ (105).

Key words: fluorescent probe, fluorescence analysis, albumin, protein.

Introduction. Presently, fluorescent probes have become one of most information-revealing and sensitive tools in protein research, and their applications are described in detail in numerous reviews and monographs [1-6]. The high sensitivity of the optical parameters of fluorescent probes to changes in their microenvironment is used in investigations of the conformational transitions and structural lability of protein molecules in enzyme-substrate and receptor interactions and changes in pH, ionic strength, temperature, and other factors of the medium. A second wide field of application of fluorescent probes, based on the competition of ligands and probes for binding with the same sites in biomolecules, includes evaluation for binding capacity of proteins with different ligands. In past years, more than fifty fluorescent dyes have been proposed for use in protein research. However, despite this, most researchers prefer to use just the few probes investigated in most detail, such as 8-anilinonaphthalene sulfonate (ANS), 2-n-toluidinonaphthalene-6-sulfonate anion (TNS), Rhodamine B, and carboxyphenylimide dimethylaminonaphthalene dicarboxylic acid (K35).

This situation, in our opinion, is attributable to a lack of concrete criteria for the choice of an optimum fluorescent probe for evaluation of a particular protein parameter. Although the set of properties that should be provided by a molecular probe for protein research is presently well understood, a comparative analysis of the luminescence and biophysical properties of various probes can provide valuable new information. There are four main parameters of fluorescent probes that are important for their application in protein research [1-6].

Sensitivity of Luminescence Determination of a Probe. In dilute solutions, the fluorescence intensity $F$ is defined as follows [7]:

$$F = 2.3I_0eqC$$ (1)
where $I_0$ is the intensity of the exciting light, $\epsilon$ is the molar extinction coefficient of the fluorophor at the excitation wavelength, $q$ is the fluorescence quantum yield, $l$ is the optical-path length, and $C$ is the concentration of the fluorescing substance. In this equation, the product $eq$ characterizes the absolute sensitivity of a fluorescent probe, which is independent of the design of the device, the shape of the cuvette, and the concentration of the substance [7, 8]. In particular, fluorescein, considered as a standard for fluorophors, has the maximum absolute sensitivity $eq = 87 \cdot 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ [7]. In complex biological systems containing various absorbing and fluorescing substances, fluorescence detection can be difficult due to the inherent luminescence and light scattering of the samples. To minimize these factors, a fluorescent probe should have a fluorescence excitation maximum in the wavelength range of $>400$ nm and a maximum Stokes loss (the shift of the emission maximum with respect to the fluorescence excitation maximum).

Optical Selection of the Fluorescence Signal from a Probe Complexed with a Protein. This selection is based on the shortwave shift of the emission maximum and the manifolds increase in the fluorescence quantum yield of a fluorescent probe upon passage from a polar to a nonpolar medium (solvatochromic effect) [4]. This effect explains the enhancement of the fluorescence of a probe upon its incorporation into hydrophobic binding sites of proteins. The stronger this effect, the greater the extent to which the total fluorescence signal from the sample is determined by the fluorescence of the bound dye. Thus, under certain conditions, the total fluorescence signal can be used to perform dynamic monitoring of the change in the hydrophobicity or the number of binding sites of a protein without physical separation of the free and bound fractions of the probe.

Sensitivity to the State of a Protein. It is obvious that the sensitivity of a fluorescent probe to the state of a protein is closely related to its sensitivity to the polarity of the microenvironment, i.e., the more strongly the microenvironment of the probe complexed with the protein differs from that in the solution, the stronger the response of the dye should be to a change in the structural state of the macromolecule. Thus, the range of applicability of a fluorescent probe is, as a rule, limited to proteins containing hydrophobic binding sites (albumin, lipoproteins, apoheemoglobin, chomatrispin, etc.) [3-6]. Another important requirement is that the fluorescent probe itself should not affect the properties of the macromolecule under investigation. This effect is usually minimum with an excess of the protein with respect to the dye, when the first high-affinity sorption center on the protein is filled. In this case no more than one dye molecule is bound to a protein molecule.

Specificity of the Interaction with a Particular Protein. It is obvious that the higher the specificity of the interaction or the binding constant of the fluorescent probe with a particular protein, the more strongly can one reduce the probe-to-protein concentration ratio and thus reduce the excess effect of the fluorescent probe on the protein molecule. Second, a high-affinity marker provides a more specific characterization of conformational changes of protein binding sites. And, finally, a high-specificity fluorescent probe can be used for investigation of a particular protein in an actual system that contains numerous other biological compounds. Thus, in evaluating the structural state of a protein, one should choose a fluorescent probe with maximum binding specificity with respect to the given type of protein. In particular, anionic dyes (ANS, TNS, dansylsarcosene, K35, etc.) have the highest affinity toward albumin [6]. As opposed to the structural lability of proteins, their binding ability is frequently evaluated in a regime of excess dye, in which the content of the bound fluorescent probe is proportional only to the number of sorption centers and is independent of the binding constant [6]. Under these conditions, introduction of various ligands has shown that the less specific marker K35 is more easily expelled from albumin binding sites compared to the high-affinity probe ANS [9].

In our opinion, evaluation of new fluorescing dyes proposed as molecular probes for protein research should be based on the use of standard evaluation systems that include certain standard or reference fluorescent probes and proteins. Based on experience in investigation of this problem in Russia, one can consider ANS and K35 as reference probes and the most investigated and readily available human serum albumin (HSA) as a reference protein.

In the present work, an investigation of the luminescence properties of a new dye — Pyron Red (PR) — is carried out and the possibilities for its use as a molecular probe for investigation of proteins are explored with regard for the above criteria.

**Experimental.** Pyron Red (styryl-$\gamma$-pyranbutyrate, $C_{24}H_{23}N_{3}O_{3}$, $M = 401$)