BINDING OF FLUORESCENT PROBE, 1-ANILINO-8-NAPHTHALENE SULFONATE, TO APO-HORSERADISH PEROXIDASE

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1. Introduction

Fluorescent probes for hydrophobic sites in proteins are attracting growing attention as tools for study of conformational states, antibody interactions, enzyme function, etc. (cf. review by Edelman and McClure [1]). One of the most widely used compounds of this class is ANS (1-anilino-8-naphthalene sulfonate); it is virtually non-fluorescent in water (quantum yield ~ 0.002), whereas in certain solvents and when adsorbed on hydrophobic regions in protein, it has a fluorescence with a quantum yield of more than 0.5. Stryer [2] found that in both apomyoglobin and apohemoglobin, ANS binds to the same sites as heme, although the binding constants differ by an order of magnitude. Excitation energy was transferred with 100% efficiency from the protein part to the complexed dye. This is a very interesting observation, but data on the influence of this type of binding on such properties as stability to denaturing agents and radiation would be easier to interpret if the same phenomenon took place in an enzyme.

Theorell and coworkers [3, 4] and later investigators have demonstrated that it is possible to split horseradish peroxidase (HRP) into hemin and the apoenzyme and then to recombine the latter with hemin with practically no loss of enzyme activity. As shown below, ANS may take the place of heme in the split enzyme. This system, in which a longwave ultraviolet absorbing, fluorescent molecule is introduced into the vicinity of the active site of an enzyme, has been subjected to studies of radiation effects [5, 6].

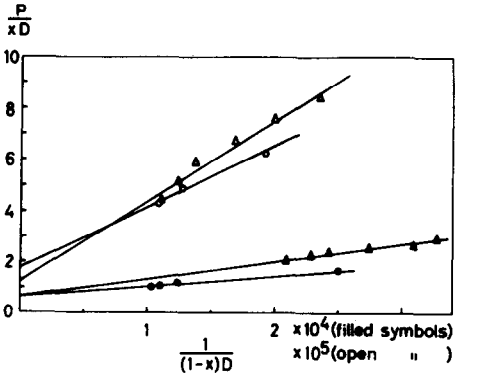

Fig. 1. Extrapolations according to Eq. (1). P, total protein concentration; D, total ANS concentration; x, fraction of ANS bound to apo-HRP.

2. Materials and methods

Apo-HRP was produced according to Theorell [7] from Boehringer horseradish peroxidase POD-I and from Sigma "Peroxidase from Horseradish Type VI", both with an R.Z. value of 3.0. The intactness of the protein part of the split enzyme was checked by recombination with hemin, which restored more than 90% enzyme activity. Peroxidase from the two sources was identical in all respects studied here. ANS was recrystallized several times from the magnesium salt, and has been used in earler investigations [8]. Fluorescence was recorded with a modified Farrand spectrofluorimeter previously described equipped with 5 nm slits [9]. Calculations and corrections for geometric factors, light intensity, and detector sensitivity were

also made essentially as described in [9]. Quantum yields and emission spectra were determined relative to known standards (cf. for example Chen et al. [10]). All experiments were run at 25°C in 0.1 M phosphate buffer pH 7. F_0 below, the fluorescence of completely bound ANS was directly determined with a 2.5×10^{-4}M apo-HRP solution for ANS concentrations of 10^{-5} M and below; the values for higher concentrations were obtained by extrapolation. Correction for the slight nonlinearity was made by comparison with a similar titration curve of ANS in bovine serum albumin. This procedure introduced an error of less than three percent, and was necessary because of the relatively high dissociation constant found for the ANS/apo-HRP system.

Table 1
Quenching of 350 nm emission.

Fraction of protein molecules binding ANS	Relative emission intensity at 350 nm (excitation at 275 nm)
0	100
0.10	92
0.14	85
0.30	67

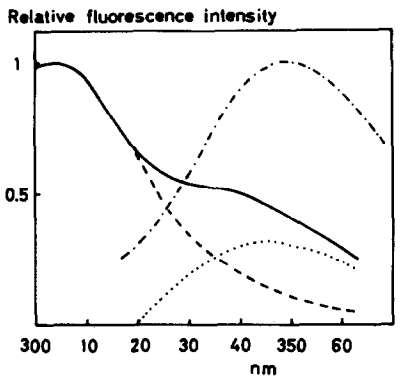

Fig. 2. Emission spectra of apo-HRP (——), ribonuclease (- - -), and the difference between their spectrum (.....). Emission spectrum of free tryptophan (-.-.-.-) is given for comparison. Excitation at 275 nm.

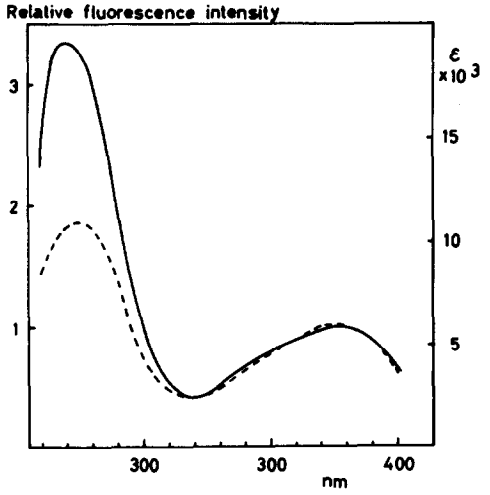

Fig. 3. Extiction coefficient (cm^2 $mmole^{-1}$) for bound ANS (- - -) plotted together with the fluorescence excitation spectrum (——). Emission measured at 470 nm.

3. Results and discussion

As was first derived by Klotz [11] and later elaborated by Weber and Young [12], the complex situation existing when a protein binds small molecules may be described by two constants: N, the total number of binding sites in the protein molecule, and K, the statistical dissociation constant. If the proper combinations of concentrations are selected, a single set of experiments may yield both of these constants by linear extrapolation according to the equation:

$$\frac{P}{xD} = \frac{1}{N}\left(1 + \frac{K}{(1-x)D}\right) \qquad (1)$$

where P is the protein concentration, D is the dye concentration, and x is the fraction of dye bound to the protein. The factor x is obtained as F/F_0, where F is the fluorescence of the test solution. F_0 has been defined above.

N and K were determined in two different experiments with each of the two different preparations of apoperoxidase (fig. 1). D, which was held constant in each titration, varied between 0.9 and 10×10^{-5} and P between 0.3 and 4×10^{-5} M. The values found were $N = 1.1 \pm 0.5$ and $K = 4.1 \times 10^{-5}$ M. Hemin displaced the dye very efficiently, thus permitting assay of enzyme activity after treatment.

Native peroxidase shows very little fluorescence due to the quenching influence of heme. When this group is split off, an ultraviolet fluorescence with a quantum yield of about 1.5% is observed. Although Maehly and Paléus [13] did not find tryptophan in horseradish peroxidase, it is evident from fig. 2 that the emission spectrum though similar to that of a non-tryptophan containing protein, ribonuclease, contains a small tryptophan component. When ANS is added to the apoenzyme, two effects are immediately noticeable which are not found with the native enzyme: (a) the tryptophan emission is quenched (table 1), and (b) the quantum yield of ANS fluorescence on direct excitation increases to 0.85.

The quantum yield of transfer to ANS may be calculated as

$$q_T = \frac{F_o - F}{aF_o} \qquad (2)$$

where a is the fraction of protein molecules binding ANS. From the data given in table 1, we obtain q_T = 1.0, which proves that the tryptophan emission actually originates from the apoperoxidase molecule. Since tyrosine and not tryptophan is responsible for the major part of the ultraviolet absorption of this protein, calculation of transfer efficiency from these quenching data will only give a false picture of the situation. Fig. 3 shows that the fluorescence excitation spectrum is identical to the absorption spectrum of the bound dye at wavelengths longer than 320 nm, but excitation energy absorbed by the protein contributes to the ANS fluorescence at shorter wavelengths. However, since the extinction coeeficient of apo-HRP at about 270 nm is 5 times larger than that of ANS, it is evident that this transfer is a relatively inefficient process. Throughout the protein absorption band, an average value of $q_T = 0.11 \pm 0.01$ was calculated from data summarized in fig. 3.

The above results contain evidence for the binding of 1-anilino-8-naphthalene sulfonate to the site normally occupied by heme in horseradish peroxidase. The binding energy (ΔF^o = 5.98 kcal mole^{-1} at 25°C) is intermediate between the corresponding values for ANS binding to apomyoglobin and apohemoglobin but is closer to that of hemoglobin. As regards fluorescence and excitation transfer between aromatic amino acids and ANS, the situation appears to be more complex with apoperoxidase than with the proteins studied by Stryer [2]. The apoperoxidase fluorescence is predominantly that of tyrosine, and the major part of excitation energy induced by absorption in the 270 nm band is not transferred to bound ANS. The low transfer efficiency found in this investigation is partly due to the poor overlap between tyrosine emission and ANS absorption, but it is probably also connected with the disturbance of the phenolic ring orbitals noticed in the absorption spectrum (ϵ_{max} at 266 nm). These results are in agreement with the findings of Weber and Teale [14], that transfer between aromatic amino acids and heme is exceptionally inefficient in peroxidase as compared to other heme proteins.

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