PERTURBATION STUDIES ON SOME BLUE PROTEINS

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1. Introduction

The effect of interaction among molecules undergoing optical transitions and surrounding solvent or other solute molecules is generally indicated as perturbation.

When applied to macromolecules these techniques allow to find out whether the aromatic side chains of the molecule are accessible to the solvent or there are presumably regions not exposed to it.

The most widely used among the perturbation techniques is the solvent perturbation of absorption [1, 2]..Recently Lehrer [3] proposed the name 'solute perturbation of fluorescence' for quenching effects due to solutes like iodide, bromide and others. This author developed also a modified Stern–Volmer equation which allows quantitative determination of the exposed residues in a macromolecule.

We applied this technique to the 'blue' copper proteins already studied in our laboratory since we found that they show unusual fluorescence most probably linked to the hydrophobic microenvironment of the fluorophores [4–7].

In fact the fluorescence of native azurin and plastocyanin is not affected by iodide. In the case of stellacyanin which contains three tryptophans it was possible to demonstrate that two of them are exposed to the solvent whereas the third is deeply buried in the protein core.

2. Materials and methods

Azurin, plastocyanin and stellacyanin were purified following the methods described elsewhere [4, 5] and that described by Graziani et al. (unpublished work). Solvent perturbation of absorption was performed using a DK2A Ratio Recording Spectrophotometer and four 1 cm cuvettes. The number of exposed chromophores was calculated according to Herskovits and Sorensen [8]. Fluorescence experiments were performed at 20°C with a Turner model 210 Spectro which gives spectra corrected for the energy of excitation [9]. The fluorescence data were plotted in terms of a simple Stern–Volmer equation, using the integrated fluorescence values:

$$F_0/F = 1 + K_Q (I) \qquad (1)$$

where F_0 and F are the fluorescence values in the absence and in the presence of a concentration (Q) of the quencher and K_Q is the Stern–Volmer constant evaluated from the slope of the F_0/F versus I plots. When these plots were non linear the equation proposed by Lehrer was used:

$$F_0/\Delta F = \frac{1}{(I) fa\, K_Q} + \frac{1}{fa} \qquad (2)$$

where fa is the fraction of fluorescence accessible at infinite quencher concentration. The data were fitted with a least squares procedure.

3. Results

Two out of the three proteins studied, namely azurin and plastocyanin, were insensitive in the native state to the presence of KI up to 0.5 M. Under denaturing condition however the fluorescence of

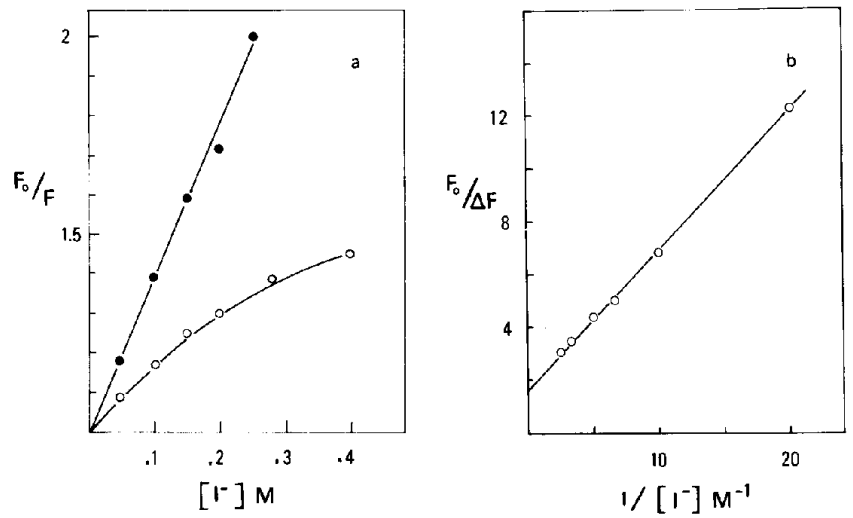

Fig. 1. Stern–Volmer and modified Stern–Volmer plots of iodide quenching on stellacyanin: a) Stern–Volmer plot, (●–●–●) denatured stellacyanin; (○–○–○) native stellacyanin, b) Modified Stern–Volmer plot for native stellacyanin.

both proteins was strongly quenched by iodide. Plots of F/F_o vs. I^- concentration gave straight lines.

Different results were obtained with stellacyanin. This protein showed a quenching by iodide also in the native state. As indicated in fig. 1, a, a downward curved line resulted by plotting the results obtained with the native protein, while a straight line was obtained for the denatured stellacyanin. A replot of the data for native stellacyanin according to eq. 2 is presented in fig. 1, b. The straight line obtained showed an intercept on y axis of 1.5. The fluorescence spectra of stellacyanin in the presence and absence of 0.3 M KI are presented in fig. 2. The fluorescence of

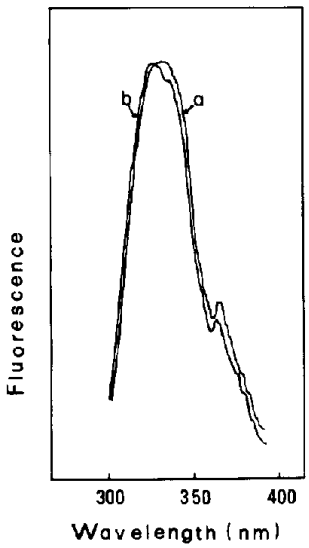

Fig. 2. Fluorescence spectra of 10^{-5} M stellacyanin in 0.01 M potassium phosphate buffer in the absence (a) and in the presence (b) of 0.3 M KI. The fluorescence intensity of spectrum (b) was instrumentally amplified up to that of (a).

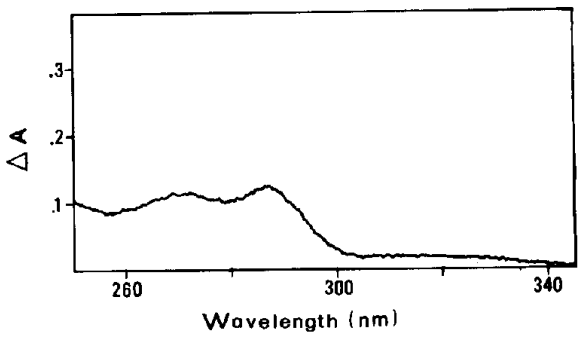

Fig. 3. Solvent perturbation of stellacyanin. Both reference and sample compartments contained two 1-cm quartz cuvettes. The buffer used throughout was 0.01 M potassium phosphate buffer pH 7. The forward cuvettes contained 1.3 × 10^{-4} M stellacyanin in the presence (sample) and absence (reference) of 20% v/v ethylene glycol. The backward reference cuvette contained 20% v/v ethylene glycol, whilst the backward sample cuvette was filled with buffer only.

the quenched sample was instrumentally normalized to that of the unperturbed sample. Iodide causes a shift in the emission maximum of about 5 nm toward the blue.

Fig. 3 shows a solvent perturbation of the absorption made on native stellacyanin. The perturbant used was ethylene glycol (20% by volume). According to Herskovitz and Sorensen [8] the difference spectrum obtained accounts for 2 tryptophans and 5 tyrosines. A similar experiment made using dimethylsulfoxide as the perturbing agent gave the same result with respect to tryptophans, but only 2 tyrosines appeared to be involved.

4. Discussion

The mononuclear blue proteins so far studied seem to share a lot of common properties. Among them we have studied in some detail the intrinsic fluorescence, which appears always 'anomalous' and quenched by the presence of copper (4–7). The 'anomalous' fluorescence seems to be correlated to a hydrophobic environment around the fluorophores. This fluorescence in azurin and stellacyanin arises from tryptophan, while in plastocyanin it arises from tyrosine.

Since azurin contains only one tryptophan, the results obtained in the present paper could have been anticipated. In fact the fluorescence of the native protein is not affected at all by the addition of a fluorescence perturbant like iodide. When the native structure of the protein is lost, its fluorescence becomes 'normal' in terms of maximum emission and quantum yield. Iodide quenches this fluorescence according to the Stern–Volmer equation. The situation for plastocyanin and stellacyanin is different. The former contains three tyrosines and the latter three tryptophans. It seemed of interest to ascertain how many of these aromatic amino acids were involved in the anomalous f uorescence of each protein; mainly for plastocyanin, since some evidence has already been obtained regarding the involvement of only one tryptophan in the interaction with copper, in the case of stellacyanin.

As far as the fluorescence perturbation experiments are concerned, each tyrosine in plastocyanin is equivalent. In fact no quenching is induced by iodide in the native plastocyanin, while the denatured protein is quenched according to a pure Stern–Volmer mechanism.

Stellacyanin behaves in a different way. Its fluorescence is affected by iodide both in the native and in the denatured state of the protein. A difference arises when the quenching data are analyzed in terms of the Stern–Volmer equation. The denatured protein gave a straight line indicating the same accessibility of all tryptophans, whereas a curved line was obtained for the native protein. Replot of the latter data as suggested by Lehrer [3] indicated that 66% of fluorescence is affected by iodide.

The quenching induced by iodide may be either static of collisional [10]; more frequently the quenching may be of a mixed type. In any case assuming that every tryptophan contributes in the same way to the overall fluorescence the above reported results suggest that only two of the three tryptophans are quenched by iodide. These tryptophans seem to be more solvated than the third one since the iodide quenching causes a blue shift of the fluorescence maximum (fig. 2). This result is confirmed by solvent perturbation of the absorption (fig. 3). The difference spectrum obtained results from perturbation of tyrosines and tryptophans absorption. The former did not give any significant contribution to the fluorescence. While the number of perturbed tyrosines varied from 2 to 5 depending on the perturbing agent used, the perturbed tryptophans were always two. It seems reasonable to conclude that stellacyanin has one tryptophan not freely accessible to the solvent or to small solute molecules like iodide, ethylene glycol or dimethyl sulfoxide. This hydrophobic tryptophan should be involved in the interaction with copper [5]. In any case it seems that solute fluorescence perturbation is a very useful tool to discriminate among fluorophores in a globular protein.

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