PHOTOCHEMICAL EFFECTS ASSOCIATED WITH THE COPPER ABSORPTION BANDS OF THE NATIVE HEMOCYANIN FROM *OCTOPUS VULGARIS*.

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1. Introduction

Visible light irradiation of proteins in the presence of suitable photosensitizers has been used for producing the photooxidative modification of the histidyl, methionyl, cysteinyl, tyrosyl and tryptophyl residues [1]. The usefulness of this technique is often limited by the poor selectivity of the photoreaction, since the sensitizer can randomly attack several or even all the potentially susceptible side chains. On the other hand, it has been shown [2,3] that, when the sensitizer is naturally or synthetically attached to specific sites of the protein molecule, only the aminoacid residues which are located in close proximity of the sensitizer undergo photooxidation.

This approach has been used by us to probe the topography of the active site of *Octopus vulgaris* hemocyanin. This protein has two absorption bands in the visible region, peaking at 348 nm ($\epsilon^{348}_{25400\,\mathrm{m.w.}}$=8900) and at 570 nm ($\epsilon^{570}_{25400\,\mathrm{m.w.}}$=540) (fig.1), both typical of the oxygenated protein. Therefore, the possibility of using the copper-oxygen system as a built-in probe of the protein active site has been explored. The modification of some histidyl residues upon methylene blue-sensitized photooxidation of *Murex trunculus* hemocyanin has been reported [4]; however, the interpretation of the data is hindered to some extent by the simultaneous destruction of other aminoacid side chains.

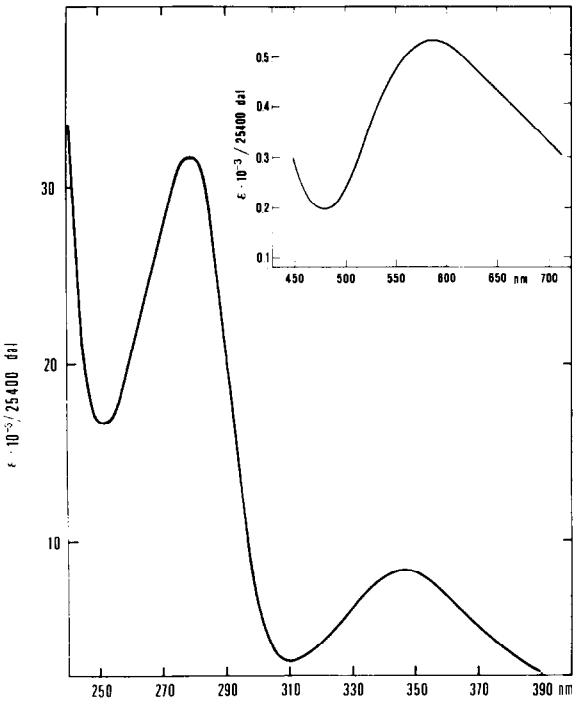

Fig.1. Visible and u.v. spectra of oxyhemocyanin from *Octopus vulgaris*. Abscissa: wavelengths. Ordinate: extinction coefficient per 25 400 daltons.

2. Experimental procedure

Octopus vulgaris hemocyanin was prepared as described elsewhere [5]. Irradiation experiments were performed by exposing a protein solution, at a concentration of ~4 mg/ml, to the light of a 450 W Xenon arc lamp (Osram XBO); wavelengths below 315 nm were cut off by means of an aqueous acetone filter. The solutions were kept at pH 8.0 (phosphate buffer μ=0.05) and at 10°C ±0.5°C by circulating water through the cell holder. Under these conditions, the hemocyanin solutions exhibited $A_{348\ nm}$~1.400. The irradiation was carried out up to 360 min. At intervals, the irradiated solutions were assayed for the absorption spectrum and the aminoacid composition of the protein. The spectral data were corrected for light scattering by the expression $A = k\lambda^{-4}$; the aminoacid analyses were carried out according to Spackman, Stein and Moore [6]. Tryptophan was quantitated in the intact protein by a spectrophotofluorimetric procedure [7].

3. Results

In fig.2, the decrease in the optical density at 348 nm and in the histidine content of hemocyanin

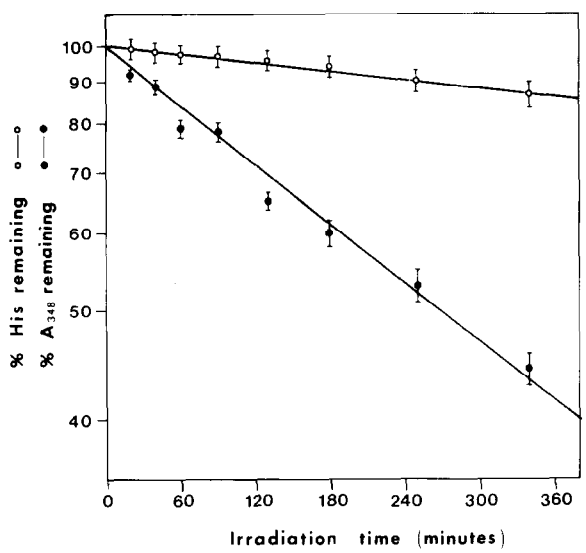

Fig.2. Semi-log plot of the effect of light (wavelengths >315 nm) to histidyl residues and 348 nm absorption band. Abscissa: time in minutes. Ordinate: histidine % (○). A_{348}% •.

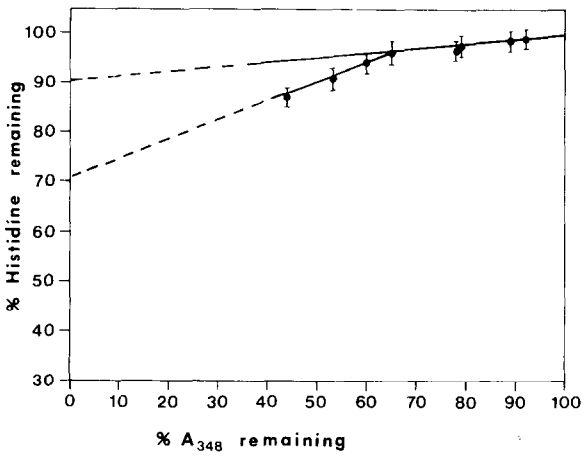

Fig.3. Plot of residual absorption at 348 nm against residual histidines in samples of irradiated hemocyanin.

are reported as a function of the irradiation time. The data are expressed as percentage of the corresponding values for the unirradiated protein. The photoinduced reaction appears to involve only the histidyl residues. In fact, no appreciable change in the content of the other aminoacid residues was detected after irradiation. Moreover, no trace of the common photo-oxidation end-products of some susceptible aminoacids, such as *N*-formyl-kynurenine, methionine sulfoxide, and cysteic acid was found.

The correlation plot between the decrease of the extinction at 348 nm and of the histidine content (see fig.3) yields a correlation factor of 0.8. It can be deduced from the plot that a 100 per cent decrease of the absorption at 348 nm corresponds with the destruction of a maximum number of six histidyl residues (out of a total number of 22 histidines) per two copper atoms.

4. Discussion

The results presented in this paper show that the near-uv and the visible absorption bands of hemocyanin do induce photochemical effects. The photoreaction specifically involves a few imidazole moieties of the protein. It is likely that these residues are the ligands of the copper ions at the active site of hemocyanin: in actual fact, the photosensitizing function

is performed by some constituents of the active site, while it is known [8] that photosensitized reactions usually occur within an approximate 5 Ångstroms distance from the photosensitizer.

Now, the photoreaction causes the destruction of the active site. Therefore, the calculated number of modified histidines (6 residues per 2 Cu atoms) represents a minimum number of ligands for the metal ions. These results are in agreement with the data obtained by acidbase titration of hemocyanin [9].

Since the mechanism of the photoprocess promoted by the copper-oxygen system of hemocyanin is still unknown, it cannot be decided whether the observed selectivity for histidines is a characteristic feature of this kind of photoreaction or is due to the absence of other photosusceptible aminoacid residues in the neighborhood of the active site. Further experiments are in progress in order to refine the described results.

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