PHOTO-OXIDATION OF HORSE HEART CYTOCHROME *C*. EVIDENCE FOR METHIONINE-80 AS A HEME LIGAND

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1. Introduction

In a previous paper [1], we showed that the irradiation of a protein containing a photosensitizer covalently linked to known positions in the molecule, causes the selective modification of only those potentially photo-oxidizable amino acid residues which are in close proximity to the sensitizer. Therefore, once the modified side chains have been identified, their location in the three-dimensional network of the protein molecule can be deduced. In principle, it should be possible to extend this method to proteins naturally possessing a chromophoric group which can act as a photosensitizer. As a first approach we attempted to explore the environment of the porphyrin prosthetic group in horse heart cytochrome *c*. Since porphyrins efficiently mediate the photo-oxidation of amino acids [2], it was expected that the porphyrin group of this protein would likewise serve as such a photosensitizer. Cytochrome *c* was chosen also because its primary structure is known, and its biological, physical and chemical properties have been studied in detail [3].

The results presented in this communication show that only those susceptible amino acids which are adjacent to the heme group are photo-oxidized upon irradiation of ferricytochrome *c*. Furthermore, our data provide compelling evidence that Met-80 serves as a protein ligand for the Fe^{3+} ion in ferricytochrome *c*.

2. Materials and methods

Horse heart cytochrome *c* (type III, Sigma Co.) was converted to the oxidized form by treatment with excess ferricyanide, followed by dialysis against cold distilled water. Purification of ferricytochrome *c* was achieved by chromatography on Amberlite CG-50 [4]; only the monomeric fraction eluted with 0.25 M NaCl was used.

Photo-oxidations were performed at 37°C by exposing 4 ml of a 0.1 mM protein solution to the light of four 300-W tungsten bulbs using the experimental arrangement previously described [5]. In some experiments, an equimolar amount of hematoporphyrin was added to the irradiated solution. The reaction of ferricytochrome *c* with cyanogen bromide and the identification of the *N*-terminal amino acids in the cleavage products were carried out by the procedure of Chu and Yasunobu [6]. The heme undecapeptide was prepared as outlined by Harbury and Loach [7]. The amino acid composition of this peptides as isolated from the irradiated cytochrome *c* agreed very well with the expected values. Amino acid analysis were performed as described previously [1].

3. Results

Cytochrome *c* was irradiated for 1 min both in deionized water solution, pH 5.9, where only methionine is susceptible to porphyrin-sensitized photo-oxidation [2], and in 0.1 M phosphate buffer solution, pH 8.2, where all five photo-oxidizable amino

Table 1

Amino acid content of unirradiated and photo-oxidized ferricytochrome *c*. The amino acids were determined with a Carlo Erba 3A27 analyzer after 22-hr hydrolysis in 6 N HCl at 110°C. The table includes only those amino acids which are known to be affected by photo-oxidation [12]. No appreciable change was found in the other amino acids. The values in the table denote number of residues per molecule.

Amino acid	Unirradiated	Irradiated pH 5.9	Irradiated pH 8.2	Irradiated pH 8.2 + hematoporphyrin[c]
Histidine	2.9	3.0	1.9	0.1
Tryptophan[a]	1.0	1.0	1.0	0.0
Tyrosine	3.9	4.1	3.9	0.3
Methionine[b]	1.8	0.9	1.1	0.0
Methionine sulphoxide[b]	0.0	0.9	1.0	1.8

[a] Determined on the intact protein by the method of Goodwin and Morton [13]. [b] Determined by automatic chromatographic analysis after alkaline hydrolysis [5]. [c] The irradiation was carried out for 15 min at 37°C.

acids are susceptible [8]. As shown in table 1, one Met residue was converted to the sulfoxide on illumination at pH 5.9; while in buffer at pH 8.2, one Met and one His residue were selectively oxidized. The other His and Met residues, as well as the single Trp and all of the Tyr residues which are present in cytochrome *c*, were recovered unchanged. This same result was obtained with samples of protein irradiated up to 120 min. However, when the irradiations were performed at pH 8.2 in the presence of hematoporphyrin all of the photoreactive residues were modified (table 1, column 4). Both the product produced at 5.9 and that produced at pH 8.2 were homogeneous as shown by chromatography on Amberlite CG-50 (fig. 1).

In order to identify the selectively modified Met residue, both photo-oxidized cytochromes were treated with cyanogen bromide. Since methionine sulfoxide is resistant to attack by cyanogen bromide [9], the polypeptide chain should be cleaved only at the unreacted methionine. Subsequent end group analysis did indeed show that 0.86 moles of *N*-terminal glutamic acid (which follows Met-65) were present per mole of photo-oxidized protein, while no trace of *N*-terminal isoleucine (which follows Met-80) was detected. Amino acid analysis of the heme undecapeptide obtained from cytochrome *c* irradiated at pH 8.2 showed that no histidine was present, indicating that His-18 was the selectively oxidized histidyl residue.

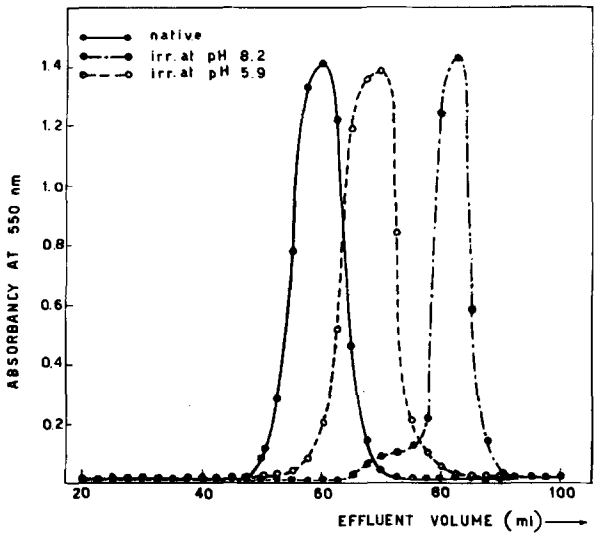

Fig. 1. Chromatography of un-irradiated ferricytochrome *c* and of ferricytochrome *c* photo-oxidized at pH 8.2 and at pH 5.9 (0.5 μmoles) on a column (0.9 × 40 cm) of Amberlite CG-50. The eluent was sodium phosphate buffer, pH 8.71. Na^+ concentration: 0.34 g ion/l.

4. Discussion

The present work shows that illumination of ferricytochrome *c* leads to the selective destruction of His-18 and Met-80, whereas illumination of the pro-

tein in the presence of added porphyrin, which is then free to contact the protein at any point, results in the destruction of all Met, His, Trp and Tyr residues. This suggests that all of the residues susceptible to photo-oxidation except His-18 and Met-80 must be shielded from any contact with the prosthetic group in ferricytochrome *c*. This conclusion is further supported by the fact that the number of amino acid residues modified is not increased by prolonging the irradiation time. Moreover, the occurrence of intermolecular photosensitization is unlikely since X-ray crystallography [10] indicates that the heme group is inserted in a cleft in the cytochrome *c* molecule and is therefore sheltered from the solvent.

The selectively photo-oxidized His residue, His-18, has been shown to be one of the two protein ligands for the Fe^{3+} in ferricytochrome *c* [3]. Several alternatives have been suggested for the other ligand, including a second His residue, a Tyr oxygen, a Met sulfur, a Lys amino group and the indole moiety of a Trp residue [11]. The results of our photo-oxidation experiments appear to rule out most of these proposals, since only one His residue and no Trp or Tyr residues appear to be located near the heme group.

Furthermore, our results suggest that Met-80, the other selectively photo-oxidized amino acid residue, serves as the second ligand in ferricytochrome *c*. Further work is in progress in order to establish these conclusions more definitely.

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