PHOTOOXIDATION OF THE THIOETHER BRIDGES OF HORSE HEART CYTOCHROME *C*

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1. Introduction

A number of studies with chemically modified forms of horse heart cytochrome *c* allowed the elucidation of the role performed by several amino acid residues in the redox process and biological function of the hemoprotein [1]. The conclusions derived from these investigations are in satisfactory agreement with those based on the three-dimensional structure of ferryicytochrome *c*, as recently obtained by X-ray diffraction studies [2]. Until now, however, no information is available about the role of the 2 thioether bridges, covalently linking the side chains of cysteines 14 and 17 with the heme group. Only recently, it was suggested [3] that treatment of cytochrome *c* with low concentrations of iodine may induce the oxidation of these thioethers, but no data on the redox properties and the functional parameters of the modified hemoprotein were reported.

Since the presence of thioether bridges in proteins is rather unusual, it appears of interest to assess their importance for the conformational and biological properties of cytochrome *c*. A simple photooxidative procedure, which leads to the conversion of the 2 thioethers to sulfoxides, will be described in this paper. Such a modification does not significantly affect the absorption and circular dichroism spectrum of cytochrome *c*, whereas its biological activity is decreased by about 50%.

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2. Materials and methods

Irradiations of ferricytochrome *c* (Sigma, type III) were carried out at 25° by exposing an aqueous solution, 1 mM protein, pH 5.5, containing a 1:1 molar ratio of methylene blue, to the light of a 450 W Xenon lamp; the experimental arrangement was the same as described elsewhere [4]. A Balzers interference filter was used to isolate the 640 nm wavelength. Under these conditions, the incident light was totally absorbed by the dye.

The heme undecapeptide was prepared as outlined by Harbury and Loach [5]; its digestion with pronase was performed by standard procedures [6]. The reaction of cytochrome *c* with iodoacetic acid to obtain the alkylation of His-33 and Met-65 was carried out by previously published methods [7]. The methods for performing the amino acid analyses and for determining the electron transport activity have been described in previous papers [8, 9]. In the case of the alkylated derivatives of cytochrome *c*, unaffected methionines were determined after performic acid oxidation [10]. Infrared spectra were monitored by a Perkin Elmer, model 141, grating spectrometer.

3. Results and discussion

When ferricytochrome *c* was irradiated with visible light for 30 min in the presence of methylene blue, a single methionyl residue was converted to the sulfoxide, as shown by amino acid analysis (table 1). A 5-fold increase in the time of cytochrome *c* exposure to light brought about no further change in the amino

Table 1
Amino acid analyses of different samples of cytochrome *c*.

Amino acid	Native	Irradiated for 30 min	Irradiated for 3 hr	Treated with iodoacetic acid[c]	Treated with iodoacetic acid and irradiated 30 min
Histidine	2.9	2.9	3.1	2.6	2.6
Tyrosine	3.9	3.7	3.8	3.7	4.0
Tryptophan	1.0	0.8	1.1	1.0	0.9
Methionine	1.8	0.9	1.0	1.1	1.1
Methionine sulfoxide[a]	0.0	1.1	1.1	0.0	0.0
Carboxymethyl-methionine[b]	–	–	–	0.9	0.9

The amino acids were determined with a Carlo Erba 3A27 analyzer. The table includes only those amino acids which are susceptible to photooxidation [13]. No change was found in the content of the other amino acids. The values in the table denote number of residues per molecule.
[a] Photooxidation product of methionine.
[b] Determined after oxidation with performic acid [10].
[c] The reaction was carried out as described in [7].

Table 2
Effect of modified cytochromes on the 0_2 consumption of cytochrome *c* depleted mitochondria.

Cytochrome *c*	Activity ($\mu M \times min^{-1}$)	Percentage
Native	226	100
Irradiated 30 min	124	55
Carboxymethylated	220	98
Carboxymethylated and irradiated 30 min	125	55
Sample no. 2 reduced	215	96
Sample no. 4 reduced	220	98

The incubation medium contained: 250 mM sucrose, 5 mM Tris-HCl pH 7.4, 2 μM rotenone, 3 mM succinate, 5.2 mg of mitochondrial protein per ml, and 0.01 mM native or modified cytochromes. Irradiation and carboxymethylation was carried out as specified in Methods. Reduction of photooxidized cytochromes was carried out with 2-mercaptoethanol (50-fold molar excess over cytochrome) for 12 hr under a N_2 stream in aqueous solution, pH 5.5.

acid composition of the protein. The uniquely modified methionine was identified as Met-65 by CNBr fragmentation of the irradiated protein and subsequent amino end-group analysis [11, 12] : actually, from native cytochrome *c*, we obtained 0.86 moles of N-termainal glutamic acid (which follows Met-65) and 0.79 moles of N-terminal isoleucine (which follows Met-80); on the other hand, the photooxidized product yielded only 0.75 moles of N-terminal isoleucine, clearly showing that Met-65 had been modified. The selective attack on Met-65 is not in contrast with previous findings, even though the heme group, at pH 5.5, acts as a specific sensitizer for the photooxidation of Met-80 [11, 12]. In actual fact, a sufficiently high concentration of methylene blue was chosen to prevent light absorption by the heme and its consequent photoexcitation. Furthermore, the selective interaction of the dye with Met-65 is consistent with the location of this residue on the surface of the protein molecule, in contact with the solvent [1, 2].

In table 2, the activity of native and modified cytochrome *c* is reported in terms of the ability to restore electron transport and, thus, 0_2 consumption in cytochrome *c* depleted mitochondrial membranes. The depression of the activity to 55% upon conversion of Met-65 to the sulfoxide disagrees with the finding that the biological activity is fully retained by cytochrome *c* alkylated at the same residue [7]. A 45% decrease of catalytic efficiency was also observed upon irradiation of cytochrome *c*, which had been alkylated at Met-65, in order to prevent photooxidative attack on this residue [9] : the amino acid analysis of this irradiated sample did not differ significantly from that obtained for the alkylated non-irradiated protein (table 1). In all cases, treatment of

photooxidized cytochromes with 2-mercaptoethanol (which is known to reduce sulfoxides to thioethers [13]) resulted in a complete recovery of biological activity.

These data suggest that some oxidative modification, in addition to that affecting Met-65, must occur in cytochrome *c* as the consequence of visible light irradiation in the presence of methylene blue; such a modification would be responsible for the observed 50% loss of activity. The most probable sites of attack are the thioether bridges, between the 2 cysteinyl residues and the heme, which would not show up in the amino acid analyses. Direct evidence that this is the case was achieved by isolating the heme group and the attached cysteines from native and photooxidized cytochrome *c*.

After digestion of the heme undecapeptide with pronase (see Methods), the hydrolysate was repeatedly extracted with ether. The red material thus obtained was free from amino acids other than cysteine, as detected by thin layer chromatography [14] and quantitative amino acid analysis. The infrared spectrum (Nujol) of the product from irradiated cytochrome *c* showed 2 strong bands at 1040 and 1115 cm^{-1}, which were absent in the non-irradiated product. Infrared transitions in this frequency range are typical of sulfoxides. Therefore, we conclude that these 2 thioethers are the site of photooxidative attack.

This chemical modification is not accompanied by appreciable changes in either the absorption or the circular dichroism spectrum, both in the visible and in the far UV regions. Consequently, the ability of modified cytochrome *c* to mediate electron transport, although at a reduced rate, is to be ascribed to the lack of significant conformational rearrangements. However, it is not possible to exclude that the retention of about 50% protein activity is due to the photooxidation of only 1 out of the 2 thioether bridges.

It is interesting that, despite their close distance from the heme, no photooxidation of the thioethers occurs under conditions in which the heme acts as a photosensitizer for its fifth and sixth ligands [11, 12]. This finding can be explained by assuming that a specific spatial orientation of the sensitizer with respect to the cysteinyl residues is required in order to induce the photooxidation of their sulfur atoms. Such a requirement would be fulfilled much better by mobile free methylene blue than by the rigidly bound heme group. In agreement with this suggestion is the high stereospecificity of photosensitized reactions occurring inside a protein matrix [15].

References

[1] R.E. Dickerson, T. Takano, O.B. Kallai and L. Samson, Proceedings of the Wenner-Gren Symposium on Structure and Function of Oxidation Reduction Enzymes, August 1970, in press.
[2] R.E. Dickerson, T. Takano, D. Eisenberg, O.B. Kallai, L. Samson, A. Cooper and E. Margoliash, J. Biol. Chem. 246 (1971) 1511.
[3] F. Lederer and J. Tarin, European J. Biochem. 20 (1971) 482.
[4] G. Jori and G. Cauzzo, Photochem. Photobiol. 12 (1970) 231.
[5] H.A. Harbury and P.A. Loach, J. Biol. Chem. 235 (1960) 3640.
[6] M. Nomoto and Y. Narahashi, J. Biochem. 46 (1959) 653.
[7] K. Ando, H. Matsubara and K. Okunuki, Biochim. Biophys. Acta 118 (1966) 257.
[8] G. Lenaz and D.H. Maclennan, Methods in Enzymology 10 (1967) 499.
[9] G. Jori, G. Galiazzo, A. Marzotto and E. Scoffone, J. Biol. Chem. 243 (1968) 4272.
[10] C.H.W. Hirs, Methods in Enzymology 11 (1967) 197.
[11] G. Jori, G. Gennari, G. Galiazzo and E. Scoffone, FEBS Letters 6 (1970) 267.
[12] G. Jori, G. Gennari, M. Folin and G. Galiazzo, Biochim. Biophys. Acta 229 (1971) 525.
[13] G. Jori, G. Galiazzo, A. Marzotto and E. Scoffone, Biochim. Biophys. Acta 154 (1968) 1.
[14] E. Stahl, Dunnschicht Chromatographie (Springer Verlag, Berlin, 1962) p. 503.
[15] G. Jori, G. Gennari, C. Toniolo and E. Scoffone, J. Mol. Biol. 59 (1971) 151.