Inorganica Chimica Acta *125* (1986) 129–133

Photoredox Reactions in Cytochrome c and Cytochrome c_{551}

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(Received March 14, 1986; revised May 21, 1986)

Abstract

A previously obtained photomodified cytochrome c is analyzed in order to characterize the first coordination sphere of the heme iron. Electron spin resonance and electrochemical measurements show that iron is exa-coordinated in the photomodified cytochrome as well as in the native one and are in favour of tyrosine-67 being the sixth heme ligand. An investigation on the photoredox behaviour of cytochrome c_{551} (*Pseudomonas aeruginosa*) is also reported. The results obtained are discussed on the basis of the difference in the heme environment with respect to cytochrome c, so obtaining further confirmation of the conclusions drawn in the case of the mitochondrial protein.

Introduction

Although the redox properties of cytochrome c are at present under investigation by a number of research groups [1, 2], the intimate mechanism by which the heme iron alternates its oxidation state during electron transport in the mitochondrial respiratory chain still remains unclear.

Wc havc previously reported on the photoredox behaviour of cytochrome c in aqueous solution [3]. In that work we pointed out that: (i) the irradiation gives rise to the reduction of Fe(III) to Fe(II); (ii) the only photoreactive species is state III [4] of cytochrome c which contains methionine-80 bound at the sixth coordination position of iron; (iii) the primary photochemical act consists of an electron transfer from methionine-80 sulfur atom to iron; (iv) the photoreduced cytochrome, when reoxidized, leads to a modified cytochrome which exhibits an absorption spectrum lacking the 695 nm band, indicating that the methionyl residue is no longer coordinated to the heme iron.

Cytochrome c_{551} is known to play a respiratory role in various bacteria analogous to that of mitochondrial cytochrome c in eukariotes [5]. Despite this, bacterial protein differs from cytochrome c in the primary structure; it resembles the mitochondrial protein in the tertiary structure and has the same iron ligands [6, 7]. This homology makes the two proteins well suited for a parallel investigation on their chemical as well as biochemical properties. On this basis, in the present paper we compare the photoredox behaviour of the cytochromes c and c_{551} hoping to gain further insight into the photoreaction mechanism as well as the nature of the axial ligands in the photomodified protein from cytochrome c [3].

Experimental

Horse heart cytochrome c was purchased from Sigma Chem. Co. Samples of cytochrome c purified on Sephadex G 25 resin gave results identical to those obtained with unpurified protein.

Horse heart cytochrome c was purchased from Sigma Chem. Co. *Pseudomonas aeruginosa* cytochrome c_{551} was a gift of Professor Israel Pecht from the Department of Chemical Immunology, The Weizman Institute of Science, Rehovot, Israel. All other chemicals were reagent grade commercial products. UV–Vis spectra were recorded with a Jasco Uvidec 650 spectrophotometer. X-band electron spin resonance spectra were recorded with a Bruker 220 SE spectrometer which was calibrated using α,α'-diphenyl-pycrylhydrazyl (DPPH). pH measurements were performed with a Beckman Select Ion

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0020-1693/86/$3.50

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5000 pH-meter. Cyclic voltammetry and differential polarography experiments were performed by using a 4,4'-dipyridine modified gold electrode. The current–potential curves were recorded with an Amel mod. 460 Universal Stand. A three electrode system was used: the working electrode was a gold electrode Amel mod. 492 (area = 3.464 × 10^{-2} cm^2) and potentials were measured against a saturated calomel reference electrode (SCE) with an auxiliary platinum electrode. The voltammetry cell was thermostated at 25.0 ± 0.5 °C. The solutions were deaerated with pure argon prior to analysis and an argon atmosphere was maintained above the solutions during all the experiments. The irradiation equipment was as previously described [3]. Native protein solutions were irradiated at room temperature and the photoreaction monitored by electronic spectrophotometry. The photomodified cytochrome from cytochrome c was isolated, purified and lyophilized as described in the previous paper [3] and stored at –20 °C. Solutions were prepared by dissolving the lyophilized product in distilled water and then used within 24 h. Any attempt to separate the photoreaction product of cytochrome c_{551} was unsuccessful. Deaerated samples were obtained by bubbling pure argon into the solutions through a glass capillary for one hour. In the electron spin resonance (ESR) experiments the solutions were cooled after each irradiation inside the spectrometer cavity and the spectra recorded at 95 K.

Fig. 1. Electron spin resonance spectrum at 95 K of a 3 × 10^{-2} mol dm^{-3} aerated solution of native cytochrome c (pH ≃7) after irradiation with λ > 315 nm light at room temperature. For specific conditions, see 'Materials and Methods'.

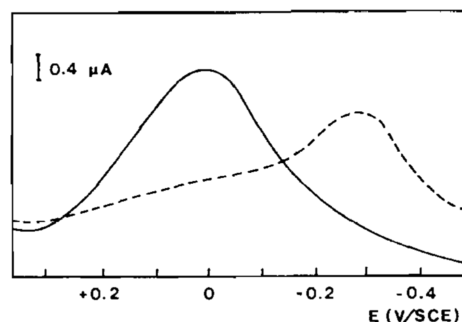


Fig. 2. Differential pulse polarography of native (——) and photomodified (-----) 5 × 10^{-3} mol dm^{-3} cytochrome c solutions containing 5 × 10^{-3} mol dm^{-3} 4,4'-bipyridine and 0.1 mol dm^{-3} $KClO_4$, pH 7. For specific conditions, see 'Materials and Methods'.

Results

Cytochrome c

Because of very short spin–lattice relaxation times, no electron spin resonance signals were obtained at 95 K with horse heart ferricytochrome c (nC) aqueous solutions. On the other hand, when nC aqueous solutions were irradiated with λ > 315 nm light in the ESR cavity at room temperature and then frozen to 95 K, an ESR spectrum resulted (Fig. 1). The spectrum did not change when oxygen or ferricyanide was added to the solutions and remained unaltered for several days in the pH range 5–11. An identical ESR spectrum was observed when the solutions were deaerated before irradiation.

Solutions prepared by dissolving isolated and lyophilized photomodified cytochrome (pmC) [3] showed similar ESR spectra at 95 K except for a broad (~180 G) absorption at $g = 2$, due to the presence of aggregates formed during the lyophilization process.

Differential pulse polarographic and d.c. cyclic voltammetry measurements were performed on nC and pmC solutions in the presence of 4,4'-dipyridine using a gold electrode to prevent absorption phenomena on the electrode surface [8]. The results obtained are reported in Fig. 2 where $E°$ of pmC is shifted with cathodic direction by about 250 mV with respect to that of nC. d.c. cyclic voltammetry experiments essentially confirm these results; in addition, they show the reversibility of the electrodic process.

As previously reported [3], re-oxidation of nC solutions with oxygen or ferricyanide, previously irradiated under anaerobic conditions, gives rise

to an absorption spectrum which shows essentially the features of the native Fe(III) protein, except for the 695 nm band which regains only in part its original intensity.

The irradiation of aerated solutions of nC resulted in an irreversible decrease of the 695 nm band which is characteristic of the binding of the methionine-80 sulfur atom to iron. The rate of the absorbance diminution at 695 nm decreases with increasing irradiation wavelength, becoming negligible at $\lambda > 450$ nm. This behavior exactly parallels the increase of the absorption bands characteristic of the Fe(II) cytochrome formed upon irradiation of oxygen-free solutions of nC [3].

The irradiation of aqueous solutions of pmC did not lead to appreciable photoreaction.

Cytochrome c_{551}

5×10^{-5} mol dm^{-3} deaerated solutions of *Pseudomonas aeruginosa* cytochrome c_{551} (c_{551}) were irradiated at pH $\simeq 5$ (natural pH) with light of $\lambda > 315$ nm. The spectral variations observed during the irradiation (Fig. 3) show an absorbance increase at 620 nm indicating that a Fe(III) cytochrome high-spin species was being formed. A similar spectral behaviour was observed in the pH range 4–9.

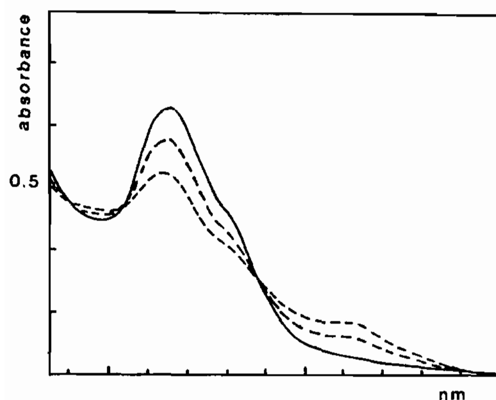


Fig. 3. Spectral changes observed upon $\lambda > 315$ nm irradiation of a 5×10^{-5} mol dm^{-3} deaerated aqueous solution of cytochrome c_{551} at pH $\simeq 5$: ——, before irradiation; -----, after irradiation.

The ESR spectrum obtained after 30 min of irradiation of deaerated solutions of c_{551} presents an evident signal with $g = 4$ which is typical of a ferric high-spin species. Unfortunately, the spectrum is complicated by a broad and intense band with $g = 2$ which is indicative of the occurrence of aggregation phenomena.

Deaerated aqueous solutions of c_{551} containing a 100 fold excess of sodium azide were irradiated at $\lambda > 335$ nm*. In these conditions the spectral variations indicated that Fe(II) cytochrome c_{551} was the photoreaction product. The spectrum of the irradiated solutions reverted to that of the reactant c_{551} when oxygen was bubbled into the solution.

Irradiations of azide containing c_{551} solutions were also carried out in an ESR cavity in the presence of phenyl-tert-butyl-nitrone (PBN) as a spin trap. The ESR spectrum obtained is typical of a $\cdot N_3$ radical–PBN paramagnetic adduct [9], so revealing the formation of $\cdot N_3$ radicals.

Discussion

We have previously reported [3] that the irradiation of nC in a deaerated aqueous solution at near neutral pHs leads to the reduction of the heme iron. This variation in the oxidation state of iron is accompanied by an irreversible change in the protein structure, yielding a modified cytochrome (pmC) that is chromatographically separable from the native one. The modification is likely to occur as a consequence of reactions initiated by the methionyl radical cation formed in the primary electron transfer process. The characterization of the pmC structure, as well as the identification of the processes by which the modification occurs are, however, out of the aim of the present investigation, in which the effect of the primary photoredox process on the axial coordination of the heme iron is essentially taken into consideration.

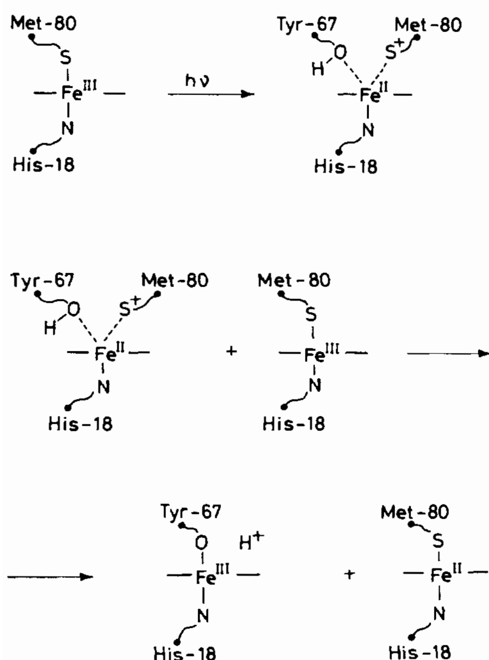
The results obtained in the previous study indicated that the peculiarity of pmC is the absence of the methionine-80 residue in the sixth axial position of the heme iron. On the other hand, the ESR signal which is seen to increase during the irradiation of deaerated solutions of nC (Fig. 2) is typical of low-spin heme complexes, indicating that the hexacoordination is retained also in pmC. The g values ($g_z = 2.50, g_y = 2.23, g_x = 1.81$) are quite different from those reported for nC at 1.6 K ($g_z = 3.06$, $g_y = 2.25$, $g_x = 1.25$) [10], thus confirming that pmC does not contain methionine-80 bound to the sixth position of the heme iron. On the other hand, the g values are very close to those obtained for ferric porphyrin complexes containing imidazole in one axial position and a phenoxy or a mercapto group in the other [11]. Similar values are reported for heme proteins having histidine and OH as axial ligands [10]. The previously reported spectrophotometric titration at 620 nm [3] indicated that pmC is converted to a high-spin form via a two-step process with pK values of 2.5 and 5.3. The fact that the first pK does not vary with respect to that of nC indicates, according to the ESR data, that histidine-18 is the fifth ligand also in pmC. The

*Irradiation at wavelengths shorter than 335 nm gave complications which can be ascribed to photoprocesses other than the photoredox one.

spectral titration curve for nC appears to represent a single-step process, suggesting a different coordination in the sixth heme iron position with respect to pmC. A correlation between the nature of the sixth ligand and the p*K* value is, however, not practicable.

The absence of free cysteine side chains in the protein and the invariance of the ESR spectrum shape in the pH range 5–11 allow one to rule out the axial coordination of S^- and OH^-, respectively. On the other hand, the hydroxyphenyl side chain of tyrosine-67 might be in a favourable position [4] to bind to iron in pmC. The results of differential pulse polarographic experiments (Fig. 2) are also in favour of this hypothesis: the coordination of tyrosine-67 to heme iron, followed by the de-protonation of the phenolic group, could be responsible for the stabilization of Fe(III) with respect to Fe(II), evidenced by the negative shift of 250 mV in the $E_{1/2}$ value. The phenolate coordination to iron porphyrin complexes was previously observed to cause a similar shift in the iron redox potential [12].

The increase of ESR signals observed during the irradiation of deaerated solutions of nC is clear evidence that the pmC is obtained independently of the presence of oxygen. This indicates that the Fe(II) cytochrome species formed in the primary photochemical act undergoes, in deaerated solution, a rapid oxidation by an oxidant other than oxygen. The difference in the redox potentials of nC and pmC suggests that the former acts as an oxidizing agent of the reduced pmC as soon as this is produced under irradiation (Scheme 1).



Scheme 1.

The 695 nm absorption is considered as the finger print of the native cytochrome c in its oxidized state. A decrease or the disappearance of this band is an indication either of iron reduction or of a modification of the protein. For this reason, particular attention has been devoted to this band, in the present as well as in previous work [3]. First, we observed that irradiation of aerated solutions of nC led to a decrease of the 695 nm band. Since the presence of oxygen prevents the accumulation of ferrous species, the observed behaviour entails a photoinduced modification of the protein. Second, the not complete return to the original intensity after the photoreduction–reoxidation sequence indicates that the ferrous species formed in deaerated solution indeed originates from the unreacted Fe(III) native cytochrome as indicated in Scheme 1. Finally, the fact that the rate of increase of the Fe(II) cytochrome bands at 520 and 550 nm in deaerated solution has a similar dependence on the irradiation wavelength demonstrates that the photomodification process occurs as a consequence of the intramolecular photoreduction of the heme iron.

The above statements are confirmed by the results obtained with cytochrome c_{551}. This protein resembles cytochrome c in having a similar tertiary structure and the same axial ligands. On the other hand, it does not contain a tyrosine residue in proximity of the heme group [4, 7]. Despite the similarity in the axial coordination which favours a common primary photoprocess occurring in both cytochromes, no experimental evidence of Fe(II) is obtained during irradiation of c_{551} in the absence of oxygen. The ESR and electronic spectra obtained after irradiation are typical of high-spin ferric cytochrome species. These results indicate that, due to the absence of a residue capable of acting as a trap for the ferrous intermediate, it undergoes rapid autooxidation, similar to that observed by irradiating cytochrome c at pH $\simeq 4$, when a H_2O molecule is likely to bind to the heme iron [4].

In conditions where azide replaces methionine as the heme axial ligand in c_{551}, the results obtained indicate that, just as reported for cytochrome c, an electron transfer from N_3 to iron is the primary photoprocess. Accordingly, the spectral changes observed during the irradiation of oxygen-free solutions of ferricytochrome c_{551} are indicative of the formation of ferrocytochrome c_{551}, from which, after reoxidation, the native ferric protein is obtained.

Acknowledgements

The authors wish to acknowledge the support from the Italian Ministry of Education. Thanks are also due to Mr. Luciano Righetti for his contribution in the experimental work.

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