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LETTER

Photoredox properties of free and caged iron(III) cytochrome *c*

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Cytochromes play a fundamental role in biological electron transfer reactions. Physiological functions of cytochrome *c* are conditioned by reversible oxidation-state changes of its iron central atom. It has been believed that cytochrome *c* transfers electrons via an outer-sphere mechanism when acting in the mitochondrial respiratory chain [1].

Along with the studies of outer-sphere long-distance electron-transfer reactions of cytochrome *c* [2–4], attention has been focused on the changes located to the iron central atom and its nearest environment. A substantial amount of knowledge clarifying the relationships between the composition of the chromophore and intramolecular photoredox reactions of cytochrome *c* and its derivatives has been obtained by Bartocci *et al.* [5, 6].

Our study is devoted to the investigation of the photoredox chemistry of horse-heart cytochrome *c* in two different forms. These being in the form of a free solvated complex and, secondly, as a complex caged in a phospholipid liposome bilayer or in sodium dodecylsulfate micelles.

Experimental

Horse-heart cytochrome *c* (Serva) and sodium dodecylsulfate, SDS (Fluka, purissimum) were used as received. Phospholipid liposome, PLL, was prepared from egg yolk by extraction, ultracentrifugation and ultrasonication according to the standard procedure [7]. Double distilled water and methanol (Lachema, reagent grade) distilled from $Mg(OCH_3)_2$ were used. All other chemicals used were of analytical grade (Lachema).

In all systems investigated, a water–methanol mixture (9:1 vol./vol.) was used as solvent; the iron(III) cytochrome *c*, Fe(III)cyt, concentration was 5×10^{-5} mol dm^{-3}. All solutions were left unbuffered at their natural (neutral) pH. The absence of buffers was so as not to introduce further ions into the systems. At such neutral pH, methionine and histidine are coordinated to the central atom via sulfur and nitrogen atoms, respectively [5]. The systems containing PLL were prepared by ultrasonication and handled as described in refs. 7 and 8.

The course of redox changes was monitored by electronic absorption spectroscopy in the visible region based on different absorption spectra of the parent Fe(III)cyt and photochemically produced iron(II) cytochrome *c*, Fe(II)cyt. Formaldehyde CH_2O was released by a stream of argon from the irradiated systems. Its identification as 3,5-diacetyl-1,4-dihydrolutidine was performed by the spectrophotometric method reported in ref. 9.

Irradiations were performed with a medium-pressure mercury lamp (125 W, RVK Tesla, Czechoslovakia) in a three-chambered photoreactor. The wavelengths in the range 320–600 nm were allowed to pass through a solution filter [10] into the part containing Fe(III)cyt. The intensity of incident light, determined by a Reinecke's salt actinometer [11] was of the order 10^{-3} Nhν min^{-1}. Irradiated solutions were deoxygenated by bubbling pure argon 30 min before and during irradiation, and were kept at 20 ± 2 °C.

Electronic absorption spectra (2 ml samples withdrawn from the photoreactor at suitable intervals of irradiation) were recorded on a M-40 spectrophotometer (Zeiss Jena, GRD) using 1 cm cells.

Results and discussion

The experimental results obtained may be summarized as follows.

(1) Irradiation of Fe(III)cyt in water–methanol solutions gave rise to the spectral changes which clearly indicate the course of photoreduction of Fe(III)cyt to Fe(II)cyt. The nature of the spectral changes was identical with that published by Bartocci *et al.* [5]. The molar absorption coefficients of Fe(III)cyt (parent compound) and Fe(II)cyt (prepared by quantitave reduction of Fe(III)cyt with a large excess of solid L-ascorbic acid) calculated from

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the spectra are 9900 and 21 500 mol^{-1} dm^3 cm^{-1}, respectively, at 552 nm (a peak maximum of Fe(II)cyt).

(2) On introducing gaseous oxygen (or air) in the dark into the systems, following a partial photoreduction of Fe(III)cyt to Fe(II)cyt the reappearance of the original spectrum of the parent Fe(III)cyt was observed.

(3) In the systems containing Fe(III)cyt and liposome (c(PLL) = 2.6×10^{-4} mol dm^{-3}) irradiation did not lead to any observable net photoreduction of Fe(III)cyt.

(4) The addition of oxalato anions $C_2O_4^{2-}$ (as a source of the anions the compound $K_2C_2O_4$ was used) to Fe(III)cyt solutions (up to 5×10^{-3} mol dm^{-3} $C_2O_4^{2-}$) had no effect on the Fe(III)cyt electronic absorption spectra (measured in the region 500–900 nm). When irradiating such oxalato anions containing systems, Fe(III)cyt was photoreduced and the rate of Fe(II)cyt formation increased with increasing concentration of the $C_2O_4^{2-}$ anions. Variation of liposome concentration (0; 1.3×10^{-4}; 2.6×10^{-4} mol dm^{-3}) in the systems of Fe(III)cyt (5×10^{-5} mol dm^{-3}) and $C_2O_4^{2-}$ (5×10^{-3} mol dm^{-3}) did not significantly influence the course of Fe(III)cyt photoreduction (Fig. 1).

(5) Irradiation of systems containing Fe(III)cyt in the presence of an over-critical micellar concentration of sodium dodecylsulfate (SDS) did not result in observable photoreduction of Fe(III)cyt either in the absence or in the presence of oxalato anions (up to 5×10^{-3} mol dm^{-3} $C_2O_4^{2-}$).

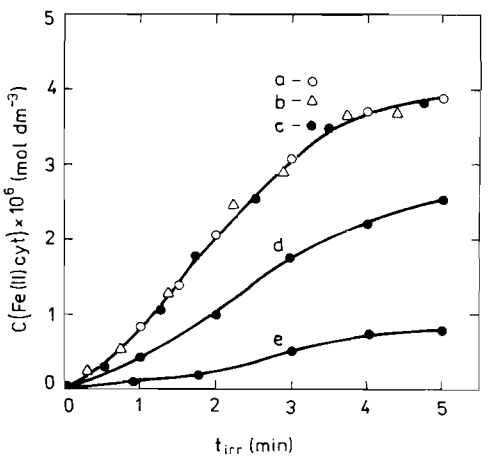


Fig. 1. Dependence of the concentration of iron(II) cytochrome c, Fe(II)cyt, on irradiation time, t_{irr}, for water–methanol (9:1 vol./vol.) solutions containing iron(III) cytochrome c (5×10^{-5} mol dm^{-3}), liposome (a = 0; b = 1.3×10^{-4}; c,d,e = 2.8×10^{-4} mol dm^{-3}), and oxalato anions $C_2O_4^{2-}$ (a,b,c = 5×10^{-3}; d = 1.8×10^{-4}; e = 1×10^{-4} mol dm^{-3}). Irradiation wavelengths 320–600 nm, temperature 20 ± 2 °C.

(6) Irradiation of systems containing Fe(III)cyt and oxalato anions (5×10^{-3} mol dm^{-3}) in the presence of oxygen resulted in a non-stoichiometric oxidation of the oxalato anions. During one hour of irradiation 1.8×10^{-4} mol $C_2O_4^{2-}$ was being oxidized which corresponded to approximatively 30 catalytic cycles.

(7) The electronic absorption spectra of the systems under investigation (Fe(III)cyt in the presence of methanol, liposome, oxalato anions) contained the 695 nm band, which was present also after the reoxidation of Fe(II)cyt to Fe(III)cyt.

Iron(III) cytochrome c can be photoreduced when being irradiated. In the primary photoredox step the photoreduction of the iron(III) central atom may be accompanied by an outer-sphere photooxidation of a molecule (ion) of the Fe(III)cyt environment [2–4], inner-sphere photooxidation of an axial ligand coordinated to the central atom [5, 6], photooxidation of part of the globin chains, or some of these modes may be realized simultaneously.

In the systems containing Fe(III)cyt in a water–methanol mixture, we believe that a methanol molecule is oxidized, as is usual in irradiated solutions of Fe(III) complexes in the presence of methanol [12–14]. The small size of the methanol molecules allows them to penetrate into the vicinity of the central atom. The presence of formaldehyde CH_2O in irradiated systems confirms a such proposal. From the experimental results obtained it cannot be unambiguously stated, however, whether a methanol molecule is oxidized in the primary photochemical step or in a secondary thermal redox reaction.

Phospholipid liposomes (PLL) are known to incorporate porphyrinatoiron biocomplexes into bilayers with the average particle size being a few tens of nanometers [8]. The preventive effect of PLL on the Fe(III)cyt photoreduction may be of consequence with one of the following processes:

(a) participation of PLL in redox processes, e.g. reoxidation of Fe(II)cyt by PLL;

(b) encapsulation of a cytochrome and a methanol molecule in the PLL cage and recombination of the primary photoredox products in the cage without their separation;

(c) separation of Fe(III)cyt from its redox partner due to an encapsulation of sole cytochrome in the PLL cage.

The results gathered in (4) allow the elimination of the alternatives (a) and (c). Moreover, the participation of PLL in redox processes of porphyrinatoiron compounds was not observed [8]. It seems to be probable that the electron transfer from the axially coordinated sulfur atom (methionine), nitrogen atom (histidine), or oxygen atom (methanol) to

the central atom Fe(III) is a reversible process with no net chemical redox change observed. It is worth mentioning that the radical $\dot{C}H_2OH$ which might be formed in the photoredox step

$$Fe(III)cyt + CH_3OH \xrightarrow{h\nu} Fe(II)cyt + \dot{C}H_2OH + H^+$$

is not only a good reductant ($E°(CH_2O/\dot{C}H_2OH) = -0.92$ V), but also a good oxidant ($E°(\dot{C}H_2OH/CH_3OH) = +1.29$ V) [15] and if it cannot escape, it is able to oxidize the central atom Fe(II) back to Fe(III) as at pH = 7, $E°$(Fe(III)cyt/Fe(II)cyt) = 0.26 V [3].

Unlike an electrically neutral methanol molecule, the anion $C_2O_4^{2-}$ bears the negative charge and, in addition, it is a irreversible redox agent. The coincidence of the electronic absorption spectra of Fe(III)cyt solutions in both the absence and presence of oxalato anions suggests that these anions do not penetrate to the iron central atom and coordinate to it (the axial coordination of an azido anion in Fe(III)cyt gave rise to spectral changes [5]). Systematic studies on the relationships between the structure of cytochromes *c*, localization of their large positive charge (the charges of Fe(III)cyt and Fe(II)cyt are 7+ and 6+, respectively [3]), binding sites of negatively charged redox counter-partners, and mechanisms of electron-transfer processes have been demonstrated. It has been shown [16] that there are at least six binding sites for anions on the surface of Fe(III)cyt and the kinetics of the electron-transfer processes is different for each of the binding sites. Ultrasonication of Fe(III)cyt, anions $C_2O_4^{2-}$, and PLL produces systems containing Fe(III)cyt–$C_2O_4^{2-}$ pairs embedded in bilayers of PLL. The ratio of $C_2O_4^{2-}$: Fe(III)cyt increases with the increase of $C_2O_4^{2-}$ concentration which is reflected in the dependence of the rate of Fe(III)cyt photoreduction on the concentration of $C_2O_4^{2-}$ anions. Based on the data summarized in (4) it can be deduced that the formation of mutually bonded Fe(III)cyt and $C_2O_4^{2-}$ anions is not influenced to a measurable extent by PLL and that the transfer of an electron from the bonded anion $C_2O_4^{2-}$ to the central atom Fe(III) yields the irreversible formation of Fe(II)cyt.

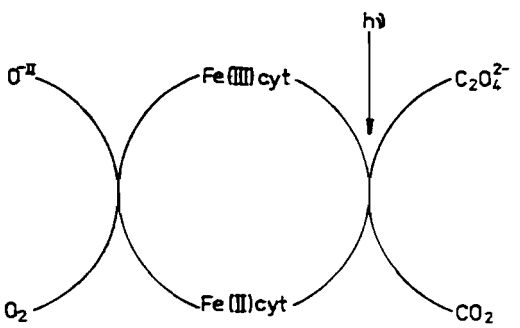
A similar effect was observed within the study of the photoredox behaviour of the systems containing the complex $[Fe(benacen)(CH_3OH)_2]^+$, oxalato anions and PLL in a water–methanol mixture (benacen = *N*,*N*′-ethylenebis(benzoylacetoneiminato) dianion). It was found [17] that PLL does not influence the course of Fe(III) photoreduction, and the quantum yield of Fe(II) was determined to be 0.16 in either the absence or presence of PLL.

Sigmoidal dependence of the concentration of a product versus reaction time are quite common in the chemical reactivity of biocomplexes. Evaluation of kinetic data fitting such dependences is not a simple matter and also explanation of the kinetics of the processes exhibited in Fig. 1 would require a lot of further detailed study.

Photochemical behaviour of the systems containing sodium dodecylsulfate, SDS, is rather different to those containing PLL. The critical micellar concentration of the anionic detergent SDS was determined to be 8×10^{-3} mol dm^{-3} [18, 19]. SDS specifically surrounds cationic Fe(III)cyt and micelles with a diameter (including the core and the Stern layer) of several nanometers are formed [18]. The distance between a Fe(III)cyt encapsulated in a micelle and anionic $C_2O_4^{2-}$ is thus large enough to prevent an effective electron transfer from an anion $C_2O_4^{2-}$ to an excited Fe(III)cyt, and there was no observed net chemical change. Molecules of SDS themselves do not quench excited iron complexes [18].

Point (6) of the results concerns the photocatalytic properties of Fe(III)cyt. The reactions of the catalytic cycle are shown in Scheme 1. Within one hour of irradiation the number of redox cycles of cytochrome *c* is about 30. One of the possible reasons why this value is low may be the relatively low quantum yield of the photoreduction of Fe(III)cyt. This was not determined exactly but is of the order of 10^{-4}. It should be added, however, that some side reactions (for example, partial oxidation of the globin chains of cytochrome *c* by traces of singlet oxygen formed by a transfer of energy from an excited Fe(III)cyt to a dioxyen molecule in its ground triplet state) may occur in the irradiated systems. Cytochrome *c* is thus able to act as a catalyst of both the spontaneous (biological) and photochemical electron-transfer reactions.

The presented experimental results, in accordance with literature data, show that the porphyrin ring of cytochrome *c* is not involved in redox decomposition reactions (in each case electronic absorption spectra correspond to porphyrinatoiron complexes).



Scheme 1.

Moreover, any significant denaturation of cytochrome *c* does not occur (in systems containing denaturated complexes, autooxidation reactions are so fast that they are unmeasurable by techniques of continuous photolysis).

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