Modification of Met-80 by carboxymethylation [3, 4] or methylation perturbs the equilibrium constant for the conformation change involved in the alkaline isomerization so that it can be observed in the neutral pH range. In the latter case just one 'alkaline' form is observed by NMR and the resonances assigned to Met-80 now show up with twice the intensity observed for the corresponding peaks in the unmodified protein. Also in the carboxymethylated form of ferricytochrome *c* the Met-80 methyl resonances are clearly visible in the same ppm range.

The NMR results were correlated with the signals appearing at alkaline pH in EPR spectra (9.2 GHz and 17 K). The two forms observed by NMR thus correspond to the $g_z = 3.39$ and $g_z = 3.57$ signals. An additional EPR species with $g_z = 3.26$ was correlated with the form of ferricytochrome *c* where the conformation change, but not the deprotonation of the group with pK = 11, has occurred. This form of cytochrome *c* can also be observed at high temperatures [5] or high urea concentrations [6] at neutral pH. As expected both carboxymethylation and methylation of Met-80 yielded the $g_z = 3.26$ species as the major form at neutral pH.

The occurrence of two alkaline forms, as described above, is most likely due to a slow exchange between the two chiral binding modes of Met-80 to the haem iron. This is also strongly supported by the modification experiments on Met-80. The anomalously broadened resonances stemming from the Met-80 methyl group (the width at half height is approximately 350 Hz) are probably due to the slow flipping rate of the nearby Tyr-67. Such an exchange can lead to appreciable broadening since the scalar relaxation of the Met-80 methyl resonance, due to the haem iron, will be modulated by the exchange rate [7]. Considering the positive charge on the sulphur atom of Met-80 in the modified proteins, it is likely that repulsion between the sulphur and the iron will lead to an increased iron–sulphur bond length. This would rapidly explain the disappearance of the 695 nm band, the decreased hyperfine shifts in NMR as well as the EPR signals appearing at alkaline pH.

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Q9

Photoredox Behaviour of Cytochrome *c* in Aqueous Solution

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It is well known that cytochrome *c* has a fundamental importance in biological electron transfer chains. Following the definition of the International Union of Biochemistry its mode of action is electron and/or hydrogen transport by virtue of a reversible valency change of its heme iron. For this reason, a number of authors are attracted by the study of the redox behavior of this compound [1]. Photochemical methods offer a valuable means of obtaining information on the redox properties of iron proteins. In fact, several papers have been recently published on the photoredox behavior of iron porphyrins [2–4], which are known to be the prosthetic groups of heme-proteins.

Here we report some preliminary results obtained in the course of an investigation on the photochemical behavior of cytochrome *c* in aqueous solution.

Results and Discussion

The irradiation in the 330–400 nm range of previously deaerated solutions of cytochrome *c* gave rise to spectral variations (Fig. 1) which clearly indicate that Fe(III) was reduced to Fe(II) without apparent involvement of the porphyrin ring. The plot of

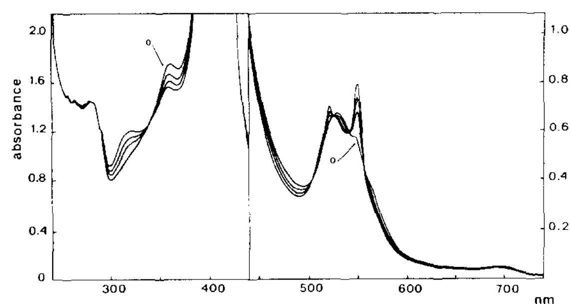


Fig. 1. Spectral variations observed during the irradiation of a 6.5×10^{-5} *M* deaerated aqueous solution of cytochrome *c* at 25 °C, pH = 7.0 initial spectrum; irradiation periods, 10 min.

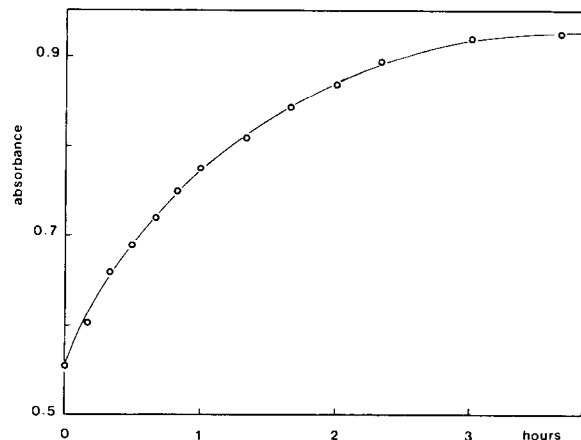
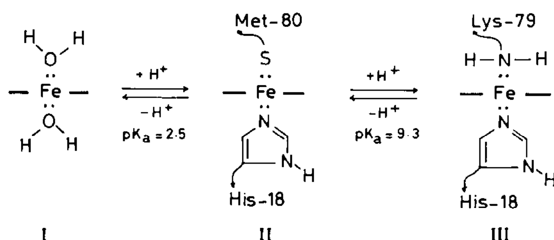


Fig. 2. Absorbance values at 548 nm of a 6.5 × 10^{-5} *M* deaerated aqueous solution of cytochrome *c*, as a function of irradiation time. Temperature 25 °C; pH = 7.

the absorbance *vs.* time observed at 548 nm (Fig. 2) shows that the photoreduction does not follow zero-order kinetics. Since the solution absorbs 100% irradiation light for the whole period of the photochemical run, the above behavior may be ascribed to a re-oxidation of the reaction product. The fact that no spectral changes were observed in the dark after irradiation suggests that the re-oxidation process occurs as a consequence of light absorption of the reduction product. This hypothesis is experimentally supported by the finding that irradiation of cytochrome *c* solutions, which were previously reduced by sodium dithionite, leads to spectral variations that are exactly opposite to those observed in the photoreduction experiments.

The photoreduction rate was observed to be strongly dependent on pH. The rate increases from pH 2 to pH 5, and then decreases from pH 8 to pH 11. It is known [1] that ferricytochrome *c* has five pH-dependent conformational states, which differ from one another depending on the nature of the axial ligands. An interpretation of three pH states of cytochrome *c* is illustrated in Scheme 1. The comparison of the equilibria shown in Scheme 1 with the observed dependence of the photoreduction on pH suggests that Species II, which contains methionine



Scheme 1.

and histidine as iron axial ligands, is the only photoreducible species. This is an indication that an electron transfer from the sulfur of the methionine to the central iron should be responsible for the primary photoreduction.

The absorption spectrum of the photochemically reduced cytochrome *c* was found to be identical to that obtained for the chemically reduced hemeprotein, thus suggesting that both methionine and histidine are retained in the axial positions of central iron also after photoreduction. This conclusion gives rise to a question as to the fate of the hole on the sulfur atom after the electron transfer to iron. Studies aiming at the resolution of this problem are now in progress in this laboratory.

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Q10

Metal Ion Insertion into N-Alkylated and N,N′-Dialkylated Porphyrins

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Addition of $FeCl_3$/Fe to N-(2,2′-bis-p-chlorophenylethenyl)tetraphenyl porphyrin yields the iron(II) insertion product which has been characterized by extensive ^1H and ^2H NMR studies. The pyrrole protons of this and other N-alkyl porphyrin Fe(II) complexes show up as four equally intense resonances with chemical shifts at 25 °C of 28, 20, 8 and –6 ppm. The NMR spectra clearly distinguish between N-alkyl porphyrin complexes of Fe(II) and Fe(III) complexes of similar symmetry in which a carbene is inserted into the Fe–N bond. Treatment of N,N′-bis(2,2′-*p*-chlorophenylethenyl)tetraphenyl porphyrin with metal carbonyls can result in the rupture of one or both N–C bonds. Thus with $Fe_3(CO)_{12}$ the iron(II) carbene complex TPPFe (C = $C\{C_6H_4Cl\}_2$) is formed while $Ni(CO)_4$ nickel is inserted into one N–C bond to form I. (*See next column*).