BBA 46263

CYTOCHROME *c* INTERACTION WITH THE MITOCHONDRIAL MEMBRANE: A SPIN LABEL STUDY

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(Received October 5th, 1971)

SUMMARY

Horse-heart ferrocytochrome *c* has been labeled with *N*-(2,2,5,5-tetramethyl-3-pyrrolidinyl-1-oxyl) iodoacetamide at methionine-65. The paramagnetic resonance spectrum of labeled ferricytochrome *c* indicates a weak immobilization of the radical ($\tau_c = 9.3 \cdot 10^{-10}$ sec) which becomes stronger upon binding of labeled cytochrome *c* to cytochrome *c*-depleted mitochondrial membranes ($\tau_c = 3.3 \cdot 10^{-9}$ sec). The hyperfine coupling constant remains, however, unchanged (16.7 $\pm$ 0.1 gauss) indicating that the cytochrome *c* binding site is highly polar. The region where cytochrome *c* is bound to the membrane is insensitive to large variations of medium viscosity.

INTRODUCTION

Recently, the interaction between proteins and membranes has been studied by labeling either the protein or the membrane with stable free radicals[1-3], termed spin labels[4], which can be utilized as "reporter molecules"[5]. The nitroxide radical is very stable to chemical modifications and its paramagnetic resonance spectrum is sensitive to the environment[1,2]. The hyperfine coupling constant A^n and the correlation time τ_c, which are functions of polarity and viscosity respectively, can supply important information regarding the microenvironment of biologically interesting molecules labeled with the nitroxide.

Horse-heart cytochrome *c* is particularly suitable for the study of membrane protein interactions: in fact, its three dimensional structure is known[8,9] and chemical methods are available for spin labeling non-functional aminoacids[10,11]. Moreover, it offers the unique possibility of disrupting and reconstituting the protein membrane complex without impairing electron transport or ATP synthesis[13]. Furthermore, the localization of methionine-65 on the portion of the molecule probably specifically involved in the binding with the membrane[8,9], gives additional interest to the study of the microenvironment affecting a spin label covalently bound to the side chain of this amino acid.

METHODS AND MATERIALS

Mitochondria from rat liver and cytochrome *c*-depleted mitochondria were prepared as previously described[12,13]. The spin label used was *N*-(2,2,5,5-tetramethyl-3-pyrrolidinyl-1-oxyl) iodoacetamide, prepared according to KABAT *et al.*[14], from 2,2,5,5-tetramethyl-3-aminopyrrolidine-1-oxyl[15], iodoacetic acid and dicyclohexylcarbodiimide. Alkylation of methionine-65 was carried out essentially according to ANDO *et al.*[10] for 64 h. Its extent was evaluated by amino acid analyses before and after performic acid oxidation[16] (Erba aminoacid analyzer). No evidence was found of alkylation of amino acids other than methionine, which was alkylated more than 50 %.

Protein content was determined by a biuret method[17].

Optical measurements were carried out in Hitachi Perkin Elmer spectrophotometer (Model 124). Paramagnetic resonance spectra were recorded on a Varian E3 spectrometer.

Cytochrome *c* (Type III from horse heart) was a Sigma product. All other reagents were analytical grade products.

The correlation time τ_c was obtained by the relation[4]:

$$\frac{1}{T_2(0)}\left[\frac{T_2(0)}{T_2(\mp 1)}-1\right]=\left[\frac{b^2}{8}\pm\frac{4}{15}\,b\Delta\gamma B\right]\tau_c$$

using the experimentally determined linewidth parameters T_2 (0) and T_2 ($\mp$1) and the reported hyperfine interactions and *g*-factor anisotropies (for values and symbols, see refs. 2 and 4).

The visible absorption spectrum of spin labeled cytochrome *c* is identical to native cytochrome *c* in the visible region between 400 and 600 nm, the contribution of the absorption of the label to the spectrum being not detectable as a consequence of its low extinction coefficient and concentration. The identity between the spectrum of native and alkylated cytochrome *c* indicates that alkylation has not occurred at residue 80, which results in a decrease of the extinction coefficient of the α and in an increase in that of the γ band[10,11].

RESULTS AND DISCUSSION

The rate of oxygen consumption of mitochondrial membranes falls to values close to zero when cytochrome *c* is extracted. Restoration of the activity can be obtained by adding cytochrome *c*[13].

In Fig. 1, the respiratory response of cytochrome *c*-depleted membranes to the addition of a non saturating amount of cytochrome *c* (native or spin labeled) is reported. No appreciable difference is observed when the labeled hemoprotein is used instead of the native one. This indicates that the labeled protein has conserved its properties intact, as it would be expected from alkylation of methionine-65 but not methionine-80[10,11]. Moreover, a reduced *minus* oxidized difference spectrum of mitochondrial membranes reconstituted with labeled cytochrome *c* has indicated that all labeled cytochrome *c* can be reduced enzymatically (succinate in the presence of 1 mM KCN was used as a reductant for cytochrome *c*).

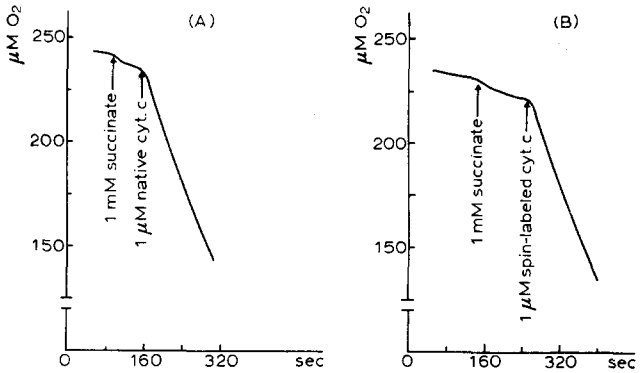

Fig. 1. Effect of the addition of native and spin-labeled cytochrome *c* on the respiration of cytochrome *c*-depleted mitochondria. The incubation medium contained: 0.25 M sucrose, 5 mM Tris–HCl, pH 7.5, 2 μM rotenone, and 5 mg protein per ml. Respiration was measured polarographically with a conventional Clark platinum electrode.

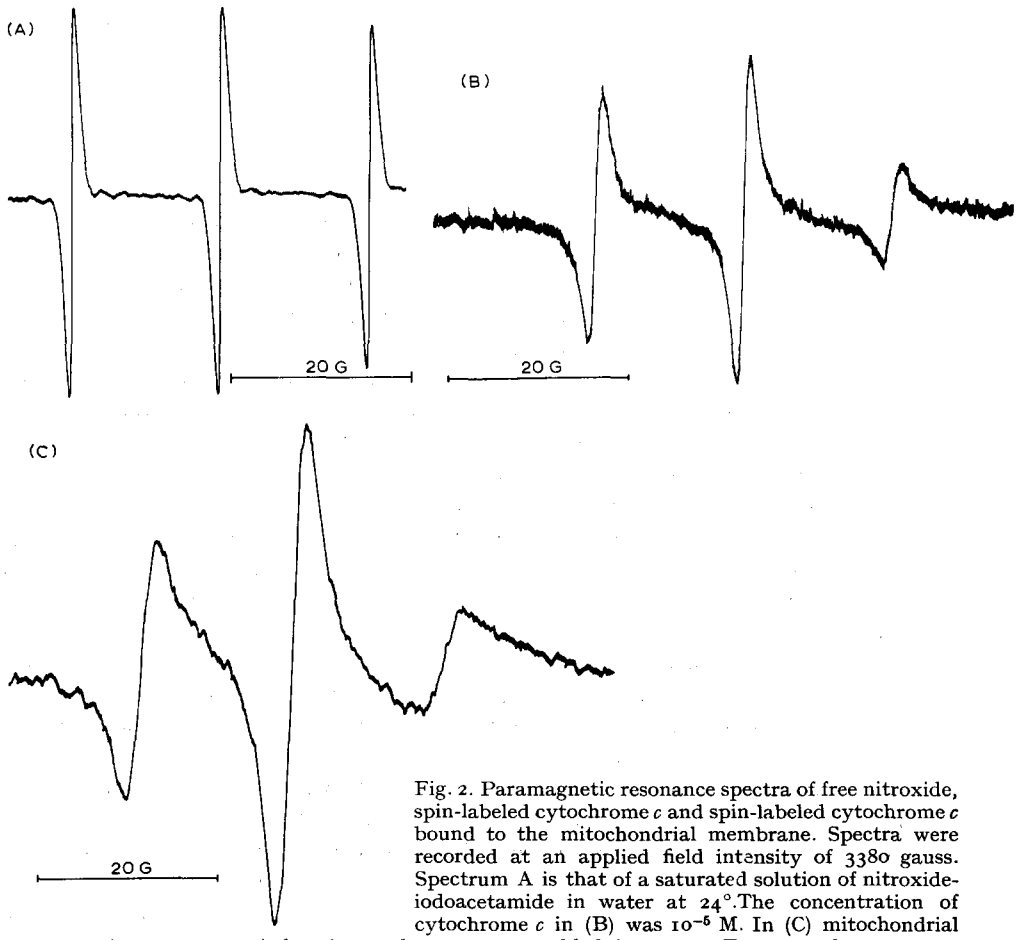

Fig. 2. Paramagnetic resonance spectra of free nitroxide, spin-labeled cytochrome *c* and spin-labeled cytochrome *c* bound to the mitochondrial membrane. Spectra were recorded at an applied field intensity of 3380 gauss. Spectrum A is that of a saturated solution of nitroxide-iodoacetamide in water at 24°.The concentration of cytochrome *c* in (B) was 10^{-5} M. In (C) mitochondrial concentration was 50 mg/ml and cytochrome *c* was added in excess. Free cytochrome *c* was then removed by centrifugation of mitochondrial membranes at 20000 × *g* for 10 min.

In Fig. 2A, the paramagnetic resonance spectrum of nitroxide radical in water appears with three almost identical lines as reported previously[4]. The calculations of the rotational correlation time according to KIVELSON[19] gives a value of $2.4 \cdot 10^{-11}$ sec, which is in good agreement with the value of $2.5 \cdot 10^{-11}$ sec obtained by DROTT *et al.*[20] from the nitroxidebromoacetamide and STONE *et al.*[4] for another simple nitroxide molecule. The hyperfine coupling constant was 16.7 ± 0.1 gauss in a series of 9 experiments.

The covalent binding of nitroxide radical with cytochrome *c* to form a carbamidomethylated product results (Fig. 2B) in a decrease in the magnitude of both the high- and the low-field lines, which are also broadened. This result indicates that the radical bound to the protein is weakly immobilized. From the experimentally determined spectrum it is possible to calculate a correlation time of $9.3 \cdot 10^{-10}$ sec, in agreement with a value of $1.0 \cdot 10^{-9}$ obtained with a yeast cytochrome *c* with the label attached to the cysteinyl residue[20]. A $\tau_c = 2 \cdot 10^{-10}$ has been found by BARRATT *et al.*[21] for cytochrome *c* randomly spin labeled at NH_2 groups.

The rotational correlation time of the protein at 24° in 0.25 M sucrose calculated by means of the Stokes–Einstein equation was $5.0 \cdot 10^{-9}$ sec assuming Dickerson values for the diameters of the molecule ($30 \times 34 \times 34$ Å)[22]. The difference between the rotational correlation time calculated from the spin label spectrum and that calculated with Stokes–Einstein equation, indicates that the motion of nitroxide radical is, to a large extent, independent from that of the protein. BARRATT *et al.*[21] and DROTT *et al.*[20] have arrived at a similar conclusion on the basis of analogous experiments.

In Fig. 2C the spectrum of spin labeled cytochrome *c* bound to the membrane of cytochrome *c*-depleted rat liver mitochondria is shown. A further immobilization of the radical with respect to free cytochrome *c* is evident from the broadening of the spectral lines and the decreased intensity of the low and high field lines with respect to the central line. The correlation time under these conditions is $2.3 \cdot 10^{-9}$ sec. This value can be compared with that of Fig. 2B and indicates that the motion of nitroxide radical becomes more hindered when cytochrome *c* is bound to the mitochondrial membrane. It is worth mentioning that a *c* of $2.5 \cdot 10^{-9}$ sec has been found by DROTT *et al.*[20] for membrane-bound bakers yeast cytochrome *c* selectively spin labeled at the cysteinyl residue by nitroxide homoacetamide. The hyperfine coupling constant remains practically unchanged with respect to both free label and cytochrome *c* bound nitroxide (16.8 ± 0.1 gauss, 9 experiments). This value should be compared with the hyperfine coupling constant of nitroxide iodoacetamide in decane which is 14.5 gauss.

If the viscosity of the medium in which cytochrome *c* is dissolved is increased by changing sucrose concentration[23] (Fig. 3) the correlation time of spin labeled cytochrome *c* is also increased. This indicates that methionine-65 and its label are exposed to the solvent, since the tumbling of the protein is already slower than that of the label (see above and BARRATT *et al.*[21] and DROTT *et al.*[20]). Upon binding of cytochrome *c* to cytochrome *c*-depleted mitochondria the correlation time of the label becomes insensitive to the viscosity of the medium. This result, together with that reported in Fig. 2C, suggests that the label is facing or interacting with the membrane. An alternative possibility can also be considered, that the cytochrome *c* region containing methionine-65 does not interact with the membrane, but with the solvent. In this case one must assume that the motion of the label reflects the motion of the protein, in

order to account for the result of Fig. 2C, and consider the decreased motion of the label as a consequence of the decreased motion of the bound protein. This interpretation would not be in agreement with the above conclusion and that of DROTT *et al.*[20] and BARRATT *et al.*[21], that the motion of the label is independent of the motion of the protein. Moreover, if the label were exposed to the solvent, in case of bound cytochrome *c*, on top of the increased correlation time due to protein immobilization, still the viscosity of the medium should affect the mobility of the nitroxide.

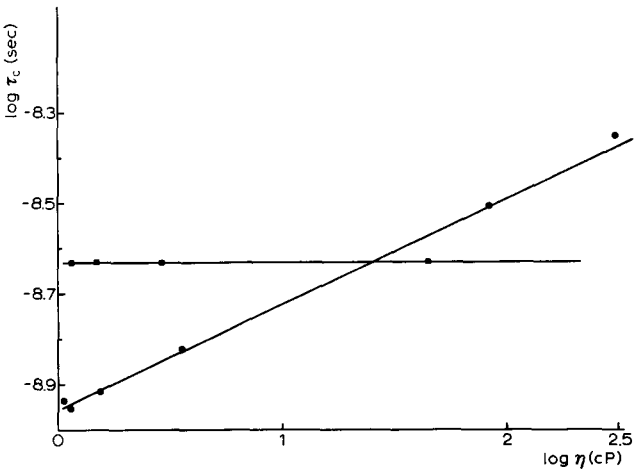

Fig. 3. Relationship between the correlation time of nitroxide radical attached to free and bound cytochrome *c* as a function of medium viscosity. Experimental conditions as in Fig. 2C. Viscosity was varied by resuspending the membranes or dissolving cytochrome *c* in sucrose solutions of known concentrations. Viscosity values were those reported in ref. 23, interpolated for the temperature of the experiments of 24°.

In conclusion, the region of cytochrome *c* which *x*-ray analyses have indicated as one of the probable candidates for binding to the mitochondrial membrane has been labeled with the free radical nitroxide iodoacetamide at residue 65. The interaction of the cytochrome *c* site which includes methionine-65 appears to occur with a polar region of the membrane. This region is, however, partially shielded from the suspending medium. Finally if cytochrome *c* is considered a portion of the mitochondrial membrane, and not simply a ligand, the general conclusion may be drawn that biological membranes contain in their interior, not only hydrophobic structures, but also hydrophilic regions.

REFERENCES

1 C. L. HAMILTON, AND H. M. MCCONNELL, in A. RICH AND N. DAVIDSON, *Structural Chemistry and Molecular Biology*, W. H. Freeman and Co., San Francisco, 1968, p. 115.
2 O. H. GRIFFITH, AND A. S. WAGGONER, *Accounts Chem. Res.*, 2 (1969) 17.
3 B. CHANCE, H. WOHLRAB, A. AZZI, S. FLEISCHER, AND C. P. LEE, in J. M. TAGER, S. PAPA, E. QUAGLIARIELLO AND E. C. SLATER, *Electron Transport and Energy Conservation*, Adriatica, Bari, 1970, p. 317.
4 T. G. STONE, T. BUCKMAN, P. L. NORDIO, AND H. M. MCCONNELL, *Proc. Natl. Acad. Sci. U.S.*, 54 (1965) 110.
5 H. R. HORTON, AND D. E. KOSHLAND, JR., *Methods Enzymol.*, 11 (1967) 856.
6 R. BRIERE, H. LEMAINE, AND A. RASSAT, *Bull. Soc. Chim. France*, 3273 (1965).

7 T. KAWMURA, S. MATSUNAMI, AND T. YONEZAWA, *Bull. Chem. Soc. Japan*, 40 (1967) 1111.
8 R. E. DICKERSON, D. EISENBERG, T. TAKANO, O. BATTIFAI, AND L. SAMSON, *Abstr. 8th Int. Congr. of Biochemistry, 1970*, p. 13.
9 R. E. DICKERSON, T. TAKANO, D. EISENBERG, O. B. KALLAI, L. SAMSON, A. COOPER, AND E. MARGOLIASH, *J. Biol. Chem.*, 246 (1971) 1511.
10 K. ANDO, H. MATSUBARA, AND K. OKUNUKI, *Biochim. Biophys. Acta*, 118 (1966) 257.
11 A. SHEJTER, AND I. AVIRAM, *J. Biol. Chem.*, 245 (1970) 1552.
12 A. SCARPA AND A. AZZI, *Biochim. Biophys. Acta*, 150 (1967) 473.
13 E. E. JACOBS, AND D. R. SANADI, *J. Biol. Chem.*, 235 (1970) 531.
14 D. KABAT, B. HOFFMAN, AND A. RICH, *Biopolymers*, 9 (1970) 95.
15 E. G. ROSANTZEV, AND L. A. KRINTKAYA, *Tetrahedron*, 21 (1965) 491.
16 D. H. SPACKMAN, W. H. STEIN, AND S. MOORE, *Anal. Chem.*, 30 (1958) 1190.
17 E. LAYNE, *Methods Enzymol.*, 3 (1967) 447.
18 T. YONETANI, *J. Biol. Chem.*, 240 (1965) 4509.
19 D. J. KIVELSON, *J. Chem. Phys.*, 33 (1960) 1094.
20 H. R. DROTT, C. P. LEE, AND T. YONETANI, *J. Biol. Chem.*, 245 (1970) 5875.
21 M. D. BARRATT, D. K. GREEN, AND D. CHAPMAN, *Biochim. Biophys. Acta*, 152 (1968) 20.
22 R. E. DICKERSON, M. L. KOPKE, J. WEINZIERL, J. WARNUM, D. EISENBERG AND E. MARGOLIASH, *J. Biol. Chem.*, 242 (1967) 3015.
23 *Handbook of Biochemistry*, The Chemical Rubber Co., Cleveland, 1968, p. J-250.

Biochim. Biophys. Acta, 256 (1972) 619–624