Vol. 92, No. 3, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 12, 1980 Pages 743-748

BINDING OF CYTOCHROME c TO CYTOCHROME c - OXIDASE IN INTACT MITOCHONDRIA. A STUDY WITH RADIOACTIVE PHOTOAFFINITY-LABELED CYTOCHROME c

Maria Erecińska*, Reiko Oshino** and David F. Wilson

Department of Pharmacology and
Department of Biochemistry and Biophysics
University of Pennsylvania, Medical School
Philadelphia, PA 19104

Received December 13, 1979

SUMMARY: [^{3}H]-p-Azidophenacylbromide-(methyl-4-mercaptobutyrimidate)-cytochrome c from Saccharomyces cerevisiae was prepared and its properties determined. The radioactive photoaffinity-labeled cytochrome c was linked by irradiation into a covalent complex with cytochrome c oxidase. Analysis of the complex on SDS-polyacrylamide gels showed that cytochrome c bound to one of the smaller subunits of cytochrome c oxidase with an apparent molecular weight of 15,000.

INTRODUCTION

We have shown previously (1,2) that photoaffinity-labeled derivatives of cytochrome c can be synthesized which upon irradiation with cytochrome c - depleted mitochondria are inserted covalently into the mitochondrial membranes. Fractionation of these membranes with salts in the presence of detergents demonstrated that the labeled cytochrome c was bound covalently to cytochrome c oxidase in a 1:1 molar complex. Preliminary experiments (2) on sodium dodecylsulfate (SDS) polyacrylamide gels suggested that one of the smaller subunits of cytochrome c oxidase was the site of interaction with cytochrome c. This paper reports the results of the studies carried out with radioactive, photoaffinity-labeled cytochrome c whose binding to cytochrome c oxidase and its subunits can be followed conveniently and sensitively on polyacrylamide gels after dissociation of the complex with SDS.

---

*ME is an Established Investigator of the American Heart Association
**Permanent address: Laboratory of Biochemistry, Kobe Yamate Women's College Kobe, Japan.
Supported by USPHS GM 12202.

0006-291X/80/030743-06$01.00/0

MATERIALS AND METHODS

Preparation of radioactive cytochrome c

Saccharomyces cerevisiae cytochrome c, 100 mg (Sigma St. Louis, Mo.,) was dissolved in 2 ml of 50mM phosphate buffer, pH 8.5 and added to a vial containing ~10 μmoles of [^{3}H]-iodoacetic acid (Amersham, Arlington, Ill.; specific radioactivity 82 mCi/mmol) which had previously been solubilized with 0.3ml of the same phosphate buffer. The mixture was transferred to a thermostated glass vessel and the vial rinsed with 0.8ml of the phosphate buffer. The incubation was carried out at 28°C for 5h with continuous, gentle stirring. The reaction was terminated by filtration through a column (20 x 1 cm) of Sephadex G-25 (Sigma, St. Louis, Mo)preequilibrated with 50mM Pi buffer (pH 8.5). The elution was carried out with the same buffer and the flow adjusted so that cytochrome c was eluted in a volume of 3-4ml. The radioactive cytochrome c was well separated from the non-reacted iodoacetic acid. The specific radioactivity of cytochrome c was 15 mCi/mmol.

Preparation of p-azidophenacylbromide-(methyl-4-mercaptobutyrimidate)-cytochrome c. The preparation was done as described in ref. 2. The number of photoactive groups was estimated from absorbance measurements at 285 nm [λ_{mM} = 20 cm^{-1} for p-azidophenacyl bromide (3)] after the subtraction of absorbance due to cytochrome c. Concentration of cytochrome c was estimated at 550 nm - 540 nm (reduced-oxidized) using a millimolar extinction coeffi-cient of 19.7 cm^{-1}.

Irradiation of cytochrome c - depleted mitochondria: Pigeon breast cytochrome c-depleted mitochondria were prepared by the method of Jacobs and Sanadi (4) as modified by Boveris et al (5). They were suspended at a protein concentration of 20 mg/ml in 0.25 M sucrose-0.01 M phosphate buffer at pH 7.4. Forty micromolar photoaffinity-labeled cytochrome c was then added and the mixture was irradiated with constant stirring for 30 min in an open 50 ml beaker (total fluid volume 6 ml) using a medium pressure, 100 W, water-cooled mercury arc. The distance between the light source and the surface of the irradiated mitochondria was approximately 5 cm. After irradiation, the suspension was centrifuged for 10 min at 8000 x g, the pellet suspended in 0.25 M sucrose 0.01 M phosphate buffer at pH 7.4 and further fractionated with detergents and salts as described by Sun et al. (6).

Spectral studies were carried out using a Johnson Foundation dual wave-length scanning spectrophotometer provided with a digital wavelength drive on one monochromator, the other being set at the reference wavelength. The operation of this instrument is described in Ref. 7.

Polyacrylamide gel electrophoresis: The isolated cytochrome c-cytochrome c oxidase complex was treated with 10 volumes of 5% trichloroacetic acid, the precipitate washed once with 10 mM phosphate buffer pH 7.0 and once with either cold chloroform-methanol (2:1)mixture or with cold acetone containing 4% water (v/v). The sediment was washed again with 10mM phosphate buffer and finally suspended in the same medium. SDS-polyacrylamide gel electrophoresis was carried out either by the procedure of Weber and Osborn [(8) 10% gels with acrylamide-bisacrylamide ratio of 19:1] or by the method of Laemmli (9) (12% gels, acrylamide-bisacrylamide ratio of 20:1). The gels were stained with Coomassie blue G and destained in a quick gel destainer. The stained gels were scanned in a Gilford spectrophotometer equipped with a scanning attach-ment. The same gels were then cut into 1mm slices and digested for 16 h at 50°C with 0.1 ml of 30% hydrogen peroxide. The radioactivity was measured using a Searle Aqueous Counting Scintillant. The molecular weights were determined by comparison with standard protein references (m.w. 13,000-45,000)

Protein concentration was determined by the biuret reaction using bovine serum albumin as the standard.

RESULTS

There are three residues in yeast cytochrome c which are susceptible to alkylation with mono-iodoacetic acid under mild conditions; cysteine-103, histidine-33, and methionine-65. Modification of the histidine and methionine residues requires higher temperature (40°C) and lower pH (5-6) as well as longer incubation time (>5h) than of cysteine-103 (10), therefore we have every reason to believe that under experimental conditions described here alkylation of cytochrome c occurred only at the latter residue. It has been shown (11) that modification of this residue caused no alteration in enzymatic activity, thermodynamic properties and absorption spectra as compared to that of the native cytochrome c. In agreement with this it was found that ^{3}H-pABB (+4-MBI)-S. cerevisiae cytochrome c was bound to cytochrome c-depleted mitochondria and restored their oxygen uptake with succinate as the substrate in exactly the same manner as did pAPB (+4-MBI)-cytochrome c from horse heart (2). After fractionation of the irradiated mitochondria with KCl in the presence of Triton X-100 (6) the photoaffinity-labeled ^{3}H-cytochrome c was found in the tightly sedimenting pellet of cytochrome c oxidase. Cytochrome c remained bound to cytochrome c oxidase during filtration on an Agarose BioGel A5m column (elution medium 0.1M phosphate buffer pH 7.4 containing 0.5% Triton X-100) and eluted mostly in a 1:1 molar complex.

The cytochrome c-cytochrome c oxidase complex and cytochrome c oxidase (isolated in parallel experiments by the identical method) were analyzed by polyacrylamide gel electrophoresis in the presence of SDS (Fig. 1). Both cytochrome c oxidase and cytochrome c-cytochrome c oxidase complex showed the presence of 7 main polypeptide bands (molecular weights of 31,000, 24,000, 22,000, 16,500, 15,000, 12,500, and 10,000). There was, however an additional small peptide band in the cytochrome c-cytochrome c oxidase sample with an apparent molecular weight of 27,000-28,000 which was not seen in the cytochrome oxidase sample. Concomitant with the appearance of the new band there

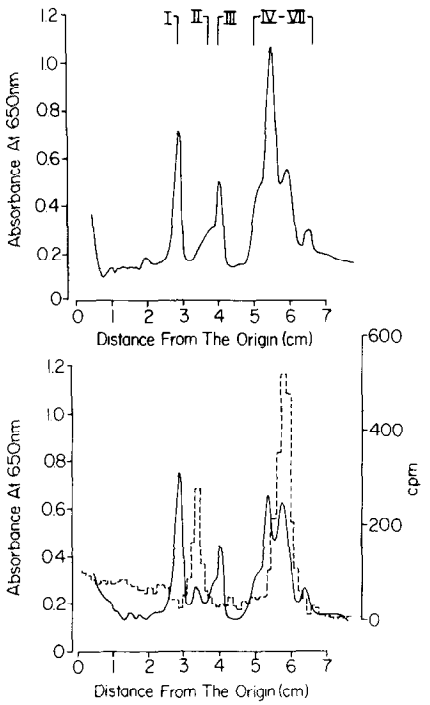

Fig. 1. Densitometer tracings of SDS-polyacrylamide gel electrophoresis of cytochrome c oxidase (upper panel) and of cytochrome c-cytochrome c oxidase complex (lower panel).

SDS-polyacrylamide gel electrophoresis was carried out according to Weber-Osborn (8) as described in the Methods. The broken line in the lower panel represents the radioactivity counts.

was a decrease in the intensity of a band with the apparent molecular weight of about 15,000. The results of Fig. 1 also show that the radioactivity of the sample was concentrated in two peaks. The first peak was associated with a band of apparent molecular weight of 12,500; the second overlapped with the new peptide band only observed in the cytochrome c-cytochrome oxidase sample. The first of the radioactive bands had the same mobility on the polyacrylamide gels as did cytochrome c standard and thus can be identified with cytochrome c tightly bound to the oxidase phospholipids (1,12). The second must be due to cytochrome c which had become covalently-linked to one of the smaller subunits of cytochrome c oxidase with molecular weight of about 15,000 and resulted in a new species with an apparent molecular weight of about 28,000. This band is unlikely to be due to cytochrome c dimer because its apparent molecular weight is too high as compared to 24,000-25,000 expected for the cytochrome c dimer.

Moreover an identical labeling pattern was obtained after filtration of the cytochrome c-cytochrome c oxidase complex on an agarose Biogel A5m column.

The results were very reproducible. Identical labeling patterns were obtained with three different preparations of ^{3}H-cytochrome c and six different preparations of cytochrome c oxidase. Variations in gel technique and gels composition did not affect the results in that the radioactivity was always found with two peptide bands of apparent mw. 12,500 (cytochrome c) and 28,000 (cytochrome c-cytochrome oxidase subunit complex).

DISCUSSION

The results of the studies reported in this paper show that in intact mitochondria cytochrome c binds to one of the smaller subunits of cytochrome c oxidase with an apparent molecular weight of 15,000. This appears to be the functional site for cytochrome c because the photoaffinity-labeled derivative of cytochrome c linked covalently to the mitochondrial membranes at this site can be oxidized by mitochondrial cytochrome c oxidase (13).

It was reported by Bisson and coworkers (12,14) that another photoaffinity labeled derivative of cytochrome c, 4-fluoro-3-nitrophenylazide-cytochrome c when reacted with isolated, detergent-solubilized cytochrome c oxidase forms a covalent complex with subunit II of this enzyme (m.w. 22,000). This complex is, however, unable to catalyze electron transfer from ascorbate to molecular oxygen. The discrepancy in the results may be due to a difference in the location and/or orientation of cytochrome c with respect to cytochrome c oxidase in intact mitochondria and in a solubilized preparation. The formation of the inactive complex might occur at a site which had been generated during isolation and may not necessarily be the same as the active ("native") site at which cytochrome c binds in cytochrome c-depleted mitochondrial membranes.

REFERENCES

1. Erecińska, M.,Vanderkooi, J.M., and Wilson,D.F. (1975) Archiv. Biochem. Biophys. 171, 108-116.

2. Erecińska, M. (1977) Biochem. Biophys. Res. Commun. 76, 495-501.
3. Hixson, S.H. and Hixson, S.S. (1975) Biochem. 14, 4251-4254.
4. Jacobs, E.E. and Sanadi, D.R. (1960) J. Biol. Chem. 235, 531-534.
5. Boveris, A., Erecińska, M., and Wagner, M. (1972) Biochim. Biophys. Acta 256, 223-242.
6. Sun, F.F., Prezbindowski, K.S., Crane, F.L., and Jacobs, E.E. (1968) Biochim. Biophys. Acta 153, 804-818.
7. Erecińska, M., Oshino, R., Oshino, N., and Chance, B. (1973) Archiv. Biochem. Biophys. 157, 431-445.
8. Weber, K. and Osborn, M. (1969) J. Biol.Chem. 244, 4406-4412.
9. Laemmli, U.K. (1970) Nature 227, 680-684.
10. Ando, K., Matsubara, H., and Okunuki, K. (1966) Biochim.Biophys. Acta 118, 240-245.
11. Motonaga, K., Misaka, E., Nakajima, E., Ueda, S., and Nakanishi, K. (1965) J. Biochem. (Japan) 57, 22-28.
12. Bisson, R., Azzi, A., Gutweniger, H., Colonna, R., Montecucco, C. and Zanotti, A., (1978) J. Biol. Chem. 253, 1874-1880.
13. Waring, A., Chance, B. and Erecińska, M. (1979) Fed. Proc. 38, 576.
14. Bisson, R., Gutweniger, H., Montecucco, C., Colonna, R., Zanotti, and Azzi, A. (1977) FEBS Lett. 81, 147-150.