A NEW PHOTOAFFINITY-LABELED DERIVATIVE OF MITOCHONDRIAL CYTOCHROME c

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SUMMARY Three lysine residues of horse heart cytochrome c were modified by reaction with methyl-4-mercaptobutyrimidate hydrochloride and the free SH group of the latter was covalently linked to p-azidophenacyl bromide yielding a photoaffinity-labeled cytochrome c. The photoaffinity-labeled cytochrome c was bound by irradiation into a covalent complex with cytochrome c oxidase.

INTRODUCTION

As a part of our studies on the mechanism of interaction of mitochondrial redox components we have utilized a photoaffinity-labeled derivative of cytochrome c, 2,4-dinitroazidophenyl cytochrome c, to determine the immediate environment of this carrier in the mitochondrial inner membrane (1,2). It was found that the labeled cytochrome c was bound covalently to cytochrome c oxidase in a 1:1 molar complex. Since the overall yield of the complex was relatively small (< 5%), in this communication we report the preparation of another photoaffinity-labeled derivative of cytochrome c which upon irradiation converts the oxidase almost quantitatively into a covalent cytochrome c-cytochrome c oxidase complex.

MATERIALS AND METHODS

Horse heart cytochrome c (30 mg) was dissolved in 4 ml of 50 mM phosphate buffer at pH 8.4 and reacted with 4 mg of methyl-4-mercaptobutyrimidate hydrochloride at room temperature for 30 min. At the end of the incubation time the solution was dialyzed for 4 hours at 4°C against 250 volumes of 50 mM phosphate buffer at pH 7.2 containing 0.1 mM mercaptoethanol. The dialyzed solution of cytochrome c was then reacted with 10 µmoles of p-azidophenacyl bromide (50 mM, in methanol) added in 20 µl aliquots over a period of 40 min. Total incubation time was 60 min. at 4°C. The solution was dialyzed overnight against 50 mM phosphate buffer at pH 7.2 and the small amount of precipitated protein was removed by centrifugation.

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The number of photoactive groups was estimated from absorbance measurements at 285 nm [ϵ_{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 coefficient of 19.7 cm^{-1}.

Pigeon breast cytochrome c -depleted mitochondria were prepared by the method of Jacobs and Sanadi (4) as modified by Boveris et al (5).

Irradiation of cytochrome c -depleted mitochondria: Cytochrome c-depleted pigeon breast mitochondria 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 volume of the fluid was 5 ml) using a medium pressure,100 W, water-cooled mercury arc. The distance between the light source and the surface of irradiated mitochondria was approximately 5 cm. At the end of the 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 wavelength 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.

Binding of the photoaffinity-labeled derivative was determined from the amount measured in the mitochondrial pellet formed by centrifuging the suspension at 8000 x g for 10 minutes as described previously (8).

Oxygen uptake was measured at 24°C with a Clark oxygen electrode in 0.2 M sucrose-0.05 M morpholinopropane sulfonate (MOPS) buffer at pH 7.2, with succinate as substrate.

Free SH groups were determined using 5,5'-dithiobis-(2-nitrobenzoic acid) (9).

Cytochrome a concentration was measured at 605-630 nm (reduced-oxidized) using an extinction coefficient of 26.4 cm^{-1}.

Protein was determined by the biuret reaction (10) using crystalline bovine serum albumin as the standard.

Materials: Triton X-114, Triton X-100, horse heart cytochrome c type VI, and 5,5'-dithiobis-(2-nitrobenzoic acid) were obtained from Sigma Chemical Co. (St. Louis, MO). Methyl-4-mercaptobutyrimidate hydrochloride and p-azidophenacyl bromide were products of Pierce (Rockford, ILL). Agarose Bio Gel A-5m, 100-200 mesh, was obtained from Bio-Rad Laboratories (Richmond, CA).

RESULTS

Methyl-4-mercaptobutyrimidate [$HS - (CH_2)_3 - C(=NH_2^+) - OCH_3$] is water soluble and reacts rapidly at room temperature and moderate pH (7-10) with ε-amino groups of lysine residues (11). While the presence of a $=NH_2^+$ group maintains the positive charge on cytochrome c molecule, free SH is available for re-

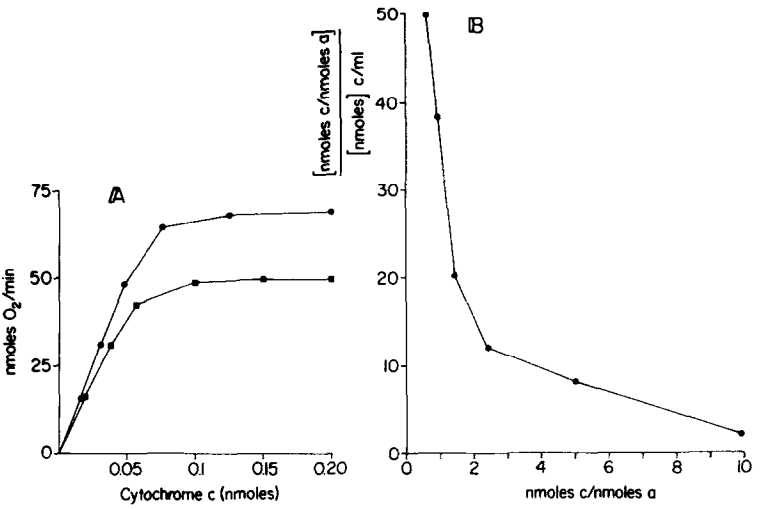

Fig. 1. A. Restoration of oxygen uptake by photoaffinity-labeled and native cytochrome c in cytochrome c-depleted pigeon breast mitochondria.

Cytochrome c-depleted pigeon breast mitochondria (0.2 mg protein/ml) were suspended in 0.2 M sucrose, 0.05 M MOPS at pH 7.2 buffer and respiration was measured with 10 mM succinate as the substrate.

Fig. 1. B. Binding of photaffinity-labeled cytochrome c to cytochrome c-depleted pigeon breast mitochondria.

Cytochrome c-depleted mitochondria (3.2 nmoles of cytochrome a) were incubated in 0.2 M sucrose, 0.05 M MOPS at pH 7.2 buffer with various amounts of cytochrome c for 2 min at room temperature. The mitochondria were removed by centrifugation at 8000 x g for 10 min at 4°C and the pellet suspended in 0.1 M phosphate buffer at pH 7.2 containing 1% Triton X-100. The concentrations of cytochrome c in the pellet and in the supernatant were measured spectrophotometrically at 550-540 nm.

acting with the photoaffinity-label, p-azidophenacyl bromide (3). Under experimental conditions described here, three lysine residues were modified as shown by the presence of three free SH groups (9) per cytochrome c molecule. Reaction with p-azidophenacyl bromide occurred at 4°C within 30-60 min., and no free SH-group could be detected after overnight dialysis of the cytochrome c solution.

By increasing the concentration of methyl-4-mercaptobutyrimidate as many as 6-8 lysine residues could be modified. However, appropriate increase in

the concentration of p-azidophenacyl bromide led to substantial precipitation of cytochrome c and consequent small yield of the highly labeled derivative. Moreover, the results obtained using cytochrome c with as many as six lysine groups modified were essentially the same as those described below, when only 3 residues were photoaffinity-labeled. It should be mentioned here that only longer incubations (24 hours and more) with p-azidophenacyl bromide at room temperature led to the photoaffinity labeling of methionine 65 of the cytochrome c polypeptide chain (M. Ereciński unpublished data).

The photoaffinity-labeled cytochrome c was as stable as native cytochrome c and could be stored at 4°C in light-shielded vessels.

Fig. 1 shows that the photoaffinity-labeled cytochrome c was bound to cytochrome c-depleted mitochondria and restored their oxygen uptake with succinate as substrate. The K_D value was approximately 1×10^{-8} M and there were two high affinity binding sites for the labeled cytochrome c per cytochrome a on the mitochondrial membrane. The photoaffinity-labeled cytochrome c restored oxygen uptake at approximately the same concentration as did the native cytochrome c, but the restoration was not complete; it was usually 70-75% of that observed with native cytochrome c.

The mitochondria irradiated in the presence of the photoaffinity-labeled cytochrome c were separated into various fractions by salts, in the presence of Triton X-100 as described by Sun et al (6). It can be seen from the spectrum shown in Fig. 2 that there were approximately 1.5 molecules of cytochrome c per molecule of cytochrome a present in the tightly sedimenting pellet of the oxidase fraction. Treatment with higher concentration of Triton X-100 resulted in solubilization of the cytochromes and the clear supernatant could be applied to an agarose Bio Gel A-5m column. The very first fraction which eluted from the agarose column with 0.1 M sucrose-0.1 M phosphate buffer pH 7.2 containing 0.5% Triton X-100 consisted of cytochrome c-cytochrome c oxidase complex in a ratio of 2 cytochrome c per one cytochrome a. It was followed by a cytochrome c oxidase-cytochrome c complex in 1:1 molar ratio and finally by a relatively small

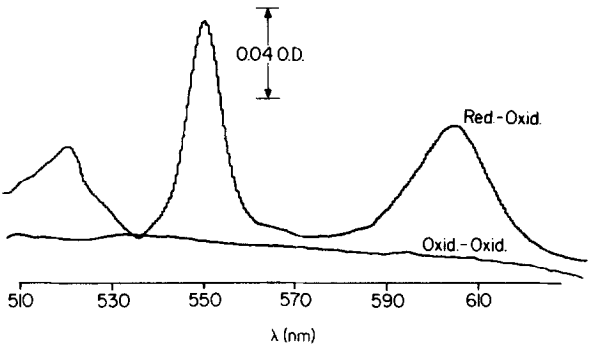

Fig. 2. Absorption spectrum of the cytochrome c oxidase fraction obtained by detergent fractionation of pigeon breast mitochondria irradiated in the presence of photoaffinity-labeled cytochrome c.

The cytochrome c oxidase fraction was diluted to appropriate concentration in 0.1 M phosphate buffer at pH 7.2, containing 1% Triton X -100. The fully oxidized spectrum was measured and stored in the memory of a digital computer. The stored spectrum was then subtracted from any other subsequently measured. Thus, the straight baseline is the oxidized-oxidized difference spectrum. The sample was reduced by the addition of dithionite and the dithionite reduced-oxidized difference spectrum was recorded.

percentage of the mixture of cytochrome c oxidase-cytochrome c complex and free cytochrome c oxidase. About 30% of total cytochrome c eluted last, probably as the complex with lipids.

Some cytochrome c was also present in the soluble, cytochrome b-c_1 complex containing fraction. But when this fraction was applied to the same agarose column, the cytochrome b-c_1 complex free of cytochrome eluted first while the cytochrome c eluted in a single peak well separated from the cytochrome b-c_1 complex. The absence of any appreciable quantities of cytochrome c in the cytochrome b-c_1 complex was confirmed using yet another independent method (7) of fractionation of the irradiated mitochondria.

Preliminary experiments on sodium dodecylsulfate polyacrylamide gels (12.5% acrylamide - 0.6% methylenebisacrylamide, the method of Laemmli) (12) showed that when the cytochrome c oxidase-cytochrome c complex was applied to the gels, the bands corresponding to the two smallest polypeptide chains of the oxidase (m.w.

9000 and 11,000) were almost completely missing. There were two bands (approximate m.w. of 24,000 and 36,000) which stained very intensely with Coomasie Blue and overlapped the bands of the same molecular weight in native cytochrome c oxidase. Experiments are in progress to demonstrate the presence of heme c in the latter two bands.

DISCUSSION

The method of preparation described above of the new photoaffinity-labeled derivative of cytochrome c is quick and simple and leads to quantitative conversion of native cytochrome c into its photoaffinity-labeled derivative. The new derivative has the same overall charge on the protein and binds to the cytochrome c-depleted mitochondria with the same affinity as does the native molecule. The increased size of the modified cytochrome c must, however, slightly impair the accomodation into the binding site which results in incomplete restoration of oxygen uptake.

It was found that after fractionation of irradiated mitochondria with detergents and salts and chromatography on an agarose column, cytochrome c was present in a mixture of complexes with cytochrome c oxidase in 2:1 and 1:1 molar ratios. This suggests that either two molecules of cytochrome c bind to the oxidase or that the binding site for the second cytochrome c is on the cytochrome c molecule bound to the oxidase. This second possibility is more appealing as the first requires the exposure of substantial part of the oxidase surface towards the outside of the mitochondrial inner membrane in order to accomodate two molecules of cytochrome c.

No covalent binding of cytochrome c to cytochrome b-c_1 complex was observed which substantiates the previous data (2) that the binding sites for cytochrome c oxidase and for the reductase on cytochrome c molecule are distinct.

Preliminary results on SDS polyacrylamide gels show that the two smallest polypeptide chains of cytochrome c oxidase are the site of cytochrome c binding. This finding is in agreement with the results of Eytan et al (13) who arrived

at the same conclusion using the membrane impermeant-reagent, p-diazonium benzene [^{35}S] sulfonate.

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