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INTERACTION OF THE "BACK" OF YEAST ISO-1-CYTOCHROME C WITH YEAST CYTOCHROME C OXIDASE

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Summary

The 102-S-(2-thiopyridyl) and 102-S-(4-azidophenacyl) derivatives of yeast iso-1-cytochrome c have been prepared, both containing one mole of label per mole of cytochrome c. When the 102-S-(2-thiopyridyl) cytochrome c is allowed to interact with purified, detergent-solubilized yeast cytochrome oxidase, covalent crosslinked products are formed containing 0.9 to 0.98 moles of cytochrome c per oxidase. When the 102-S-(4-azidophenacyl) cytochrome c is exposed to the oxidase in the presence of ultraviolet light, covalently crosslinked products containing 0.2 to 0.35 moles of cytochrome c per oxidase are formed. SDS-polyacrylamide gel electrophoresis of these crosslinked products reveals the formation of a new band of apparent molecular weight 38,000 Daltons and the concurrent disappearance of subunit III (a crosslink of subunit III and cytochrome c). These affinity and photoaffinity crosslinking results are interpreted as evidence that the "backside" of yeast iso-1-cytochrome c interacts with subunit III of yeast cytochrome oxidase.

Cytochrome c oxidase, the terminal enzyme of mitochondrial electron transport, oxidizes the soluble hemoprotein cytochrome c and uses these electrons to reduce molecular oxygen to water. In most eukaryotes, cytochrome oxidase is an oligomeric, noncovalent complex of seven nonidentical polypeptide subunits, two heme a molecules and two atoms of copper (for review, see ref. 1). The molecular mechanism of electron transfer from

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[1] Abbreviations: APA-cytochrome c - 102-S-(4-azidophenacyl) yeast iso-1-cytochrome c; APAB - azidophenacyl bromide; DTNB - dithiobis-(2-nitrobenzoate); MET buffer - 10 mM MOPS, 10 μM EDTA, 1% Tween-80, pH 7.4; SDS - sodium dodecyl sulfate; SDS-PAGE - SDS-polyacrylamide gel electrophoresis; TP-cytochrome c - 102-S-(2-thiopyridyl) yeast iso-1-cytochrome c; TMPD - N,N,N',N'-tetramethyl-p-phenylenediamine.

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cytochrome c to the oxidase cannot be understood without a knowledge of how and where cytochrome c interacts with the individual components of the complex. Covalent crosslinking of cytochrome c to the oxidase has been used in an attempt to identify which of the seven polypeptide subunits is the cytochrome c binding site (2-5). Arylazido derivatives of lysine 13 of cytochrome c (on the X-ray crystallographic "front" of the molecule; see ref. 6) have been shown to crosslink to subunit II in both the beef heart (3, 4) and yeast (5) oxidases. However, yeast iso-1-cytochrome c modified at cysteine 102 (on the "back" of the molecule) with 5,5'-dithiobis(2-nitrobenzoate) crosslinks exclusively to subunit III of the yeast cytochrome oxidase (2). In this communication, we re-examine the interaction of the "backside" of iso-1-cytochrome c with yeast cytochrome oxidase by using two independent crosslinking techniques, conducting the experiments in nonionic detergents where the detergent-solubilized enzyme is most active (7) and by examining the activity of the crosslinked complex formed.

Materials and Methods

The 102-S-(2-thiopyridyl)-iso-1-cytochrome c (TP-cytochrome c) was prepared by first reducing yeast iso-1-cytochrome c with 100 mM dithiothreitol (DTT) (Sigma) (disodium salt) for five minutes in 100 mM sodium phosphate, 40 mM EDTA, pH 8.0. The sample was applied to a G-25 Sephadex column (16 x 0.9 cm) with the same buffer. Cytochrome c was diluted to 50 μM and reacted with 1 mM 2,2'-bispyridine disulfide (Sigma) for thirty minutes at 25°C. The derivative was concentrated on CM52 cation exchange resin (Whatman), eluted with 500 mM sodium phosphate, pH 7.0, and finally desalted into 10 mM morphilinopropanesulfonate (MOPS), 10 μM EDTA, 1% Tween-80 (MET buffer) by G-25 Sephadex chromatography on a 16 x 0.9 cm column. The concentration of 2-thiopyridine covalently linked to cytochrome c was determined by dithiothreitol titrations of the TP-cytochrome c. Upon reaction with dithiothreitol, TP-cytochrome c releases 2-thiopyridone which can be determined as an increase in absorbance at 343 nm (ε = 7.06 mM) (8). Cytochrome c concentration was determined at 410 nm (ε = 106.1 mM) (9). Ratios of thiopyridine to cytochrome c ranged from 0.96 to 1.04 moles TP/cytochrome c.

The 102-S-(4-azidophenacyl) iso-1-cytochrome c (APA cytochrome c) was prepared using iso-1-ferricytochrome c that contained at least 0.98 moles of sulphydryl per mole cytochrome c. After chromatography on G-25 Sephadex (16 x 0.9 cm) in 50 mM sodium phosphate, pH 7.2, the cytochrome c was diluted to 50 μM and reacted with 250 μM 4-azidophenacyl bromide (APAB) (Pierce Chemicals) in 10% methanol, 50 mM sodium phosphate, pH 7.2. The reaction mixture was stirred in the dark under nitrogen for 90 min at 25°C.

The derivatized cytochrome c was concentrated and desalted as described above. These chromatographic procedures also remove the excess APAB. All procedures were carried out in minimal light to protect the azide group. The molar ratio of azidophenacyl moieties to cytochrome c was determined spectrally using the following expression:

$$\frac{[A_{300} - ([Cytc] \times \varepsilon_{300cyt}) / \varepsilon_{300APAB}}{(Cyt\ c)} \qquad \text{where } (cytc) = (A_{410}/\varepsilon_{410c})$$

$[\varepsilon_{300APAB} = 20.1\ (10),\ \varepsilon_{300ferricytochrome\ c} = 13.0\ (9)]$

This was confirmed by determining the amount of unmodified cysteine 102 on dithiothreitol-reduced samples of APA cytochrome c by the method of Ellman (11) at 450 nm (2). The reaction of ferricytochrome c with APAB was complete by 90 minutes as judged by the amount of APAB incorporation into cytochrome c (0.98 to 1 mole/mole cytochrome c) and by DTNB titration of the remaining thiol groups (0.0 to 0.02 moles per mole of cytochrome c).

Cytochrome oxidase was prepared from commercial baker's yeast (13). The cytochrome oxidase was passed through a 5 x 0.5 cm column of P-100 (Bio-Rad) into MET buffer, and its concentration determined by dithionite reduced minus ferrocyanide oxidized difference spectra as previously described (13).

TP cytochrome c was incubated with cytochrome oxidase (5-10 μm) in a 2 1/2 molar excess for 45 minutes. Samples were subjected to CM-52 chromatography on 0.5 x 1 cm columns in MET buffer, collowed by Sephacryl S-200 (Pharmacia) chromatography (16 x 0.9 cm columns) into 10 mM sodium phosphate, 2% sodium cholate (Sigma), pH 7.2. Cytochrome oxidase as well as the crosslinked cytochrome oxidase-c complex elute in the void volume of the S-200 columns while cytochrome c is retained. Before applying the samples to SDS-PAGE, portions of the crosslinked and control samples were treated with 10 mM N-ethylmaleimide for 60 minutes at room temperature to prevent disulfide exchange in SDS. Other aliquots were treated with 10 mM dithiothreitol for 60 minutes at room temperature to observe the reversibility of crosslinking. Samples were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using the step system of Cabral and Schatz (14). Activities of crosslinked complex were determined by a spectral method (6) and by a polarographic method (12).

Cytochrome c oxidase (5-10 μM) was incubated with a five-molar excess (25-50 μM) of APA cytochrome c in the dark and subsequently exposed to a hand-held ultraviolet light (366 nm max, Mineralight) at a distance of 1 cm from the lamp in glass tubes for 15 minutes. Samples were treated the same way as described above, except without N-ethylmaleimide or dithiothreitol treatment before SDS-PAGE.

Scanning spectra were obtained from an Aminco DW-2 UV visible spectrophotometer interfaced with a Hewlett-Packard 9845S computer.

Results and Discussion

Yeast iso-1-cytochrome c is unique in that it has a single cysteine residue at position 102 (15) on the X-ray crystallographic back of the molecule (6). Using sulphydryl reagents, one can prepare derivatives of cytochrome c containing one mole of label per mole cytochrome c. TP-

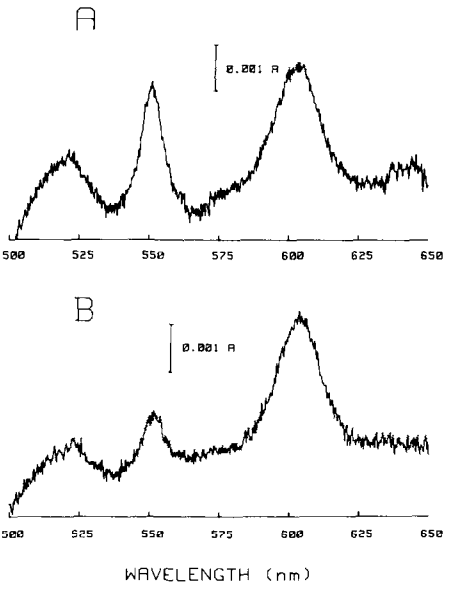

Figure 1. Panel A: Difference spectra of TP cytochrome c-oxidase crosslink. Sample contains 0.95 moles of cytochrome c per mole oxidase. Panel B: Difference spectra of APA cytochrome c-oxidase crosslink. Sample contains 0.35 moles of cytochrome c per mole oxidase.

cytochrome c has been prepared containing 0.96 to 1.04 moles of thiopyridine per mole of cytochrome c.

Exposure of a 2 1/2-fold excess of TP cytochrome c to cytochrome c oxidase followed by chromatographic isolation of crosslinked products results in crosslinked products that contain 0.90 to 0.98 moles of cytochrome c per mole oxidase as examined by difference spectra (Figure 1A). When the crosslinked products are examined by SDS-PAGE (Figure 2, lanes A and B), subunit III all but disappears concommitant with formation of a new band of molecular weight 38,000 daltons. Treatment with an excess of dithiothreitol before running SDS-PAGE resulted in loss of the new band and regeneration of subunit III plus free cytochrome c (Figure 2, lanes C, D and E). Note that subunit II and not subunit III contains the most reactive thiol group in the oxidase complex with respect to reactivity to N-ethylmaleimide and iodoacetamide (2).

Is the crosslinked cytochrome c at or near a functional binding site? This can be examined by two techniques. First, the oxidation of added sub-

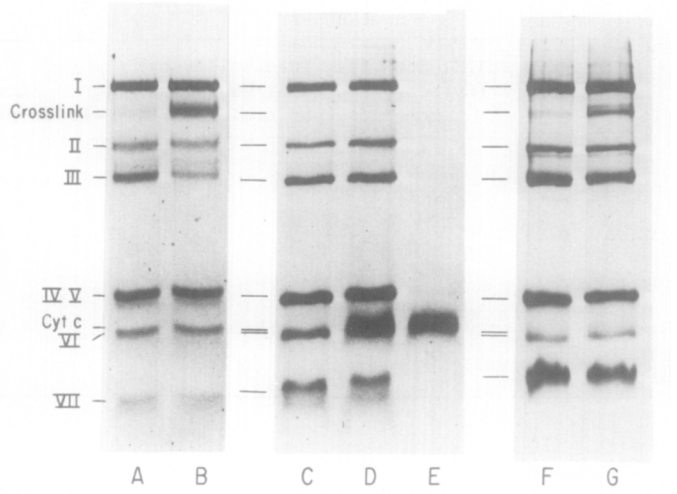

Figure 2. SDS-PAGE of crosslinked products. (I) TP cytochrome c-oxidase crosslinking. Lane A, nonreduced control; Lane B, TP cytochrome c crosslink; Lane C, dithiothreitol-reduced control; Lane D, dithiothreitol-reduced TP cytochrome c-oxidase crosslink; Lane E, cytochrome c. (II) APA cytochrome c-oxidase crosslinking. Lane F, UV flashed control; Lane G, APA cytochrome c-oxidase crosslink.

strate (ferrocytochrome c) by oxidase should be inhibited if the crosslinked cytochrome c blocks the substrate binding site (spectral assay of ferrocytochrome c oxidation). Secondly, if the crosslinked cytochrome c is positioned correctly, artificial electron donors (such as TMPD) should reduce the cytochrome c on the oxidase (19), and a transfer of electrons through the oxidase and a consumption of molecular oxygen should result (polarographic assay). Examination of crosslinked products in the spectral assay reveals that the oxidation of added cytochrome c is inhibited from 85 to 95% of that of control (Table I). This inhibition of oxidase activity corresponds well with the amount of cytochrome c covalently associated with the oxidase. In the polarographic assay, the crosslinked mixture (50 nM oxidase, 125 nM TP-cytochrome c) consumed molecular oxygen at a rate comparable to the control (50 nM oxidase, 125 nM native cytochrome c). Cytochrome oxidase alone without the addition of cytochrome c had almost no oxygen consumption (less than 3% of the above control). The cytochrome c crosslinked to the oxidase through a disulfide bond to subunit III inhibits oxidation of added ferrocytochrome c

TABLE I

Sample	Specific Activity (Spectral Assay) (Cytochrome c oxidized per heme a,a3 per sec)	Active in Polarographic Assay
Cytochrome oxidase	100-155	No
Cytochrome oxidase plus 2.5/1 excess of yeast iso-1-cytochrome c	---	Yes
Cytochrome oxidase plus 2.5/1 excess of TP-cytochrome c	8-15	Yes

and transfers electrons from TMPD through the oxidase and on to molecular oxygen. Hence, subunit III is very close to or comprises part of a binding site for cytochrome c on the oxidase.

The thiopyridyl reaction that generates such high efficiency crosslinking is a forced equilibrium reaction. As an alternative approach, photoaffinity crosslinking can be used that sacrifices efficiency for nonspecificity. Aryl azides, upon exposure to ultraviolet light, photodecompose to highly reactive nitrene intermediates which are capable of relatively random insertion into neighboring chemical bonds (16, 17). The use of APA-cytochrome c to probe the binding site on oxidase provides a reaction that requires no specific nuclephile for the crosslinking reaction. When a 5-fold excess of APA cytochrome c is exposed to the oxidase in the presence of ultraviolet light (366 nm max), and crosslinked products are isolated by chromatographic means, 0.25 to 0.45 moles of cytochrome c become associated per mole of oxidase, as determined by difference spectra (Figure 1B). The chromatographic procedures remove most of the noncovalently bound cytochrome c as evidenced by the minimal association of native iso-1-cytochrome c with cytochrome oxidase treated in the same manner (0.06 moles cytochrome c per mole of oxidase). SDS-polyacrylamide gel analysis of isolated crosslinked samples reveals the formation of a new band of apparent molecular weight 38,000 Daltons as well as a de-

crease in subunit III (Figure 2, lanes F and G). This new band corresponds to the sum of the molecule weights of subunit III and cytochrome c. The photoaffinity-labeled cytochrome c crosslinks to the same subunit of the oxidase complex as the affinity-labeled TP-cytochrome c. Note that this crosslinking experiment is irradiated only once in the presence of a single treatment with APA-cytochrome c. Higher crosslinking efficiencies are achievable with multiple changes of photoaffinity-labeled cytochrome c's (3-5).

The highly conserved lysine-rich region that surrounds the heme crevice of cytochrome c on the front of the molecule (6) has been shown to be involved in the ion-paired association of cytochrome c to the cytochrome oxidase by kinetic approaches (16, 17) and by differential chemical modification (18, 19). Aryl azide derivatives of lysine 13 have been shown to crosslink to subunit II in the yeast oxidase (5). With this work, we confirm and extend the original observation of Birchmeier, et al. (2). The backside of yeast cytochrome c interacts with subunit III of yeast cytochrome c oxidase. Using a new thioactivated cytochrome c, we have been able to achieve close to equimolar crosslinking bewteen cytochrome c and subunit III of the oxidase. The crosslinked cytochrome c blocks the binding of additional substrate and is capable of passing electrons to oxidase. Identical crosslinked products are formed when photoaffinity labeling techniques are used. Taken together, the data suggest that the cytochrome c binding site may consist of more than one subunit of the cytochrome oxidase. Furthermore, the evidence that both the "front" and "back" of cytochrome c interact with oxidase suggests that the binding site may be a pocket rather than a flat surface on the enzyme.

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