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Biochemistry

Interaction of integral and peripheral membrane proteins: Affinity labeling of yeast cytochrome oxidase by modified yeast cytochrome *c*

(reversible chemical crosslinking/two-dimensional electrophoresis/sulfhydryl reactivity/enzyme activity/mitochondrially made proteins)

W. BIRCHMEIER*, C. E. KOHLER, AND G. SCHATZ

Biocenter, University of Basel, CH-4056 Basel, Switzerland

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ABSTRACT To identify possible substrate-binding subunit(s) of yeast cytochrome *c* oxidase (ferrocytochrome *c*:oxygen oxidoreductase, EC 1.9.3.1), the purified enzyme was reacted with yeast iso-1-cytochrome *c* whose single free sulfhydryl group at position 107 had been activated with 5,5′-dithiobis(2-nitrobenzoate). The resulting cytochrome *c* derivative appeared to function as an "affinity-label" of cytochrome oxidase, since it rapidly inactivated the enzyme. Inactivation was competitively prevented by underivatized cytochrome *c*. When the "affinity-labeled" oxidase was analyzed by two-dimensional polyacrylamide electrophoresis in dodecyl sulfate (separation in the second dimension being carried out in the presence of excess sulfhydryl compound), it was found that the derivatized cytochrome *c* had specifically formed a mixed disulfide with the mitochondrially made subunit III (apparent molecular weight 24,000) of the oxidase. Similar results were obtained when underivatized iso-1-cytochrome *c* was crosslinked to the oxidase by oxidative disulfide bridge formation in the presence of *ortho*-phenanthroline and Cu^{++}.

These data indicate that the hydrophobic mitochondrially made subunit III of yeast cytochrome *c* oxidase is in close proximity to a cytochrome *c* binding site on the enzyme. Since cytochrome *c* and the mitochondrially made cytochrome oxidase subunit III are typical peripheral and integral membrane proteins, respectively, the present study suggests a useful approach for analyzing specific interactions between these different classes of membrane proteins.

Cytochrome *c* and cytochrome *c* oxidase (ferrocytochrome *c*:oxygen oxidoreductase, EC 1.9.3.1) are currently two of the best known proteins of the mitochondrial inner membrane (see ref. 1 for a review). Cytochrome *c* is a water-soluble globular protein; *in vivo*, however, it is firmly bound to the outer face of the mitochondrial inner membrane (2), transferring electrons from cytochrome c_1 to cytochrome oxidase. Since cytochrome *c* can easily be extracted from mitochondria in a lipid-free form with salt solutions (1), it has been defined as a typical peripheral membrane protein (3). In contrast, cytochrome oxidase can be solubilized only by disrupting the mitochondrial inner membrane with detergents. At least some subunits of this oligomeric enzyme (compare *text* below) thus behave as typical integral membrane proteins.

Cytochrome oxidase from yeast can be resolved into seven polypeptides with the following apparent molecular weights: I, 43,000; II, 34,000; III, 24,000; IV, 14,000; V, 12,000; VI, 12,000; and VII, 4,500 (4). Similar results have been obtained for cytochrome oxidase from *Neurospora* (5) and from bovine heart (cf. ref 6). In yeast and *Neurospora*, subunits I, II, III are synthesized on mitochondrial ribosomes, whereas subunits IV to VII are made on cytoplasmic ribosomes (5, 7). The three mitochondrially made subunits of the yeast enzyme are very hydrophobic (8) and hence characteristic integral membrane proteins. The cytoplasmically made subunits are generally more hydrophilic, but subunits V and VII are nevertheless difficult to handle in the absence of detergents (9). While it is not yet known how these four smaller subunits are linked to the apolar membrane core, the holoenzyme spans the mitochondrial inner membrane (10) and is thus best defined as an integral membrane complex. The function of the individual cytochrome oxidase subunits is largely unknown. Since antibodies directed either against the mitochondrially made subunit II or against the cytoplasmically made subunits inhibit the activity of the purified yeast enzyme, we concluded that both classes of polypeptides probably participate in catalysis (11).

MATERIALS AND METHODS

Purification of Yeast Cytochrome Oxidase. Highly purified enzyme was prepared from commercial baker's yeast by a slight modification of our published procedure (ref 4; D. Deters, H. Homberger, and U. Müller, manuscript in preparation). The enzyme (containing up to 15 nmol of heme *a* per mg of protein) was then filtered through a 0.9 × 60 cm column of Sephadex G-100 in 10 mM NaP_i–2% sodium cholate, pH 7.2. This filtration did not affect enzymic activity.

Preparation of the Thionitrobenzoate Derivative of Yeast Cytochrome *c*. Iso-1-cytochrome *c* from *Saccharomyces cerevisiae* was a gift from Sankyo Co., Tokyo, through Dr. Matsubara. The heme moiety and the sulfhydryl group in position 107 were first fully reduced by incubation for 10 min at room temperature with 0.3 M 2-mercaptoethanol, pH 7.6, followed by gel filtration through Sephadex G-25 in 10 mM NaP_i, pH 7.6. The reduced cytochrome *c* was then incubated for 10 min at room temperature with 10 mM 5,5′-dithiobis(2-nitrobenzoate) in 0.2 M NaP_i, pH 7.6, and again filtered through Sephadex G-25 in 10 mM NaP_i, pH 7.2. Protein-bound thionitrobenzoate was determined spectrophotometrically during the course of its release by 2-mercaptoethanol (12). Because of the overlap of the absorption band of thionitrobenzoate and the Soret band of cytochrome *c*, an ϵ of 7.5 mM^{-1} cm^{-1} at 450 nm was used (13).

Miscellaneous. Protein and heme *a* of cytochrome oxidase were determined as in ref 4. Enzymic activity of cytochrome oxidase was measured spectrophotometrically at 550 nm in the presence of 32 μM ferrocytochrome *c* (horse heart, Sigma type III) in 40 mM NaP_i–0.5% Tween 80–0.2 mM EDTA, pH 7.4. Cytochrome *c* was reduced with 0.3 M 2-mercaptoethanol for 10 min at room temperature followed by gel filtration on Sephadex G-25. Removal of all traces of reductant was essential for testing the enzymic activity of mixed disulfides between cytochrome oxidase and cytochrome *c*. Dodecyl sulfate polyacrylamide electrophoresis was carried out according to Cabral and Schatz (14) using a slab gel apparatus designed at the

* Present address: Department of Biology, University of California at San Diego, La Jolla, Calif. 92093.

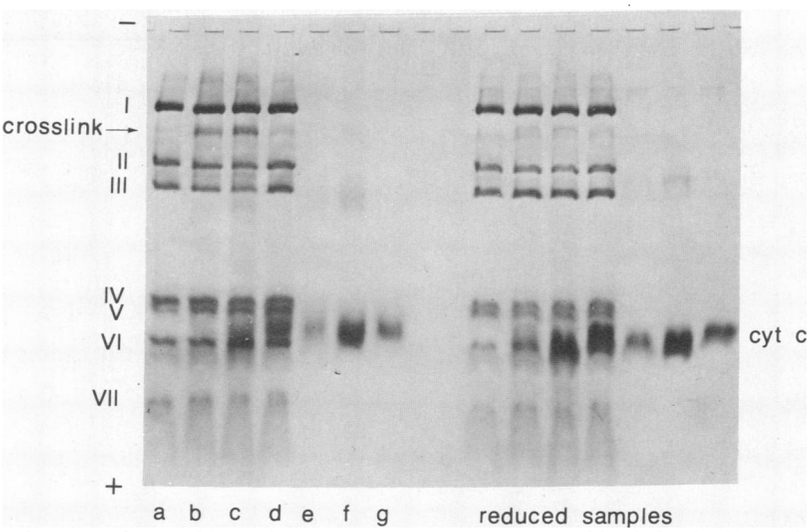

FIG. 1. Crosslinking of cytochrome oxidase to thionitrobenzoate-cytochrome *c* as analyzed by dodecyl sulfate polyacrylamide electrophoresis. The reaction was carried out with 0.01 mM cytochrome oxidase (based on a molecular weight of 140,000) for 15 min at 35° in 0.01 M NaP_i–2% sodium cholate, pH 7.2. The samples were then incubated with 20 mM *N*-ethylmaleimide for 15 min at room temperature before the addition of dodecyl sulfate to prevent disulfide exchange during denaturation. (a) Cytochrome oxidase alone, (b) cytochrome oxidase plus 1 equivalent of modified yeast cytochrome *c*, (c) cytochrome oxidase plus 2.5 equivalents of modified yeast cytochrome *c*, (d) cytochrome oxidase plus 1 equivalent of (thiol-free) horse heart cytochrome *c* that had been treated with 5,5′-dithiobis(2-nitrobenzoate) in the same way as the yeast protein. The samples (e), (f), and (g) correspond to (b), (c), and (d), respectively, except that cytochrome oxidase was omitted. The unlettered samples correspond to (a)–(g), except that they were treated with 0.3 M 2-mercaptoethanol for 15 min before electrophoresis.

Brookhaven National Laboratories. Two-dimensional electrophoresis was done as described (15) except that slab gels were used for both dimensions.

RESULTS

Interaction of yeast iso-1-cytochrome *c* with 5,5′-dithiobis(2-nitrobenzoate) as described in *Materials and Methods* yielded a cytochrome *c* derivative containing 0.95–1.05 equivalents of thionitrobenzoate per mol of cytochrome *c*. Thus, virtually all of the cytochrome *c* molecules carried one thionitrobenzoate moiety covalently linked via a disulfide bridge to the single free cysteinyl residue in position 107 (16). Cysteinyl-bound thionitrobenzoate is an excellent leaving group, provided free thiols are present for exchange (12); the cytochrome *c* derivative mentioned above is therefore an activated form of cytochrome *c* which should form cytochrome-*c*-S-S-cytochrome-oxidase complexes provided free sulfhydryl groups are available near a cytochrome *c* binding site on the oxidase.

Indeed, when thionitrobenzoate-cytochrome *c* was incubated with an equimolar amount of cytochrome oxidase (based on a molecular weight of the oxidase of 140,000) and the mixture was subsequently analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis, a new protein band migrating between subunits I and II of the oxidase was observed (Fig. 1). This new band (apparent molecular weight 38,000) disappeared almost completely upon treatment of the crosslinked samples with 2-mercaptoethanol†. Apparently, this band consists of smaller components held together via a disulfide bridge. The 38,000 dalton species was not observed after addition of unmodified yeast cytochrome *c* to the oxidase. Moreover, the appearance of the crosslinked band required a certain time of incubation of the two proteins at elevated temperatures (e.g., 35°, compare *text* below). Since mixing at 0° was ineffective, the new band is therefore not simply the result of disulfide exchange during subsequent quenching of the reaction or the denaturation with dodecyl sulfate.

The new protein band could also be generated by complexing the oxidase with unmodified iso-1-cytochrome *c* and inducing disulfide bridge formation with copper phenanthroline (Fig. 2). As expected, however, this reagent catalyzed not only the covalent binding of cytochrome *c* to the oxidase but also dimerization of yeast cytochrome *c* itself.

When four gel strips selected from those shown in Figs. 1 and 2 were analyzed for the distribution of protein stain, the emergence of the new crosslinked band seemed to be accompanied by a specific loss of subunit III. Conversely, loss of the new band upon reduction with excess 2-mercaptoethanol was paralleled by a restoration of normal levels of subunit III (Fig. 3). This suggested that the new band might represent a mixed disulfide of iso-1-cytochrome *c* and subunit III. Its apparent molecular weight of 38,000 further strengthened this possibility, since the value is close to the sum of the apparent molecular weights of iso-1-cytochrome *c* (12,000) and subunit III (24,000). More direct evidence for the composition of the crosslinked band was obtained by two-dimensional gel electrophoresis (cf. ref. 15). In a first dimension, the crosslinked band was resolved from the other cytochrome oxidase subunits by dodecyl sulfate polyacrylamide gel electrophoresis in the absence of thiol compounds. In the second dimension, the band was separated into its component polypeptides by electrophoresis in the presence of excess mercaptoethanol. The cleavage products of the 38,000 dalton band were subunit III and cytochrome *c* (Fig. 4).

Does the crosslink between cytochrome *c* and subunit III of the oxidase result from interaction through functional binding sites or does it merely reflect nonspecific disulfide formation with the most reactive cysteinyl residue of the oxidase? As shown in Fig. 5, virtually all of the cysteinyl residues of native cytochrome oxidase were rather unreactive towards commonly used thiol reagents. On a molar basis, the seven subunits together incorporated less than one equivalent of radioactive

† In the experiment documented here, the preparations of cytochrome oxidase and cytochrome *c* each contained traces of an impurity whose electrophoretic mobility resembled that of the new crosslinked band. However, the quantitative scannings of the stained gels (Fig. 3) make it quite clear that these minor contaminants cannot account for the appearance of the prominent new band.

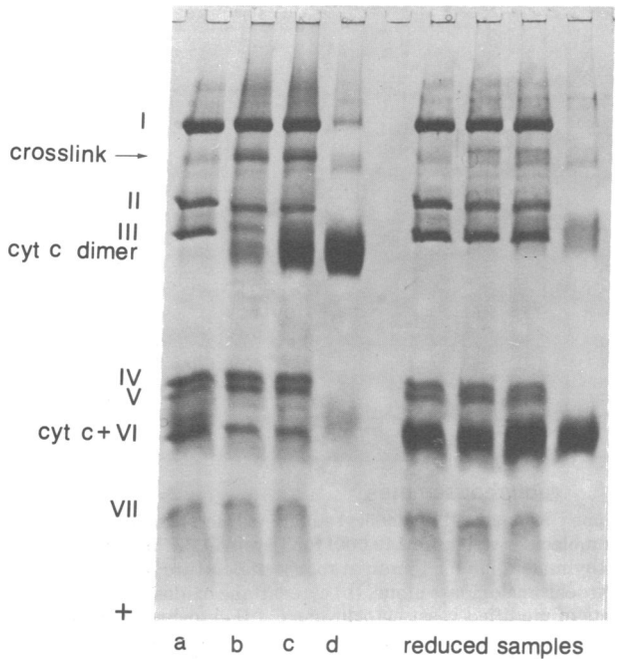

FIG. 2. Crosslinking of cytochrome oxidase to unmodified yeast cytochrome *c* with *o*-phenanthroline and copper. The reaction was carried out for 15 min at 35°. It was started by the addition of a mixture of *ortho*-phenanthroline and copper sulfate (final concentrations 4 and 2 mM, respectively) to 0.01 mM cytochrome oxidase (based on a molecular weight of 140,000) and 0.01 or 0.02 mM yeast cytochrome *c* in 0.01 M NaP_i–2% sodium cholate, pH 7.2. The reaction was stopped by incubation with 20 mM EDTA and 20 mM *N*-ethylmaleimide for 15 min at room temperature and the samples were analyzed by dodecyl sulfate polyacrylamide gel electrophoresis. The samples are: (a) both proteins incubated without copper phenanthroline, (b) cytochrome oxidase incubated with 0.01 mM cytochrome *c* and copper phenanthroline, (c) cytochrome oxidase incubated with 0.02 mM cytochrome *c* and copper phenanthroline, (d) as (c) but without oxidase. The samples without letters on the right-hand side of the figure correspond to (a)–(d) except that 0.3 M 2-mercaptoethanol was added to the samples for 15 min before loading them onto the gel.

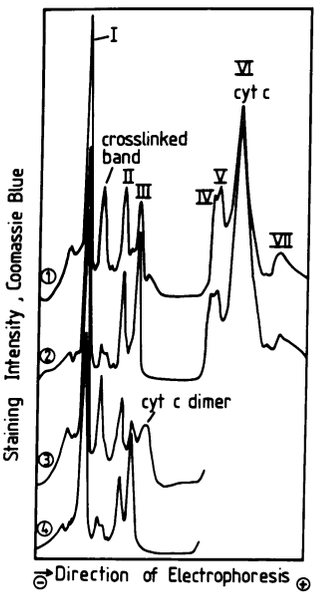

FIG. 3. Scans of the Coomassie blue stain on four gel strips selected from those shown in Figs. 1 and 2. Scan 1 is (c) of Fig. 1 (cytochrome oxidase crosslinked with a 2.5-fold molar excess of thionitrobenzoate-cytochrome *c*). Scan 2 is the same sample but treated with 2-mercaptoethanol following the initial incubation. Scan 3 is (c) of Fig. 2 (cytochrome *c* oxidase crosslinked with copper-phenanthroline to a 2-fold molar excess of unmodified yeast cytochrome *c*). Scan 4 is the same sample but incubated with 2-mercaptoethanol before electrophoresis.

iodoacetamide or *N*-ethylmaleimide despite the fact that either reagent was used at a concentration two to three orders of magnitude higher than that at which activated iso-1-cytochrome *c* readily formed a crosslink with subunit III. Moreover, the highest incorporation of thiol blockers occurred into subunit II, a subunit not involved in a crosslink; subunit III bound at least five times less thiol blocker. The crosslinking to cytochrome *c* must thus be a rather specific event. This view is further supported by the experiment with copper phenanthroline as the disulfide-forming catalyst (Fig. 2); it shows that even disulfide formation between the highly reactive sulfhydryl groups of yeast cytochrome *c* is not greatly favored over the crosslink to subunit III. We conclude that specific noncovalent binding of thionitrobenzoate-cytochrome *c* to the oxidase is a prerequisite for disulfide formation.

Covalent crosslinking of cytochrome *c* to a functional binding site on cytochrome oxidase would be expected to cover this area and block the oxidation of additional ferrocytochrome *c* molecules. Indeed, incubation of cytochrome oxidase with thionitrobenzoate-cytochrome *c* under crosslinking conditions rapidly lowered the activity of cytochrome oxidase as measured by oxidation of horse heart ferrocytochrome *c*. Unmodified yeast cytochrome *c* (Fig. 6) or thiol blockers such as iodoacetamide or *N*-ethylmaleimide (not shown) did not markedly affect the activity. The residual enzymic activity of around 40% after exposure to thionitrobenzoate-cytochrome *c* corresponds approximately with the extent of noncrosslinked subunit III (see also *Discussion*). It is significant, however, that in the presence of an excess of horse heart cytochrome *c*, both the rate and the extent of inactivation by the modified yeast cytochrome *c* is diminished. Probably the horse heart cytochrome *c* competes

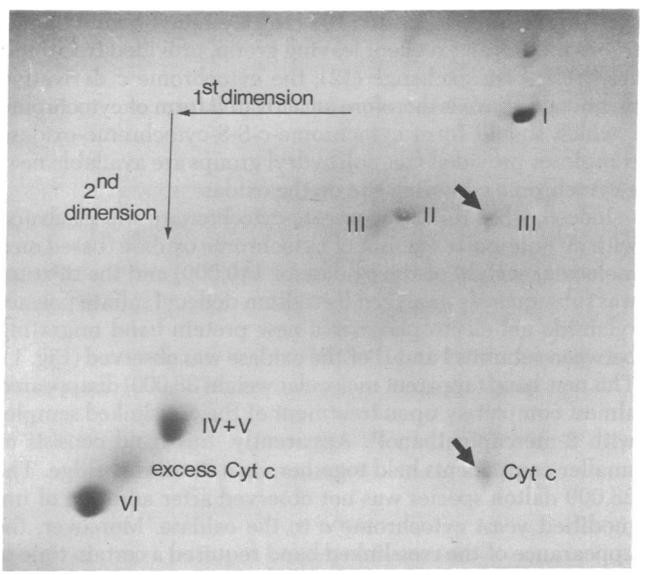

FIG. 4. Two-dimensional polyacrylamide electrophoresis in dodecyl sulfate with a sample of cytochrome oxidase crosslinked to yeast cytochrome *c* under the conditions of Fig. 1c. A strip of the first dimension slab gel run in the absence of any thiol compound was cut out, incubated with 0.7 M 2-mercaptoethanol, and electrophoresed perpendicularly in a second dimension essentially according to ref. 15. The two short arrows indicate the two cleavage products of the crosslinked band, i.e., cytochrome *c* and subunit III.

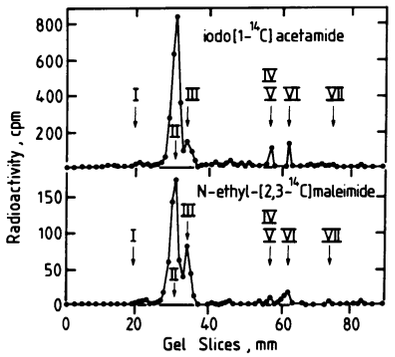

FIG. 5. Radioactive labeling of cytochrome oxidase with sulfhydryl reagents. Purified cytochrome oxidase (1.1 mg/ml in 0.01 M NaP_i–2% sodium cholate, pH 7.5) was incubated at room temperature with 1.4 mM iodo[^{14}C]acetamide (2.1 Ci/mol) or 5 mM *N*-ethyl[2,3-^{14}C]maleimide (49 Ci/mol). After 60 min, the reactions were stopped by adding 50 mM thioglycollate. The enzyme was then dissociated with dodecyl sulfate and electrophoresed on 20% polyacrylamide gel slabs. Slab strips were sliced and counted. With *N*-ethylmaleimide the total amount of radioactivity incorporated into protein corresponded to 0.93 mol of reagent per mol of cytochrome *c* oxidase (molecular weight 140,000, taken as the combined molecular weights of one set of seven subunits). The incorporation of iodoacetamide was five times lower.

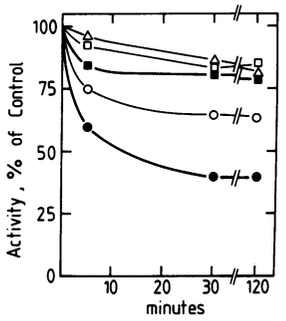

FIG. 6. Inactivation of yeast cytochrome oxidase by thionitrobenzoate-cytochrome *c*. Protection by unmodified horse heart cytochrome *c*. Cytochrome oxidase (0.01 mM based on a molecular weight of 140,000) was incubated at 35° in 10 mM NaP_i–1.2% sodium cholate, pH 7.4, with the different cytochrome *c* preparations indicated below. Enzymic activity of the oxidase is expressed relative to the activity of enzyme incubated in the absence of any cytochrome *c*. Oxidase in the presence of: (●) 0.031 mM thionitrobenzoate derivative of yeast cytochrome *c*; (△) 0.031 mM unmodified yeast cytochrome *c*; (□) 0.14 mM horse heart cytochrome *c*; (○) 0.031 mM thionitrobenzoate yeast cytochrome *c* plus 0.14 mM horse heart cytochrome *c*; (■) gives the values of the preceding competition experiment corrected for the slight inhibition of the enzyme by horse heart cytochrome *c* (compare trace □).

with the modified yeast cytochrome *c* for a binding site on the oxidase.

DISCUSSION

The binding of cytochrome *c* to cytochrome oxidase seems to involve mainly electrostatic interactions. Cytochrome *c*, with its basic surface, apparently binds to acidic groups of membrane-bound or solubilized cytochrome oxidase (1, 17). This interaction is not absolutely specific, however, since cytochrome *c* may also bind to other acidic membrane sites (18) and since the cytochrome *c* binding site on the oxidase interacts also with other basic polypeptides such as polylysine (19). In attempting to identify the cytochrome *c* binding subunit(s) of the oxidase by crosslinking, the possibility of chemically fixing nonspecific associations must be excluded. However, the crosslink reported here meets the following criteria, which render such an artifact unlikely: (*i*) it inactivates cytochrome oxidase; (*ii*) inactivation is competed for by unmodified cytochrome *c*; (*iii*) the crosslink involves only one of the seven cytochrome oxidase subunits. The activated cytochrome *c* derivative used here thus acts as a typical affinity label for cytochrome oxidase.

With our cytochrome oxidase preparation we were unable to push the crosslinking reaction to completion although generally two-thirds of all subunit III molecules were converted to the crosslinked derivative. This structural information is complemented by the finding that the crosslinking reaction destroyed no more than two-thirds of the enzymic activity of the oxidase. A likely explanation for the incomplete reaction would be a partial oxidation of the critical sulfhydryl group of subunit III, e.g., during the isolation of cytochrome oxidase. However, alternate explanations cannot be excluded at present.

It is important to consider whether the crosslink reported here is consistent with present information on the interaction between cytochrome *c* and cytochrome oxidase. Even though the crystal structure of iso-1-cytochrome *c* from yeast has not yet been determined, a comparison with the published structure of horse heart cytochrome *c* (17) suggests that the reactive cysteinyl residue 107 in the yeast protein (16) is situated on the right-hand surface of the molecule as it is commonly depicted (17). Significantly, several independent considerations have suggested that this right-hand side of the molecule might be important for binding to cytochrome oxidase. For example, chemical modification studies with horse heart cytochrome *c* have indicated that derivatization of lysyl 13 (which is located near the right upper front of the molecule) severely impairs binding to cytochrome oxidase (20, 21). Also, structural considerations led Dickerson to suggest (22) that a cluster of lysyl residues on the right-hand back side of the molecule may be critical for binding. The crosslink to the oxidase via cysteinyl 107 is therefore consistent with these views.

Several uncertainties will have to be resolved, however, before the results obtained here can be unequivocally related to the catalytic mechanism of cytochrome oxidase. First, experiments from different laboratories have recently indicated that cytochrome oxidase may have more than one binding site for cytochrome *c* (24, 25). Second, Phan and Mahler have recently claimed that active cytochrome oxidase which lacks all three mitochondrially made subunits can be prepared (25, 26). Efforts in our laboratory to reproduce this observation have so far been unsuccessful. Third, Vanderkooi *et al.* have reported that binding of yeast iso-1-cytochrome *c* carrying an electron spin label on the cysteinyl residue 107 did not result in any measurable immobilization of the spin label (27). Finally, it must be kept in mind that cytochrome *c* is large enough to cover more than one cytochrome oxidase subunit; subunit III could thus be adjacent to the actual binding site or even only a part of the binding site for cytochrome *c*.

A conservative interpretation of our results would therefore be that subunit III is at the very least situated close to one of the cytochrome *c* binding sites on cytochrome oxidase and almost certainly on the same side of the mitochondrial inner membrane as cytochrome *c* itself, i.e., on the outer side. Independent support for this conclusion comes from experiments with several surface labeling reagents that do not appear to penetrate lipid bilayers (28). More recent studies with cytochrome oxidase from bovine heart indicate that at least one of the subunits of apparent molecular weight around 20,000 (10) faces the outer side of the mitochondrial inner membrane. However, a comparison between the results obtained with the enzymes from yeast and

bovine heart will remain speculative until the chemical structures of the cytochrome oxidase subunits have been clarified.

The present study suggests a generally useful approach for analyzing the interaction of integral and peripheral membrane proteins. Lectin binding sites and receptors for polypeptide hormones are two obvious possibilities. The information obtained from such studies may thus lead to a better understanding of the three-dimensional architecture of biological membranes.

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