Detection of Bovine Heart Mitochondrial Cytochrome *c* Oxidase Dimers in Triton X-100 and Phospholipid Vesicles by Chemical Cross-Linking†

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ABSTRACT: Bovine heart cytochrome *c* oxidase is a multisubunit enzyme whose oligomeric state is dependent on its detergent or phospholipid environment. We have utilized the cleavable, heterobifunctional cross-linking reagent *N*-succinimidyl 3-[(4-azidophenyl)dithio]propionate (SADP) to detect cytochrome *c* oxidase dimers. Monomeric or dimeric enzyme dispersed in Triton X-100 (as assessed by sedimentation velocity measurements) was reacted with SADP. A unique intersubunit cross-link having an apparent molecular mass of 136 kDa was identified in the dimeric enzyme; this product was insensitive to limited proteolysis by trypsin and contained a cross-link between two adjacent monomers. Two-dimensional $NaDodSO_4$–PAGE (the second dimension containing β-mercaptoethanol to cleave the cross-linking reagent) indicated that subunit I was the major component of the dimer-specific cross-link. The dimer-specific cross-link created by SADP was observed in phospholipid vesicles [cardiolipin/phosphatidylcholine (1:20, w/w)] containing dimeric (2 μM heme aa_3) enzyme; a low yield of dimer-specific cross-link was observed in liposomes containing 6 μM (heme aa_3) monomeric enzyme. The 136-kDa cross-link was not observed in liposomes containing 2 μM (heme aa_3) monomeric enzyme. These results indicate that subunit I from each monomer may provide one site of interaction between monomers in the dimeric form of the enzyme and that cytochrome *c* oxidase monomers may reassociate to form dimeric complexes in phospholipid vesicles.

Cytochrome *c* oxidase is an integral membrane protein which functions as the final electron donor to molecular oxygen in the respiratory chain. In bovine heart mitochondria, this 13-subunit complex conserves the free energy of electron transfer by translocating protons from the mitochondrial matrix to the intermembrane space (Saraste, 1990).

The oligomeric state of the functional complex in the mitochondrial inner membrane is controversial. Cryo-electron microscopy of cytochrome *c* oxidase in two-dimensional crystalline arrays within collasped vesicles suggests a dimeric structure for the enzyme (Deatherage et al., 1982a,b; Valpuesta et al., 1990). Upon isolation, the oligomeric state of cytochrome *c* oxidase appears to depend on the detergent used to solubilize the protein: both monomers and dimers have been observed in Triton X-100 (TX-100)[1] solubilized enzyme (Robinson & Capaldi, 1977; Robinson & Talbert, 1986), while sedimentation equilibrium studies suggest a monomeric structure for dodecyl maltoside-solubilized oxidase (Suarez et al., 1984).

Limited data are available regarding the oligomeric requirements for the enzyme's cytochrome *c* oxidation and proton translocating activities. The minimum subunit requirement for electron-transfer activity in prokaryotes such as *Paracoccus denitrificans* includes only subunits I and II (Ludwig & Schatz, 1980; Hendler et al., 1991). Target size analysis of bovine heart and bacterial cytochrome *c* oxidase using radiation inactivation suggests that the functional unit for cytochrome *c* oxidation is monomeric (Suarez et al., 1984; Sone & Kosako, 1986) while the dimeric form of the enzyme acts as the functional unit for proton translocating activity (Sone & Kosako, 1986).

Understanding the properties of cytochrome *c* oxidase incorporated into liposomes has been limited by the lack of biophysical techniques to assess the aggregation state in a lipid environment. Although the kinetic studies of Antonini et al. (1987) suggest electron redistribution between cytochromes *a* of adjacent monomers, no direct chemical evidence for dimer reassociation in a phospholipid vesicle is available.

Our approach to addressing the question of the cytochrome *c* oxidase oligomeric state in liposomes utilizes heterobifunctional cross-linking reagents to identify dimer-specific subunit interactions. We have observed on sodium dodecyl sulfate–polyacrylamide gel electrophoresis ($NaDodSO_4$–PAGE) a 136-kDa cross-linked product found only in TX-100-solubilized dimers and in phospholipid vesicles which contain either dimeric enzyme or high (6 μM) concentrations of monomeric cytochrome *c* oxidase. Two-dimensional $NaDodSO_4$–PAGE of the 136-kDa dimer-specific cross-link shows that the cross-link contains subunit I, emphasizing that subunit I–subunit I contacts between monomers of cytochrome *c* oxidase may stabilize the dimeric form of the enzyme in membranes/phospholipid bilayers.

MATERIALS AND METHODS

Enzyme Preparations. Cytochrome *c* oxidase was isolated from bovine heart mitochondria (Azzone et al., 1979) using the method of Kuboyama et al., (1972) or Yonetani (1967). Cytochrome *c* oxidase concentration (heme aa_3) was deter-

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[1] Abbreviations: AMAS, succinimidyl maleimidoacetate; kDa, kilodalton(s); $NaDodSO_4$, sodium dodecyl sulfate; $NaDodSO_4$–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; *S*-SMPB, sulfosuccinimidyl 4-(*p*-maleimidophenyl)butyrate; SADP, *N*-succinimidyl 3-[(4-azidophenyl)dithio]propionate; SP, *N*-succinimidyl propionate; Tris, tris(hydroxymethyl)aminomethane; TX-100, Triton X-100.

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mined by using extinction coefficients of 24 mM^{-1} (van Gelder, 1966) for heme aa_3 at 605 nm for reduced minus oxidized spectra and 33 mM^{-1} for reduced heme aa_3 at $\Delta A_{605-630nm}$ (Briggs & Capaldi, 1977). The heme aa_3 concentration was determined by averaging the concentration obtained from both extinction coefficients. Protein was estimated by the method of Lowry et al. (1951). Phospholipid analysis was performed as described in Organisciak and Noell (1976).

Sedimentation velocity measurements were performed as described in Robinson and Talbert (1986) using a buffer containing 90 mM NaCl, 20 mM KH_2PO_4, pH 8.0, and 1.6 mM TX-100. Dimeric cytochrome c oxidase was prepared by the method of Kuboyama et al. (1972). Monomeric enzyme was prepared by the method of Georgevich et al. (1983) using the Kuboyama et al. preparation as the starting material. Alternatively, our Yonetani (1967) preparation of the enzyme was found to be a monomer by sedimentation velocity.

Gel Filtration Chromatography. Cytochrome c oxidase (4 mg of monomeric or dimeric enzyme) was incubated with 320 mol of TX-100/mol of heme aa_3 for 30 min at 0 °C and chromatographed over Sepharose 2B as described in Estey et al. (1990), using 20 mM KH_2PO_4, pH 8.0, 90 mM NaCl, and 1.6 mM TX-100 as an elution buffer. Fractions absorbing at 420 nm were pooled and used as the starting material for the subsequent cross-linking studies.

Chemical Cross-Linking of Cytochrome c Oxidase. Cytochrome c oxidase (0.75–1.0 μM heme aa_3) was reacted with 35 or 70 μM *N*-succinimidyl 3-[(4-azidophenyl)dithio]propionate (SADP) (Pierce Chemical) for 15 min in the dark at room temperature and then irradiated through glass with a Mineralite ultraviolet lamp [3×10^3 erg $(cm^2\ s)^{-1}$] for 30 min at 0 °C to complete photoactivated cross-link formation. The reacted enzyme was collected by ultracentrifugation and dissociated for $NaDodSO_4$–PAGE as described in Estey et al. (1990). The enzyme (1.0 μM heme aa_3) was also reacted with 47 μM succinimidyl maleimidoacetate [AMAS in dimethyl sulfoxide (Molecular Probes)] or 55 μM sulfosuccinimidyl 4-(*p*-maleimidophenyl)butyrate [*S*-SMPB (Pierce Chemical)] at 23 °C for 30 min and quenched with 180 mM Tris-HCl, pH 8.5, and 20 mM cysteine prior to electrophoresis. $NaDodSO_4$–PAGE was performed on either 12% or 16% polyacrylamide–6 M urea gels as described by Fuller et al. (1981) using Coomassie Blue as the stain.

Where indicated, dimeric cytochrome c oxidase (1.0 μM heme aa_3) was digested with 0.10 mg of trypsin/mg of heme aa_3 (TPCK-treated, Worthington Biochemicals) as described by DiBiase and Prochaska (1985). Reactions were quenched with 50 μg/mL soybean trypsin inhibitor at 0 °C for 15 min prior to chemical cross-linking.

Identification of Dimer-Specific Chemical Cross-Links by Two-Dimensional $NaDodSO_4$–PAGE. Dimeric cytochrome c oxidase was reacted with SADP as described above, and the dimer-specific chemical cross-link created by SADP was identified using two-dimensional $NaDodSO_4$–PAGE. The first dimension was run on $NaDodSO_4$–PAGE using 12% polyacrylamide–6 M urea gels as described above. The gel was first stained with Coomassie Blue, and then the 136-kDa band was excised from the gel. The slices were incubated at 37 °C (1 h) with either 10% $NaDodSO_4$ or 10% $NaDodSO_4$/25% β-mercaptoethanol and then loaded directly onto 16% polyacrylamide–6 M urea gels for the second dimension of $NaDodSO_4$–PAGE. The gel was then silver-stained using the procedure of Wray et al. (1981) to identify the subunit composition of the 136-kDa cross-link.

Preparation and Chemical Cross-Linking of Cytochrome c Oxidase Vesicles. Cytochrome c oxidase vesicles were prepared by the cholate dialysis method (Carroll & Racker, 1977) using cardiolipin and phosphatidylcholine (1:20, w/w; Sigma). The phospholipids were dried at room temperature using a rotary evaporator and then was solubilized in 23 mM (10 mg/mL) cholate + 100 mM HEPES–NaOH, pH 7.2. Liposomes (40 mg/mL phospholipid final concentration) were prepared using TX-100-solubilized cytochrome c oxidase (2 or 6 μM heme aa_3) as described in Wilson and Prochaska (1990). The amount of enzyme incorporated into the phospholipid vesicles was estimated to be greater than 80%; respiratory control ratios for these cardiolipin vesicles were 4.0–6.5 (Wilson & Prochaska, 1990).

Cytochrome c oxidase vesicles were diluted to 1 μM heme aa_3 with 20 mM KH_2PO_4, pH 8.0, and 90 mM NaCl and reacted with 70 or 210 μM SADP using the protocol described above. After the reactions were quenched, the vesicles were lysed with TX-100 (1–2 mg/mg of phospholipid), and pellets containing the enzyme were collected by ultracentrifugation at 190000*g* for 16 h. The enzyme was dissociated, and cross-linked products were separated by $NaDodSO_4$–PAGE on 12% acrylamide–6 M urea gels as described previously.

RESULTS

SADP Cross-Linking of Cytochrome c Oxidase Monomers and Dimers. Toward the goal of understanding the oligomeric state of cytochrome c oxidase in the mitochondrial inner membrane, we reacted the enzyme in well-defined oligomeric states in TX-100 with the heterobifunctional chemical cross-linking reagent SADP (Estey et al., 1990). TX-100 was chosen as the detergent because sedimentation velocity and equilibrium experiments on the enzyme have shown that in low Triton X-100 concentrations cytochrome c oxidase was a dimer, whereas in high TX-100 concentrations the enzyme was dissociated into monomers (Robinson & Talbert, 1986). SADP was used as the reagent because (1) it has broad amino acid specificity due to its amino-reactive succimidyl group and its nonspecific, highly reactive, photoactivatable azido group (Estey et al., 1990); (2) it contains a cleavable disulfide linkage which allows identification of subunits comprising newly created intersubunit cross-links, and (3) it reacts with both the hydrophilic and hydrophobic domains of the enzyme (Estey et al., 1990), thereby maximizing the chances of detecting a oligomeric state-dependent intersubunit cross-link in the enzyme.

Cytochrome c oxidase purified using the method of Kuboyama et al. (1972) in our laboratory was a dimer in low [Triton X-100] as determined by sedimentation velocity (Table I). This preparation of dimeric enzyme contained low amounts of endogenous phospholipid and exhibited heme to protein ratios which were comparable to monomeric enzyme prepared either by the method of Yonetani (1967) or by the method of Georgevich et al. (1981) (Table I). Since the oligomeric states of these enzyme preparations were not altered by solubilization of the protein in low TX-100 concentrations, these preparations were used to characterize intersubunit cross-links induced by SADP in cytochrome c oxidase monomers and dimers.

SADP cross-linking of dimeric cytochrome c oxidase resulted in the appearance of at least one new band on $NaDodSO_4$–PAGE which was not observed in cytochrome c oxidase monomers (Figure 1A). The apparent molecular mass of this dimer-specific cross-link was estimated to be 136 kDa as judged by comparison to molecular mass standards (data

Table I: Characterization of Triton X-100 Solubilized Cytochrome *c* Oxidase Monomers and Dimers[a]

enzyme preparation	sedimentation coefficient[b] (S)	oligomeric state	phospholipid content[c]
dimer, method of Kuboyama (1972)	12.7	80% dimer; 15% agggregate	13
monomer, method of Yonetani (1967)	8–9.6	100% monomer	10

[a] All enzyme fractions were incubated in 320 mol of TX-100/mol of aa_3 and chromatographed over Sepharose 2B columns equilibrated with 1.6 mM TX-100 before the determination of oligomeric state. The nanomoles of heme *a* per milligram of protein ratios ranged from 6.5 to 10.0 for the dimer preparations and from 7.4 to 8.5 for the monomer preparations (the theoretical maximum is approximately 10). [b] Sedimentation velocity measurements were performed as described in Robinson and Talbert (1986). [c] Reported as moles of phospholipid (MW = 750) per mole of cytochrome *c* oxidase monomer.

not shown). The apparent molecular mass of 136 kDa for this dimer-specific cross-link is an estimate of its size due to cross-linked proteins having differential migration in NaDodSO$_4$–PAGE (Briggs & Capaldi, 1977). The dimer-specific cross-link was formed within 5 min of irradiation; additional irradiation did not increase the yield of cross-linked product (as judged by the intensity of Coomassie Blue staining) or induce formation of the 136-kDa product in monomeric cytochrome oxidase (data not shown). Other than the formation of a 136-kDa cross-linked product, the reactivity of cytochrome *c* oxidase subunits with SADP showed few differences between monomeric and dimeric enzyme. In both monomers and dimers, subunit III was very reactive with SADP [see Estey et al. (1990)], while subunits II and Vb [nomenclature of Merle and Kadenbach (1982)] were not easily cross-linked (Figure 1). The decreased staining intensity of subunit I in the dimeric enzyme suggested that this subunit may be more susceptible to cross-linking in the dimeric enzyme.

The data in Figure 1 show that the yield of the dimer-specific 136-kDa intersubunit cross-link is low, but this was expected. We have characterized the reactivity of the monofunctional succinimidyl derivative of SADP, *N*-succinimidyl propionate (SP), with cytochrome *c* oxidase in different nonionic detergents (Estey et al., 1990). Our results showed that approximately 1.1 mol of SP was bound per mole of enzyme when the TX-100-dispersed enzyme was reacted with SP under similar experimental conditions as described for SADP. The SP was bound to all subunits of the enzyme as shown by NaDodSO$_4$–PAGE, but at different stoichiometries for each of the subunits [for complete details, see Estey et al. (1990)]. For example, we found that about 10% of the total SP was bound to subunit I; thus, approximately 0.11 mol of SP was bound to subunit I. In order to characterize the reactivity of the succinimidyl group of SADP with cytochrome *c* oxidase, we reacted the enzyme in TX-100 with [^{3}H]SP in the presence and absence of SADP and assessed the subunit distribution of [^{3}H]SP in each reaction by NaDodSO$_4$–PAGE. We found SADP effectively competed for the same subunits as [^{3}H]SP (data not shown). Also, if the specific activity of [^{3}H]SP was corrected for added SADP, equivalent amounts of reagent were bound to the enzyme under both conditions, emphasizing that SP and the succinimidyl group on SADP were competing for similar sites on the enzyme (data not shown). Thus, if subunit I was involved in a dimer-specific intersubunit cross-link, the staining intensity of the subunit I band on the gel would decrease by only 10% or so. Substoichiometric amounts of intersubunit cross-links created by bifunctional chemical reagents have been observed in other studies on cytochrome *c* oxidase (Briggs & Capaldi, 1977; Jarausch & Kadenbach, 1985) and other respiratory chain complexes (Gondal & Anderson, 1985; Dallman et al., 1992), emphasizing that the low yield of 136-kDa intersubunit cross-link is due to the reactivity of the reagent.

The possibility that the 136-kDa species was created by the cross-linking of an impurity in the dimeric enzyme preparation was investigated. Several impurities (molecular mass >75 kDa) were observed on the Coomassie Blue stained gel of untreated dimeric enzyme that were absent in our monomer preparation (see Figure 1A). However, trypsin proteolysis of the dimers prior to addition of cross-linking reagent eliminated these impurities without affecting the formation of the 136-kDa cross-link (Figure 2). Also, when the dimeric preparation of enzyme was treated at alkaline pH with high concentrations of TX-100 to form enzyme monomers (Georgevich et al., 1981) and then reacted with SADP, no 136-kDa band appeared on NaDodSO$_4$–PAGE. This result emphasized that the formation of the 136-kDa cross-link was not due to an impurity in our enzyme preparations.

If this 136-kDa species reflected an intersubunit cross-link created at the interface between two monomers of the enzyme, then alternative chemical reagents with different amino acid specificities should detect similar subunit–subunit interactions in the dimeric form of the enzyme. Figure 1B shows that two other heterobifunctional cross-linking reagents containing maleimide (reacting with sulfhydryl groups) and succinimide moieties in combination with different spacer lengths also formed the 136-kDa dimer-specific product (see Figure 1B). Both AMAS (a reagent that spans 8–9 Å), which is slightly soluble in water, and *S*-SMPB (a reagent that spans 14.9 Å), which is freely soluble in water, created the 136-kDa dimer-specific cross-link and also appeared to be more reactive with the lower molecular mass subunits (IV, Va/Vb, and VIa) than SADP. Subunits IV, VIa, and VIb were protected in dimeric enzyme from reaction with *S*-SMPB (Figure 1B). Dimeric cytochrome *c* oxidase reacted with AMAS showed a decreased extent of cross-linking of the same subunits, as well as subunit VIc.

These results identified two important characteristics of the dimeric enzyme: (1) the protein–protein interactions leading to the formation of the 136-kDa cross-link were not restricted to hydrophobic domains in the oxidase dimer and were within 8 Å of each other; (2) the formation of cytochrome *c* oxidase dimers shielded the lower molecular mass subunits (IV, Va/b, and VIa–c) from chemical modification by either water-soluble or hydrophobic reagents.

Subunit Composition of the Dimer-Specific Subunit Cross-Link. Two-dimensional NaDodSO$_4$–PAGE was used to identify the subunit composition of the 136-kDa dimer-specific cross-link. Since SADP contained an internal disulfide bridge, it was possible to reduce the chemical cross-links and identify the subunit composition of this band. The dimeric enzyme was cross-linked with SADP, dissociated for NaDodSO$_4$–PAGE in the absence of β-mercaptoethanol, and run on gels, and the dimer-specific 136-kDa band was excised from the first-dimension gel. The 136-kDa band then was incubated in β-mercaptoethanol, and the components of the cross-link were resolved on a second NaDodSO$_4$–polyacrylamide gel. Figure 3 shows the second-dimension gel stained with silver (to increase sensitivity) and demonstrates that subunit I was the predominant component of the 136-kDa cross-link,

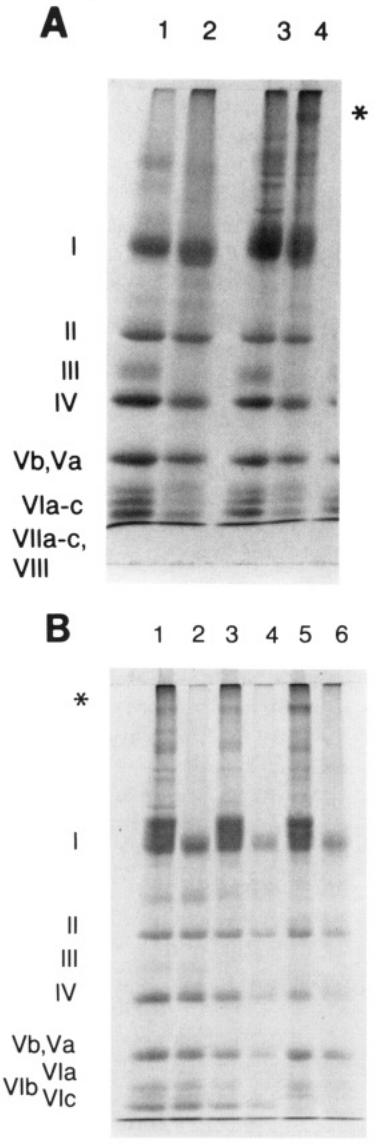

FIGURE 1: Cross-linking of Triton X-100 solubilized cytochrome *c* oxidase monomers and dimers. Cytochrome *c* oxidase monomers and dimers solubilized with TX-100 were reacted with SADP (panel A) or with AMAS or *S*-SMPB (panel B) as described under Materials and Methods and cross-linked products resolved by $NaDodSO_4$–PAGE using 12% polyacrylamide–6 M urea gels. The gels in both panels were stained with Coomassie blue. In panel A, each lane contains the enzyme irradiated as described under Materials and Methods: lane 1, monomeric enzyme; lane 2, monomeric enzyme + SADP; lane 3, dimeric enzyme; lane 4, dimeric enzyme + SADP. In panel B, AMAS, *S*-SMPB, and SADP were used to cross-link enzyme: lanes 1, 3, and 5 represent cross-linked dimeric cytochrome *c* oxidase, while lanes 2, 4, and 6 contain cross-linked monomeric enzyme. Reaction conditions for each lane of panel B were as follows: lane 1, dimeric enzyme + SADP; lane 2, monomeric enzyme + SADP; lane 3, dimeric enzyme + *S*-SMPB; lane 4, monomeric enzyme + *S*-SMPB; lane 5, dimeric enzyme + AMAS; lane 6, monomeric enzyme + AMAS. The molecular mass of the 136-kDa cross-link was estimated by migration on $NaDodSO_4$–PAGE using molecular mass standards, which included β-galactosidase, phosphorylase *b*, bovine serum albumin, egg albumin, glyceraldehyde-3-phosphate dehydrogenase, and carbonic anhydrase. The asterisk indicates the migration of the 136-kDa cross-link.

suggesting that two subunit I molecules from adjacent monomers were cross-linked together in dimeric species of the enzyme. Other subunits (except VIIa and perhaps VIII) were found in much lower stoichiometries (as judged by silver staining intensity). Treatment of the 136-kDa cross-link with $NaDodSO_4$ alone dissociated small amounts of noncovalently bound subunits (I, II, III, IV, and VIIb/c; see Figure 3), the amount of which varied between experiments. The conditions used for dissociation of the enzyme into subunits (37 °C for 45 min) may have allowed disulfide exchange between the cross-linking reagent and other adjacent cross-linked subunits to release components which were previously covalently bound (including subunit I; see Figure 3, lane 2). In any event, subunit I was the major component of the dimer-specific subunit cross-link.

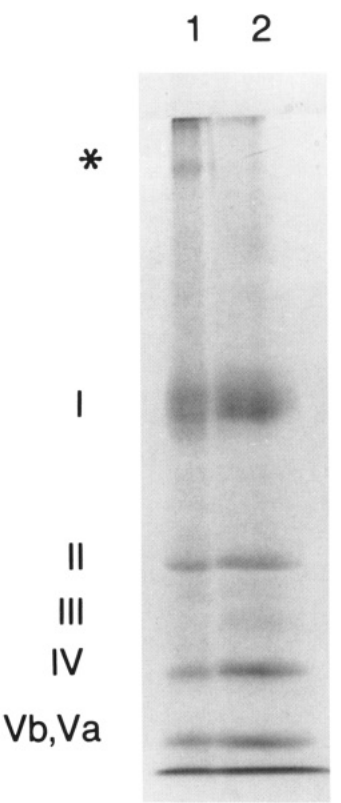

FIGURE 2: Chemical cross-linking of trypsin-treated dimeric cytochrome oxidase. Dimeric enzyme was digested with 0.1 mg of trypsin/mg of enzyme prior to chemical cross-linking with SADP. The enzyme was irradiated, and the cross-linked products were separated on $NaDodSO_4$–PAGE as described under Materials and Methods. The gel was stained with Coomassie blue. Lane 1 is trypsin-treated dimeric enzyme + SADP; lane 2 is trypsin-treated dimeric enzyme. The asterisk indicates the migration of the 136-kDa product.

Chemical Cross-Linking of Cytochrome c Oxidase in Phospholipid Vesicles. The reactivity of SADP with cytochrome *c* oxidase in phospholipid vesicles was tested to see if the reagent could identify putative dimers of the enzyme as detected by the formation of the 136-kDa dimer-specific cross-link in liposomes. Cardiolipin/phosphatidylcholine vesicles were used in these experiments because SADP could react with free amino groups in asolectin phospholipids. Figure 4 shows that cardiolipin/phosphatidylcholine vesicles prepared with 2 μM heme aa_3 monomeric enzyme did not exhibit formation of the 136-kDa band when reacted with two different concentrations of SADP. Figure 4 also shows that phospholipid vesicles containing the dimeric fraction of cytochrome *c* oxidase (2 μM heme aa_3) formed the 136-kDa cross-link in vesicles when reacted with SADP, suggesting that shielding of the enzyme by the phospholipid headgroups and/or tails did not prevent formation of the 136-kDa cross-link. When the number of cytochrome *c* oxidase monomers per phospholipid vesicle was increased by increasing the enzyme concentration (6 μM heme aa_3) at a constant phopholipid concentration (thus, at a constant liposome concentration), small amounts of the 136-kDa cross-link were created by reaction with SADP (Figure 4). These results suggest that

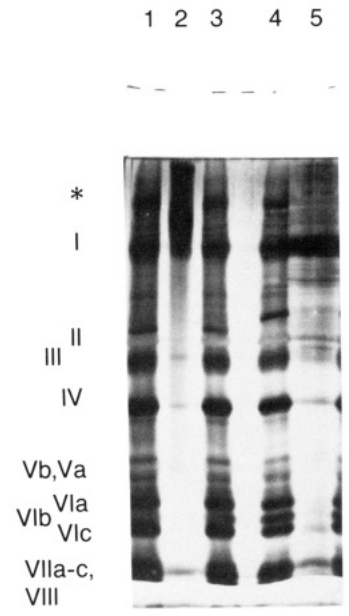

FIGURE 3: Subunit composition of the dimer-specific 136-kDa cross-link. Dimeric cytochrome *c* oxidase was reacted with SADP, and the 136-kDa product from $NaDodSO_4$–PAGE was excised from the first-dimension gel as described under Materials and Methods. The subunits in the reduced 136-kDa cross-link were separated on $NaDodSO_4$–PAGE using a second 16% polyacrylamide–6 M urea gel and were stained with silver. Lanes 1, 3, and 4 are control enzyme; lane 2 is the 136-kDa band dissociated with $NaDodSO_4$; lane 5 is the 136-kDa band dissociated with both $NaDodSO_4$ and β-mercaptoethanol to cleave intersubunit cross-links created by SADP.

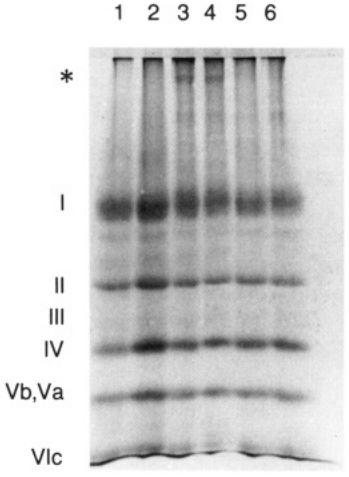

FIGURE 4: SADP cross-linking of cytochrome *c* oxidase in phospholipid vesicles. Cytochrome *c* oxidase vesicles were reacted with 70 μM SADP as described under Materials and Methods. The enzyme in all lanes was irradiated as described under Materials and Methods. Cross-linked enzyme was separated by $NaDodSO_4$–PAGE, and the gels were stained with Coomassie blue. Lane 1 is 2 μM (heme aa_3) monomeric vesicles; lane 2 is 2 μM monomeric vesicles + SADP; lanes 3 and 4 are 2 μM dimeric vesicles + SADP; lane 5 is 6 μM monomeric vesicles; lane 6 is 6 μM monomeric vesicles + SADP. The asterisk represents the migration of the 136-kDa cross-linked product.

some reassociation of cytochrome *c* oxidase into dimers may occur if high concentrations of monomers are used in the preparation of the vesicles and the number of enzyme molecules per phospholipid vesicle is increased.

DISCUSSION

The oligomeric state of cytochrome *c* oxidase in detergents such as deoxycholate, TX-100, and dodecyl maltoside has been accurately evaluated using sedimentation velocity and equilibrium methods (Robinson & Capaldi, 1977; Suarez et al., 1984). These methods cannot be used to address the aggregation state problem in phospholipid vesicles or intact membranes. Our previous studies on detergent interactions with cytochrome *c* oxidase suggested that the reactivity of the chemical modification reagents with the enzyme is very sensitive to changes in the protein's amphiphilic environment (Estey et al., 1990). Therefore, we used cross-linking reagents to identify differences in subunit interactions between monomers and dimers solubilized in the same detergent.

Our approach to assessing the oligomeric state of membrane-bound cytochrome *c* oxidase was modeled on TX-100-solubilized oxidase [see Robinson and Talbert (1986) for a full discussion]. The enzyme used in all of our studies had similar characteristics (heme to protein ratios, electron-transfer activity, and endogenous phospholipid contents) and differed only in oligomeric state when solubilized in 320 mol of TX-100/mol of heme aa_3. The observed differences in cross-linking patterns of the enzyme, therefore, could not be explained by the removal of bound phospholipid or other changes in the amphiphilic environment of the enzyme.

A 136-kDa dimer-specific chemical cross-link was identified using three chemical cross-linking reagents: SADP, AMAS, and *S*-SMPB. Neither trypsin proteolysis nor gel exclusion chromatography (to remove large protein aggregates) reduced the yield of the 136-kDa product. In addition, the 136-kDa cross-link was not formed when dimeric cytochrome *c* oxidase was converted to monomeric enzyme (Georgevich et al., 1986) (data not shown). It appears that the 136-kDa cross-link is observed only in dimeric cytochrome *c* oxidase reacted with bifunctional chemical cross-linking reagents.

There are limitations in our data which identify a 136-kDa species on $NaDodSO_4$–PAGE created by chemical cross-linking reagents in dimeric cytochrome *c* oxidase. One limitation is the accuracy of the measurement of the apparent molecular mass of the 136-kDa cross-linked product. Intersubunit cross-links have been shown to migrate anomalously on $NaDodSO_4$–PAGE (Briggs & Capaldi, 1977), so the apparent molecular mass of this species is probably inaccurate. Because of this limitation, the calculation of the putative subunit composition of the cross-linked product based on the addition of subunit molecular masses is invalid. Another limitation is that the stoichiometry and yield of the 136-kDa cross-link are low (as judged by Coomassie blue staining intensity on $NaDodSO_4$–PAGE) and only represent 5–10% of the reacted protein. This is expected as saturating reactive sites with chemical reagents is difficult especially in membrane proteins [see Prochaska et al. (1981) for example] and even more difficult using bifunctional chemical reagents, where both ends of the reagent must react with the protein. We attempted to quantitate the amount of cross-link formed using the monofunctional reagent SP, which is half of SADP. Our results suggest that about 0.1 mol of SP reacts with subunit I of the oxidase under the reaction conditions used for SADP cross-linking. Furthermore, the addition of SADP to [^{3}H]SP prior to labeling the enzyme results in a decrease in the radioactive labeling of the subunits of cytochrome *c* oxidase. This result emphasizes that SADP competes with [^{3}H]SP for reactive sites on the oxidase and that SP labeling is a good indicator of SADP reactive sites on the enzyme. Therefore, the observed stoichiometry of the 136-kDa species in Figures 1–3 is in an expected yield on the basis of the SP labeling data. Another limitation is that the 136-kDa species observed in enzyme dimers results from the cross-linking of a larger oligomeric species (trimer, tetramer) of the enzyme and is not

exclusively a marker for cytochrome *c* oxidase dimers. This is unlikely due to the gel filtration chromatography step prior to the addition of the cross-linking reagent in our experiments. Also, the amount of trimers and tetramers in the dimer preparation is small, so in order for these species to be observed on $NaDodSO_4$–PAGE as the 136-kDa species, their yield for the cross-linking reaction would have to be close to 100%. Finally, it could be argued that the 136-kDa band is an impurity in our preparations of the enzyme and that this band is highly reactive with the cross-linking reagents. The second-dimension $NaDodSO_4$–PAGE gel presented in Figure 3 shows that there is little, if any, 136-kDa impurity in the 136-kDa dimer-specific band (see lane 5). However, we cannot completely discount the idea that the cross-linking reagents reacted with this impurity, but the cross-linking of this impurity did not cause the formation of the majority of dimer-specific 136-kDa band observed on $NaDodSO_4$–PAGE.

The subunit composition of the 136-kDa cross-link showed that subunit I was the major component of the cross-link. Our previous [^{3}H]SP labeling of the enzyme (Estey et al., 1990) indicated that subunit I, although it contains nine lysine residues (Buse et al., 1985), is relatively unreactive with this reagent in comparison to the low molecular mass subunits. Predictions of the secondary structure of subunit I have identified 12 domains which potentially span the bilayer; some of these predicted α-helices are proposed to be located in the putative m_1 domain of the enzyme (Azzi & Muller, 1990; Capaldi, 1990). High-resolution electron microscopy studies suggest that the monomer–monomer contact site occurs through a cytoplasmically exposed region located directly above the membrane-spanning m_2 domain and not through direct contact in the membrane-spanning domains (Deatherage et al., 1982a,b). Although more recent structural data on the enzyme argue against the presence of discrete m_1 and m_2 domains, the volume of the membrane-spanning domain remains smaller than the volume which would be occupied by all predicted membrane-spanning helices (Valpuesta et al., 1990). It is possible that some of the predicted α-helices may fold within the matrix-exposed or cytoplasmically exposed domains in a manner that shields them from the aqueous environment.

The cross-linking data suggest that two subunit I molecules lie at or near the interface of two monomers of cytochrome *c* oxidase. Subunits VIb and VIc may also be found at the interface between monomers of the enzyme. Trypsin proteolysis of cytochrome *c* oxidase has been shown to digest selectively subunits VIb and VIc of enzyme solubilized with high [TX-100] (Ludwig et al., 1979; DiBiase & Prochaska, 1985), yet trypsin-treated dimers retained these subunits in the 136-kDa cross-link. These observations suggest that VIb and VIc were protected from proteolysis by protein interactions at the contact sites and support the previous findings of a VIb–VIb cross-link believed to be associated with the monomer–monomer contact site (Finel, 1987). Our cross-linking data using different reagents also suggest that other cytoplasmically encoded subunits may be at or lie near the site of interface between monomers of the enzyme. Subunits IV, Va/b, VIa, and VIb were slightly protected from *S*-SMPB cross-linking in oxidase dimers, and subunit VIc was protected from AMAS in dimers. The low reactivity of the cytoplasmically encoded subunits with AMAS may indicate that the 8-Å spacer arm is too short to accommodate cross-links between lower molecular mass subunits; AMAS does, however, allow the formation of the 136-kDa dimer-specific cross-link between the subunit I from each monomer. Interpretations of such cross-linking data are very limited since decreased reactivities with a particular reagent may be explained by either the inavailability of reactive groups on the protein or possibly the shielding of the subunit from the aqueous environment. Also, our results cannot discount other subunits residing at the interface of the two monomers in the dimeric structure of the enzyme. These results, even with the above-mentioned limitations, suggest that several of the cytoplasmically encoded subunits are shielded in the dimer contact site.

Information regarding the exposure of cytochrome *c* oxidase subunits to the aqueous environment in monomers and dimers was obtained by comparing their reactivities with the three cross-linking reagents. Only minor differences in the reactivity of subunits II and III with the cross-linking reagents were observed in monomers and dimers. Subunit III was very reactive with *S*-SMPB in both monomeric and dimeric enzymes. The enhanced reactivity of subunit III with this reagent is expected since subunit III contains a reactive cysteine residue (Cys-115) located in a hydrophilic loop on the cytoplasmic face of the enzyme (Hall et al., 1988). With the exception of subunit I, it appears that the mitochondrially encoded subunits share similar exposures in cytochrome *c* oxidase monomers and dimers and may form the outer core of the complex. However, the results presented here cannot unequivocally rule out that subunit III is localized at the interface of monomers in the dimer of the enzyme.

Toward the goal of assessing the aggregation state of cytochrome *c* oxidase in membranes, chemical cross-linking of cytochrome *c* oxidase in phosphatidylcholine/cardiolipin vesicles was performed. These investigations demonstrated that dimer reassociation within vesicles only occurred at higher monomer concentrations. These observations support the interpretation of Antonini et al. (1987) which suggests that the biphasic electron-transfer kinetics observed in monomeric cytochrome *c* oxidase vesicles are the result of electron redistribution between cytochromes *a* of reassociated dimers.

Taken together, these cross-linking studies suggest that some of the nuclear encoded subunits (particularly IV, VIa, VIb, and VIc) may be shielded in the dimer contact site while the mitochondrially encoded subunits II and III share similar exposure in cytochrome *c* oxidase monomers and dimers. Subunit I appears to be a major component of the 136-kDa product observed in cross-linked dimeric enzyme and lies near the contact site between two monomers in the dimeric structure of the enzyme. Subunit I, due to its large size and high α-helical content, may provide a large portion of the surface area for the contact sites (either in hydrophobic or in hydrophilic domains of the enzyme) between monomers of cytochrome *c* oxidase. These contact sites and the location of three of the redox centers of the enzyme in subunit I (Winter et al., 1980) may support the data of Antonini et al. (1987), which suggest that the dimeric structure of the enzyme regulates its electron-transferring activity. Along these lines, Bormann and Engelman (1992) have suggested that intramembrane helix–helix association in protein oligomerization occurs in signal-transducing proteins and that conformational regulation of the oligomeric state by effectors is involved with the transmembrane signaling. The control of helix–helix interactions in subunit I from each monomer of the enzyme may control electron-transfer activity by conformational change. The chemical approach to the study of monomer–monomer interactions in cytochrome *c* oxidase has allowed us to determine the oligomeric state of the enzyme in phosphatidylcholine/cardiolipin vesicles and has provided evidence to

support the idea that dimerization occurs within the phospholipid environment (Deatherage et al., 1982a,b).

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