J. Biochem. 119, 482-486 (1996)

Intra- and Inter-Complex Cross-Linking of Subunits in the Quinol Oxidase Super-Complex from Thermophilic *Bacillus* PS3[1]

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Received for publication, October 6, 1995

Gram-positive thermophilic *Bacilli* contain quinol-cytochrome *c* reductase and cytochrome *c* oxidase as two major respiratory complexes of the electron transfer chain, and these enzymes can be extracted with mild detergents as an associated quinol oxidase super-complex. The reductase is composed of three subunits; cytochrome b_6, cytochrome c_1, and FeS protein, whereas cytochrome *c* oxidase consists of four subunits numbered 1 through 4. In order to clarify the interactions between the subunits, the super-complex isolated from *Bacillus* PS3 was cross-linked with three bifunctional cross-linkers; disuccinimidyl tartrate, 3,3′-dithiobis(succinimidylpropionate), and ethylene glycolbis(sulfosuccinimidylsuccinate). The most prominent cross-linking was observed for the combination of subunit 1 *plus* 2 in cytochrome *c* oxidase, and for that of cytochrome b_6 *plus* cytochrome c_1 in the reductase. In addition to these intra-complex cross-linkings, inter-complex linking was observed for the combination of cytochrome b_6 *plus* subunit 1 with ethylene glycolbis(sulfosuccinimidylsuccinate), and for the combinations of cytochrome b_6 *plus* subunit 1 and cytochrome b_6 *plus* subunit 2 with 3,3′-dithiobis(succinimidylpropionate). Incubation in the presence of Triton X-100, which was confirmed to cleave the two enzyme complexes, selectively reduced the inter-complex cross-linking, suggesting that the chemical cross-linking reflect the spatial arrangement of subunits in the super-complex.

Key words: chemical cross-linker, intrinsic membrane protein, quinol oxidase super-complex, respiratory chain, thermophilic bacterium.

Gram-positive spore-forming thermophilic *Bacilli* such as *Bacillus stearothermophilus* and related strains contain quinol-cytochrome *c* reductase and cytochrome *c* oxidase as two major respiratory complexes of the electron transfer chain (*1*). The former enzyme, also called cytochrome bc_1 complex, purified from *Bacillus* PS3 in the presence of Triton X-100, was shown to contain two heme B, one heme C, and a Rieske-type iron-sulfur center, and to be composed of three or four subunits like other bacterial, mitochondrial, and plastidal equivalents (*2*). Recently, the gene cluster of the bc_1 complex (*qrcABC*) was cloned from *B. stearothermophilus* K1041, which was found to encode three subunits; cytochrome *c*, cytochrome b_6, and FeS protein (*3, 4*). On the other hand, cytochrome *c* oxidase was also purified in the presence of Triton X-100 and found to consist of four subunits (CO1-4: *5, 6*). CO1 contains a low-spin heme A and a Cu_B-heme A binuclear center, while CO2 contains a heme C and Cu_A, therefore the enzyme is also called the caa_3 complex. The genes for subunits of the caa_3 complex (*caaABCD*) were previously cloned from PS3 (*7*) and recently from *B. stearothermophilus* K1041 (*8*).

These two enzyme complexes can be prepared as an associated super-complex when bacterial membranes are solubilized with milder detergents such as octaoxyethyleneglycol lauryl alcohol ether ($C_{12}E_8$) and a 1:1-mixture of *n*-nonanoyl *N*-methylglucamide (MEGA 9) *plus* *n*-decanoyl *N*-methylglucamide (MEGA 10) instead of Triton X-100 (*9*). The super-complex shows high quinol oxidase activity and a reasonable molecular weight for a 1:1 association, suggesting that the super-complex is not an artefactual aggregation product in a random orientation, but rather a functional unit *in vivo* associated in the proper orientation (*9, 10*). A similar super-complex was reported for a Gram-negative bacterium, *Paracoccus denitrificans* (*11*), although the spatial arrangement of the subunits has not been clarified yet. Here, we report the intra- and inter-complex interactions between subunits in the super-complex determined by use of three cleavable bifunctional cross-linkers. We succeeded in detecting inter-complex cross-linked products.

EXPERIMENTAL PROCEDURES

Enzyme Preparation—The super-complex was prepared as previously reported (*9*) with some modifications:

[1] This study was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Culture, and Sports of Japan.

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Abbreviations: CBB, Coomassie Brilliant Blue; MEGA 9, *n*-nonanoyl *N*-methylglucamide; MEGA 10, *n*-decanoyl *N*-methylglucamide; $C_{12}E_8$, polyoxyethyleneglycol lauryl alcohol ether ($n=9$); DST, disuccinimidyl tartrate; DSP, 3,3′-dithiobis(succinimidylpropionate); sulfo-EGS, ethylene glycolbis(sulfosuccinimidylsuccinate); TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylene diamine.

Briefly, PS3 membranes (3 g of protein) were washed twice with 2% (w/v) Na-cholate at 10 mg protein/ml, and then solubilized with 6% (w/v) polyoxyethyleneglycol lauryl alcohol ether ($n=9$) ($C_{12}E_9$) containing 100 mM NaCl and 10 mM Tris-HCl, pH 8.0. The mixture was centrifuged at $100,000\times g$ for 40 min, and the supernatant was dialyzed against 10 mM Tris-HCl, pH 8.0, to reduce the NaCl concentration, and then applied to a DEAE-Toyopearl column (2.5×10 cm) equilibrated with 1% (w/v) $C_{12}E_9$ and 10 mM Tris-HCl, pH 8.0. The column was then washed with 100 ml of the same solution and 200 ml of the solution containing 40 mM NaCl. The super-complex consisting of both the bc_1 and caa_3 complexes was eluted by raising the NaCl concentration to 60 mM, and the dissociated bc_1 complex was eluted with higher concentrations of NaCl. The peak fractions of the super-complex were collected, dialyzed and then applied to a second DEAE-Toyopearl column (1.5×10 cm). The column was washed with 100 ml of buffer containing 0.5% sucrose monolaurate and 10 mM Tris-HCl, pH 8.0, to replace the detergent. The super-complex was eluted with a buffer containing 150 mM NaCl and 20 mM Na-P_i, pH 7.4 (phosphate-buffered saline, PBS), with 0.5% sucrose monolaurate.

Cross-Linking—Three bifunctional cleavable cross-linkers were used: disuccinimidyl tartrate (DST, the distance between functional moieties being 0.64 nm, according to the manufacturer's handbook), 3,3′-dithiobis-(succinimidyl propionate) (DSP, 1.20 nm), and ethylene glycolbis(sulfosuccinimidylsuccinate) (sulfo-EGS, 1.61 nm). The purified super-complex was incubated at 5 mg/ml with 5 mM DST, 0.4 mM DSP, or 3 mM sulfo-EGS in PBS for 30 min at room temperature. The reaction was quenched by adding Tris-HCl, pH 6.8, to a final concentration of 150 mM. The mixture was then applied on a mini-slab gel (60 mm×80 mm×1 mm) including 11.5% acrylamide and 6 M urea. SDS-PAGE was performed according to the method described by Laemmli (*12*) except that boiling of the protein sample was omitted. Each lane in the first-dimensional slab gel was stripped off and incubated with the reagents to cleave the linker. The cleavage conditions were as follows: DST, 15 mM sodium periodate, 1% SDS, 20 mM Na-P_i, pH 7.0, at 25°C for 2 h; DSP, 5% β-mercaptoethanol, 300 mM Tris-HCl, pH 8.8, at 37°C for 30 min; and sulfo-EGS, 1 M hydroxylamine chloride at 37°C, for 6 h. Each treated gel strip was horizontally laid on a flat stacking gel of a second-dimensional slab for SDS-PAGE including 13.5% acrylamide and 6 M urea. The gel was stained with *o*-toluidine blue to detect peroxidase activity due to heme (*13*) and/or with CBB or silver (*14*) for the detection of total proteins.

Peptide Sequencing—Proteins were electrophoresed as above and then electro-transferred to a polyvinylidene difluoride membrane for 3 h according to Towbin *et al.* (*15*). The membrane was then stained with CBB and washed extensively with water to remove glycine, and then the bands were cut out and analyzed with a pulse-liquid peptide sequencer (Applied Biosystems, model 477A).

Immunoprecipitation—Rabbit antiserum against cytochrome *c* oxidase from *Bacillus* PS3 was prepared as reported previously (*9*). The super-complex sample (20 μg of protein) was incubated with 3 μl of rabbit anti-oxidase serum in 150 μl of a buffer containing 1.4% (w/v) octylglucoside and 100 mM Na-P_i, pH 7.5, with or without Triton X-100, at room temperature for 2 h. The mixture was centrifuged for 10 min at $12,000\times g$ and 4°C. After removing the supernatant, the pellet was resuspended in 150 μl of the same buffer as above. The quinol-cytochrome *c* reductase activity and the oxidase activity for *N,N,N′,N′*-tetramethyl-*p*-phenylene diamine (TMPD) were measured spectrophotometrically at 22°C by following the increases in absorbance at 549 and 562 nm, respectively. For quinol-cytochrome *c* reductase activity, duroquinone was dissolved in ethanol at 20 mg/ml and reduced to quinol with an appropriate amount of sodium borohydride. An aliquot of hydrochloric acid was added to avoid autoxidation. The reaction was started by adding horse heart cytochrome *c* to a final concentration of 30 μM in the presence of 150 μM duroquinol, 20 μg/ml sonicated phospholipids, 1 mM KCN, 1 mM EDTA, and 20 mM Na-P_i, pH 6.6. For oxidase activity, the reaction mixture contained 0.32 mM TMPD, 0.1 M NaCl, 1 mM EDTA, and 50 mM Na-P_i, pH 6.0. The activities were calculated by using millimolar extinction coefficients, $\Delta\varepsilon_{550}=20.7\ mM^{-1}\cdot cm^{-1}$ and $\Delta\varepsilon_{562}=10.5\ mM^{-1}\cdot cm^{-1}$, respectively. Values were corrected for the slow non-enzymatic increase in the absorbance.

Materials—DST, DSP, and sulfo-EGS were obtained from Pierce. MEGA-9 and MEGA-10 were from Dojin (Kumamoto). DEAE-Toyopearl, polyvinylidene difluoride membranes, Triton X-100, and $C_{12}E_9$ were purchased from Tosoh (Tokyo), Millipore, Wako Pure Chemicals (Osaka), and Nacalai Tesque (Kyoto), respectively. Duroquinone and horse heart cytochrome *c* were purchased from Sigma. Phospholipids were extracted from *Bacillus* PS3 according to Bligh and Dyer (*16*).

RESULTS

Subunit Composition—The super-complex preparation gave nine bands corresponding to apparent molecular weights of 51, 45, 38, 29, 23, 22, 21, 14, and 11 kDa, as shown in Fig. 1, lane a. The four with apparent molecular weights of 45, 38, 29, and 21 kDa were stained with *o*-toluidine and more intense than the proteins stained only with CBB. As determined previously (*9*), the proteins of 29, 23, and 21 kDa are cytochrome c_1, iron-sulfur (FeS) protein, and cytochrome b_6 of quinol-cytochrome *c* reductase, and the proteins of 51, 38, 22, and 11 kDa are subunits 1 through 4 (CO1 through CO4) of cytochrome *c* oxidase. As reported recently, the gene cluster for cytochrome *c* oxidase (*cta* operon) contains four genes (*ctaCDEF*) encoding CO2, 1, 3, and 4 of the oxidase, in that order, in addition to two genes (*ctaAB*) encoding a putative heme A synthase and a heme O synthase, respectively (*7, 8*), whereas the gene cluster for quinol-cytochrome *c* reductase (*qcrABC*) consists of three genes encoding FeS protein, cytochrome b_6, and cytochrome c_1, in that order (*3, 4*). CO4 is usually apparent but faint and sometimes accompanied by a little smaller peptide of 10 kDa, which is probably a degradation product of CO4. CO3, that migrates between the FeS protein and cytochrome b_6 on gels, can hardly be detected unless the oxidase is separated from the reductase, because of cytochrome b_6 and the FeS protein.

In addition to these subunits, a band corresponding to a molecular weight of 45 kDa was constantly observed. The 45-kDa band was stained with *o*-toluidine like CO2, cytochrome c_1 and cytochrome b_6, suggesting that the protein

contains a covalently bound heme C or tightly bound protoheme, such as that in cyt b_6. In order to characterize the 45-kDa band material, its N-terminal sequence was determined. The main amino acids detected per Edman reaction cycle were as follows: 1, M; 2, H+L; 3, N+R; 4, G+K; 5, K+L; 6, G+Y. The amount of each amino acid was more than 80 pmol when about 20 μg of the protein was used. Based on the nucleotide sequences of the genes (*3, 4*), this result can be interpreted as a mixture of the N-terminal sequences of cytochrome c_1 (MHRGKG) and cytochrome b_6 (MLNKLY), indicating that the protein is composed of these two cytochromes, which were not dissociated in the presence of 2% SDS. They only dissociated partially in the presence of 6 M urea or on heating at 95°C for 5 min.

The 14-kDa protein detected in the super-complex was also identified in the isolated bc_1 complex (*2*). This protein was subjected to peptide sequencing and its N-terminal sequence was found to be MKFNTGLE, which could not be found in the *qcr* operon (*3, 4*).

Intra- and Inter-Complex Cross-Linking—In order to clarify the spatial arrangement of the subunits in the super-complex, three cleavable bifunctional cross-linkers, DST, DSP, and sulfo-EGS, were used. Their functional moieties are all succinimide, which is known to be highly reactive with amino groups and also with hydroxyl groups, but to a lesser extent. The distance between the succinimide groups is 0.64 nm for DST, 1.20 nm for DSP, and 1.61 nm for sulfo-EGS. Figure 1, lane b, shows a two-dimensional electropherogram of the super-complex which was cross-linked with DST and cleaved with sodium periodate. Protein bands migrated more slowly in lane b than in lane a because of the thickness of the gel strip applied to lane b. The most prominent cross-linked product was a dimer containing CO1+CO2. There were also dimers composed of cytochrome c_1+FeS protein, FeS protein+14-kDa protein, and CO3+CO4. The 10-kDa protein, migrating a little faster than CO4, was also cross-linked with CO3. It is unlikely that CO3, CO4, and the 10-kDa protein formed a ternary complex because the cross-linked product showed a molecular weight of about 30 kDa and closely comigrated with c_1 (29 kDa) but not with CO2 (38 kDa). Many other weaker spots were seen on the gel, however, no obvious cross-linking was detected between subunits belonging to different enzyme complexes.

Sulfo-EGS gave the cross-linking pattern shown in Fig. 2. The most prominent cross-linked products are the pairs of CO1+CO2 within the caa_3 complex, and cytochrome c_1+cytochrome b_6 within the bc_1 complex. In addition to these

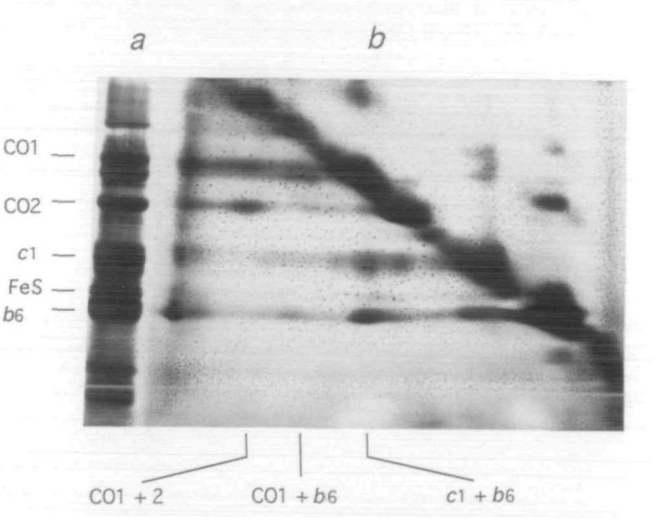

Fig. 2 **A two-dimensional electropherogram of the super-complex cross-linked with sulfo-EGS.** Experiments were carried out as described in Fig 1, except that sulfo-EGS was used as the cross-linker. In lane a, the amount of super-complex applied was 10 μg.

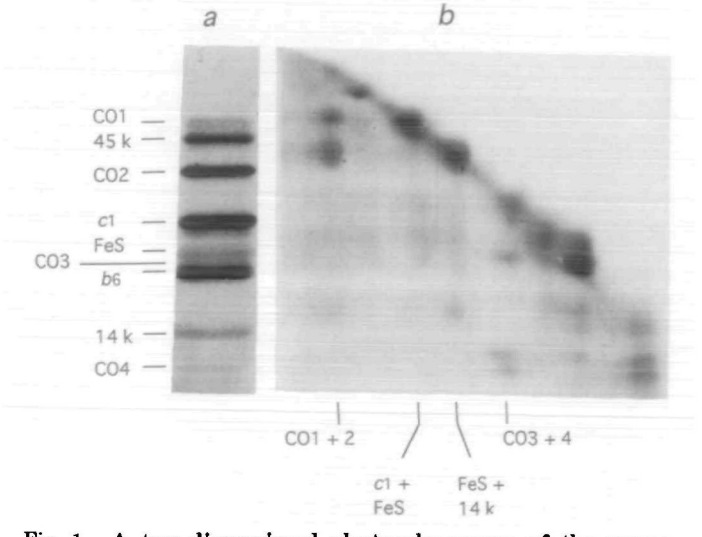

Fig. 1. **A two-dimensional electropherogram of the super-complex cross-linked with DST.** The super-complex (40 μg of protein) was cross-linked with DST and then separated by two-dimensional electrophoresis as described under "EXPERIMENTAL PROCEDURES" (lane b). In lane a, the purified super-complex (5 μg of protein) was applied to the well. The gel was stained first with *o*-toluidine and then with Coomassie Brilliant Blue.

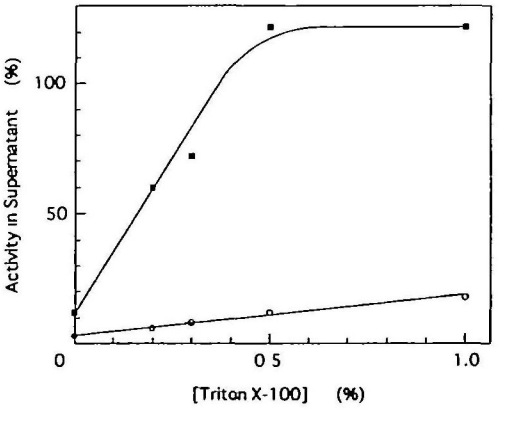

Fig. 3. **Effect of Triton X-100 on immunoprecipitation with anti-oxidase serum of the quinol-cytochrome *c* reductase and cytochrome *c* oxidase in the super-complex.** The super-complex was incubated with rabbit anti-oxidase serum in the presence and absence of Triton X-100 as described under "EXPERIMENTAL PROCEDURES." After centrifugation, the cytochrome *c* oxidase (○) and quinol-cytochrome *c* reductase (■) activities in the supernatant were assayed. The activities are expressed relative to those in the mixture before centrifugation with each concentration of Triton X-100

intra-complex pairs, an inter-complex pair of CO1+cytochrome b_6 was observed. Although the spots of CO1 and cytochrome b_6 did not look stoichiometric, the molecular weight of the cross-linked product was about 70 kDa, which reasonably agreed with the expected value for a CO1+cytochrome b_6 heterodimer with a 1:1 ratio. The CO1 spot originating from the CO1+cytochrome b_6 dimer may be overlapped with other spots of CO1 and the 45-kDa protein originating from different cross-linked products with close molecular weights, although the contribution of each product might be minor.

Effect of Triton X-100—As reported previously, the two enzymes in the super-complex can be separately eluted from ion-exchange resin columns in the presence of Triton X-100 (*2, 5*). In order to confirm that Triton X-100 cleaves the two complexes, an immunoprecipitation experiment was performed, as shown in Fig. 3. The rabbit polyclonal antiserum raised against PS3 cytochrome *c* oxidase mainly reacted with CO2 and exhibited little cross-reactivity with any subunits of the bc_1 complex (*9*). The super-complex was incubated with the antiserum, followed by the addition of various concentrations of the detergent. The incubation mixture was then centrifuged to remove aggregates of the antigen-antibody complex. In the absence of Triton X-100, cytochrome *c* oxidase and quinol-cytochrome *c* reductase activities were absent from the supernatant, indicating that both the oxidase and reductase were immunoprecipitated with the anti-oxidase serum (*9*). With an increased concentration of Triton X-100, the reductase activity appeared in the supernatant, while the oxidase activity remained low. These results suggest that the binding between the two enzymes was disrupted by Triton X-100, whereas the binding between the antigen and antibody was unaffected. The dissociation of the super-complex was also confirmed by analyzing the immunoprecipitate and supernatant by SDS-PAGE (data not shown).

Figure 4 shows the effect of Triton X-100 on the cross-linking of the super-complex with DSP. In the absence of Triton X-100, the inter-complex pairs of CO1+cytochrome b_6 and CO2+cytochrome b_6 were observed in addition to the intra-complex cross-linked pairs of CO1+CO2 and cytochrome c_1+cytochrome b_6 (Fig. 4A). In the presence of Triton X-100, the inter-complex pairs were much reduced, while the intra-complex pair, CO1+CO2, was not affected (Fig. 4B). The other intra-complex pair, cytochrome c_1+cytochrome b_6, was intermediately reduced.

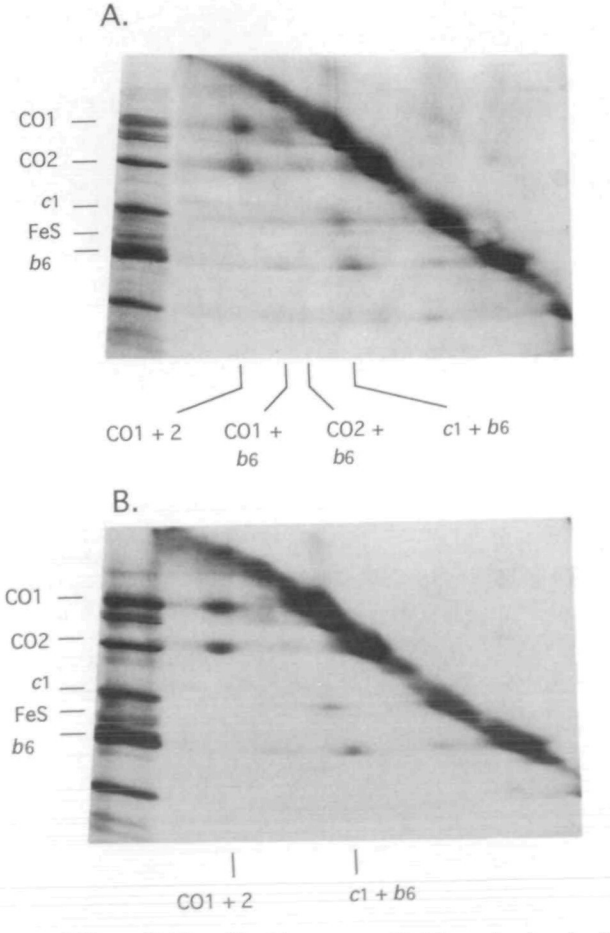

Fig. 4 **Effect of Triton X-100 on cross-linking of subunits in the super-complex with DSP.** The super-complex was cross-linked with DST in the absence (A) and presence of 0.7% (w/v) Triton X-100 (B), and then separated by two-dimensional electrophoresis as described in Fig. 1 (lane b). In lane a, the purified super-complex (5 μg of protein) was applied to the well.

DISCUSSION

The present experiments showed that the chemical cross-linkers examined produced two types of cross-linking within the quinol oxidase super-complex; intra- and inter-complex cross-linking. Intra-complex cross-linking predominantly occurred between CO1 and CO2 of the cytochrome *c* oxidase, and between cytochrome c_1 and cytochrome b_6 of quinol-cytochrome *c* reductase in the presence of DST, DSP, or sulfo-EGS. These results suggest that there are larger interfaces containing reactive amino or hydroxyl groups in these subunit combinations than other combinations. Recently, the crystal structures of cytochrome *c* oxidases at fine resolution were reported for a Gram-negative bacterium, *Paracoccus denitrificans* (*17*), and bovine cardiac mitochondria (*18*). They showed that the large surface area of CO2, both hydrophilic and hydrophobic domains, is in close contact with CO1. Since these two enzymes share homologous primary sequences, redox chromophores, and reaction mechanism with the thermophilic *Bacillus* enzyme (*1*), the essential three-dimensional structure might be common. The thermophilic *Bacillus* oxidases additionally possess a hydrophilic cytochrome *c* domain, which was kinetically suggested to play a role analogous to that of cytochrome *c* bound to the high-affinity site of aa_3-type oxidase (*19*), and consequently also suggested to further increase the surface area of CO2 in close contact with CO1. These situations seem compatible with the present finding that CO1 and CO2 are predominantly cross-linked.

No inter-complex cross-linking was observed when the protein was incubated with the short-range cross-linker, DST, while CO1 *plus* cytochrome b_6 and CO2 *plus* cytochrome c_1 or cytochrome b_6 were cross-linked with the longer-range cross-linkers, DSP and sulfo-EGS, although the extents of the reactions were less than that of the intra-complex combination. These results suggest that the extent of subunit contact between the two complexes is less than that within each complex. Previous reports showed

that the two enzymes of the super-complex can be separately eluted from ion-exchange columns in the presence of Triton X-100 (*2, 5*), whereas they are prepared as an associated super-complex when solubilized with milder detergents (*9*). The immunoprecipitation experiment confirmed that Triton X-100 destroys the contact between the two enzymes in the super-complex (Fig. 3). In parallel, the detergent selectively reduced the inter-enzyme cross-linking (Fig. 4). These results also indicate that the chemical cross-linking reflects the spatial arrangement of subunits in functional complexes.

The heterodimer of cytochrome b_6 *plus* cytochrome c_1 is the most prominent product within the bc_1 complex cross-linked with the three reagents. This suggests that the contact between these two subunits is stronger than that in the cases of the other subunit combinations. This assumption is also supported by the constant presence of the 45-kDa protein in the purified super-complex preparation. Peptide sequencing of this protein clearly showed that it was a complex composed of cytochrome c_1 and cytochrome b_6 that were so strongly associated that it was resistant even to SDS, although no chemical cross-linkers were added. The 45-kDa protein might be a complex in a 1:1 ratio, since its apparent molecular weight agrees with the sum of 29 kDa (cytochrome c_1) and 21 kDa (cytochrome b_6) more closely than any other stoichiometric ratio. The reason for the 10% difference is not well understood but it may be due to C-terminal processing of one or both of the peptides or experimental limitations of molecular weight determination with the present system.

We wish to thank Prof. M. Kawamura of the University of Occupational and Environmental Health for allowing us to use the peptide sequencer.

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