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Studies on the oligomeric state of isolated cytochrome oxidase using cross-linking reagents

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(1) Sucrose gradient centrifugation of cytochrome oxidase in the presence of Triton X-100 gave one slowly sedimenting green band. After cross-linking with dithiobis(succinimidylpropionate) (DSP), two green bands were observed, one sedimenting like the control and the other one more rapidly. Only the slowly sedimenting band was observed if the cross-linker was cleaved by dithiothreitol before centrifugation. (2) The rapidly sedimenting band in the Triton-containing sucrose gradient is probably the internally cross-linked dimer of cytochrome oxidase; the one sedimenting slowly is the monomeric enzyme. (3) Cross-linking with DSP after monomerization yields a small fraction of internally cross-linked dimers in addition to the internally cross-linked monomers. Under similar conditions, but using the shorter cross-linker disuccinimidyl tartarate (DST), no dimers are detected. (4) Both DSP and DST cross-link the dimeric enzyme so that it could no longer be monomerized by centrifugation in Triton, unless the cross-link is cleaved. (5) Polypeptide analysis using two-dimensional gel electrophoresis of cross-linked dimers and monomers suggest that subunit VIb is involved in intermonomeric cross-linking of dimeric enzyme by DSP.

Introduction

Cytochrome oxidase (EC 1.9.3.1) is the oxygen-reducing enzyme complex of the respiratory chains of mitochondria and many bacteria [1]. It is an integral membrane enzyme composed of up to 13 polypeptides per monomer in eukaryotes and possibly exists as a dimer in the inner mitochondrial membrane [2].

We have recently suggested that the dimeric state of cytochrome oxidase might be important for the proton translocation activity [3]. We have also observed that centrifugation in the presence of Triton X-100, but not of cholate, monomerizes the enzyme.

Cleavable cross-linkers were introduced by Wang and Richards [4], and employed to study near-neighbour relationships and the organization of polypeptides in different enzyme complexes [5,6]. From the many bifunctional cross-linking reagents available (for review, see Ref. 7), cleavable ones were chosen here since they allow analysis of cross-linked polypeptides.

Cytochrome oxidase has been cross-linked before to detect near-neighbour relationship of the subunits [8,9], but under conditions where the oligomeric state was not fully defined.

In this paper cross-linking is used as an additional approach to study the oligomeric state of cytochrome oxidase and the organization of monomers within the dimeric enzyme.

Abbreviations: DSP, dithiobis(succinimidylpropionate); DST, disuccinimidyl tartarate; SDS, sodium dodecyl sulphate.

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Materials and Methods

DSP and DST were purchased from Pierce. Cholic acid (Merck) was recrystallised before preparation of the sodium cholate stock solution (20%, w/v) (pH 7.5). Triton X-100 and dithiothreitol were purchased from Boehringer, ^{3}H-labeled Triton X-100 from New England Nuclear and [^{14}C]cholate from Amersham.

Cytochrome *c* oxidase was isolated from bovine heart mitochondria by a slight modification of the procedure of Yu and King [10] as previously described [3].

Cross-linking was performed at room temperature for 10 min in 200 mM potassium phosphate (pH 7.4) and detergent as specified in the figure legends. The enzyme concentration was 6 μM.

The cross-linking reagents were dissolved in dimethyl sulfoxide at 20 mg/ml prior to use, and their final concentration was 0.1 mg/ml unless otherwise mentioned. The reaction was quenched by adding 60 μl of 1 M ammonium acetate per ml. Cleavage of cross-links was achieved by adding dithiothreitol to a final concentration of 50 mM after the quenching, and incubating for 30 min at room temperature.

Sucrose gradient centrifugation was performed using an SW41 rotor. The gradients were made 5–10% in sucrose and contained 0.5 M potassium phosphate (pH 7.4) and either 2% (w/v) cholate or 0.2% (w/v) Triton X-100, in a final volume of 10.5 ml. After centrifugation for 20 h at 40000 rpm at 4°C, 0.25-ml fractions were carefully collected from the top of the tubes and mixed with 0.75 ml water before measuring the absorbance difference at 420–470 nm. Since the centrifugation was not isopycnic [3] and since fractions were collected from the top, care was taken to load equal volumes, mostly 1 ml, on already balanced tubes.

For two-dimensional SDS-polyacrylamide gel electrophoresis, 12–20% acrylamide gradients in the presence of 5 M urea were used [3]. The appropriate lane from the first gel was cut and placed horizontally on the second gel over a 3 mm thick layer of 1% agarose containing 2% β-mercaptoethanol, which was layered on the top of the stacking gel. Protein bands and spots were visualized by Coomassie blue staining.

Bound detergent was determined by incubating 9 nmol of enzyme in 1 ml of either 1% cholate containing 8 μCi [^{14}C]cholate or 0.2% Triton X-100 containing 15 μCi ^{3}H-labeled Triton X-100. The samples were centrifuged in sucrose gradients in the presence of 0.5% cholate or 0.2% Triton containing the same specific radioactivity as the incubation solutions, and otherwise in similar conditions to the gradients described above. Fractions were analysed for cytochrome aa_3 concentration using a millimolar extinction coefficient of 27 cm^{-1} for reduced minus oxidase enzyme at 605–630 nm, and for radioactivity.

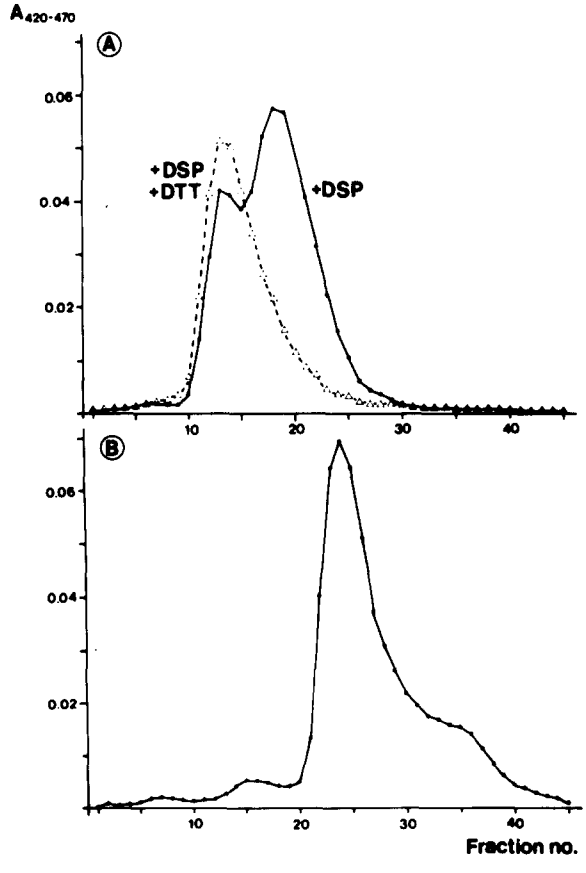

Fig. 1. Sucrose gradient centrifugation of cytochrome oxidase in the presence of Triton X-100 (A) or cholate (B) after cross-linking with DSP. The enzyme was dissolved in 0.5% (w/v) Triton/200 mM potassium phosphate (pH 7.4) and DSP was added to 0.2 mg/ml. Incubation, quenching and dithiothreitol treatment (dashed line in A) are described in Materials and Methods. The fractions are numbered from the top.

Results

We have recently suggested that sucrose gradient centrifugation in the presence of Triton X-100 and 0.5 M potassium phosphate monomerizes cytochrome oxidase, while a similar treatment in cholate does not [3]. Covalent cross-linking of the two monomers in the dimer is expected to prevent monomerization. It should therefore result in the appearance of a rapidly sedimenting band in a gradient containing Triton.

The sucrose concentrations used here are lower and the gradients more shallow than previously [3], for better separation between the sedimenting bands.

Fig. 1 shows the results of sucrose gradient centrifugation in the presence of Triton (A) and cholate (B) after cross-linking of cytochrome oxidase with DSP. In (A) there are two peaks with the cross-linked enzyme. Only one slowly sedimenting band was observed after cleavage of the cross-linker with dithiothreitol (Fig. 1A), as is the case without cross-linker [3]. When Triton was replaced by cholate in the gradient (Fig. 1B) only one main band was seen after cross-linking. This was independent of treatment with dithiothreitol (not shown). This band sedimented as the untreated dimeric enzyme [3]. The shoulder around fraction 35 (Fig. 1B) was not observed without cross-linker, and it might represent a trimer or a tetramer. A small slowly sedimenting band is also observed in Fig. 1B around fraction 15. This is probably monomeric enzyme, since cytochrome oxidase monomerized by one of the previously described procedures [3] sedimented to this position under similar conditions (not shown).

If the slow sedimenting band in the Triton-containing gradient is the monomer [3], the faster cross-linked band in Fig. 2A is very likely to be the dimeric enzyme. Cross-linking is not expected to increase significantly the amount of bound detergent, which would be an alternative explanation for the increase in sedimentation velocity.

The dimer is sedimenting somewhat more slowly in the Triton- than in the cholate-containing sucrose gradient (Fig. 1A vs. B). As the monomer behaves similarly, this is probably an effect of the detergent. This effect on the sedimentation is probably due to a difference in the amount of

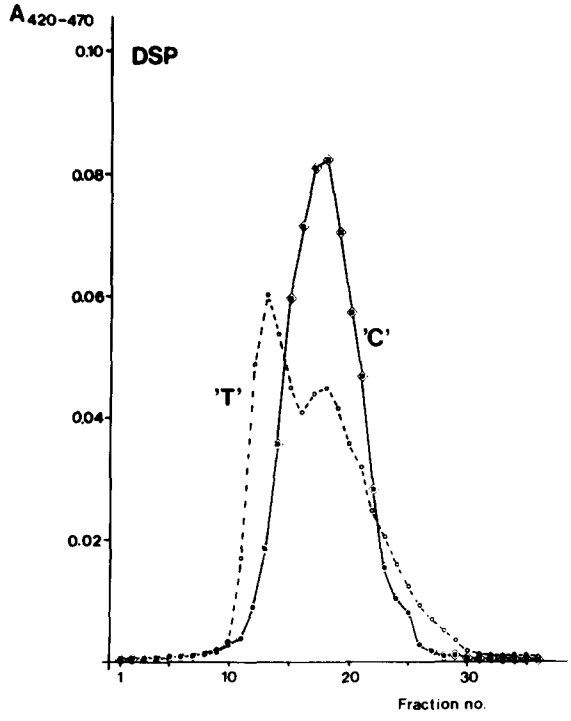

Fig. 2. Sucrose gradient centrifugation of cytochrome oxidase in the presence of Triton X-100 after cross-linking with DSP. The stock enzyme was first centrifuged in the presence of Triton ('T') or cholate ('C') and the 'green' fractions from the gradients were diluted with 2% cholate to give the final enzyme concentration of 6 μM (about 1:1 dilution). Incubation with DSP was done in these mixtures as described in Materials and Methods, followed by application to the gradients.

detergent binding to the protein and the large difference in partial specific volume between Triton and cholate [11]. In addition, the density and the viscosity of the solution and the shape of the sedimenting particle may also differ in different detergents, which also contributes to the detergent effect.

The amount of Triton bound to the monomer in the sucrose gradient was found to be 170 ($\pm$15) mol per mol enzyme. This is similar to the values reported previously for monomeric [12] and dimeric [13,14] cytochrome oxidase. Bound cholate could not be detected. This does not exclude binding of up to 80 mol per mol cytochrome aa_3, as was reported for deoxycholate [15], since the background radioactivity was high. However, the cholate concentration could not be reduced further, a difficulty typical for detergents having a high critical micellar concentration [16].

Centrifugation in the Triton-containing gradient after cross-linking could now be used to study the oligomeric state of the enzyme and to separate monomers from dimers (both are cross-linked also intra-monomerically, see below). Cytochrome oxidase was first centrifuged in cholate ('C') or Triton ('T'). Then the green bands were collected *, cross-linked with DSP, and centrifuged in the presence of Triton. The cross-linked dimeric enzyme, 'C', did not dissociate into monomers under these conditions (Fig. 2'C'). Interestingly, the originally monomeric 'T' enzyme exhibited a significant fraction of cross-linked dimers in addition to the expected monomers (faster sedimenting band in Fig. 2'T'). These cross-linked dimers could have been generated during the incubation in the reaction medium, which contained about 1% cholate. It may also be the result of cross-linking of sites in separated monomers that are at a distance less than 11 Å, which is the length of DSP [5]. The latter possibility was tested by using a shorter cross-linker. DST reacts predominantly with primary amines, as does DSP, but is only 6 Å long [17]. As shown in Fig. 3, dimeric cytochrome oxidase ('C') was completely cross-linked with DST just as with DSP (Fig. 2'C'). When the 'T' enzyme was cross-linked under similar conditions with the shorter reagent DST, cross-linked dimers were no longer observed.

Polypeptides which are cross-linked by a cleavable cross-linker can be identified by two-dimensional SDS-polyacrylamide gel electrophoresis. The cleaving reagent, dithiothreitol or β-mercaptoethanol in the case of DSP, is added only in the second dimension of electrophoresis. As a result, subunits which were cross-linked to each other migrate in the second dimension along the same vertical line and below the diagonal (see Ref. 5 for a detailed explanation of the method).

If subunits were shown to be cross-linked in the dimeric but not in the monomeric enzyme, this would suggest that they are linked across the monomer-monomer boundary in the dimer.

In order to detect the inter-monomeric cross-links, monomers and dimers were prepared and

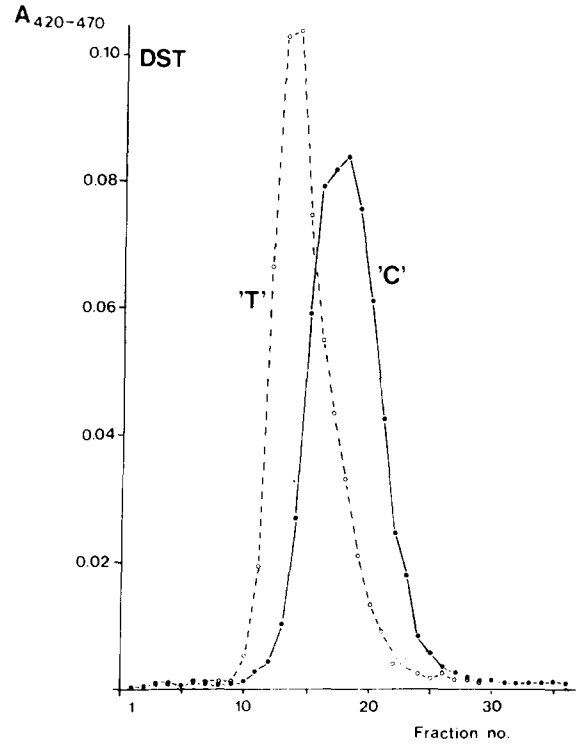

Fig. 3. Sucrose gradient centrifugation of cytochrome oxidase in the presence of Triton X-100, after cross-linking with DST. See legend to Fig. 2, except that DST was used instead of DSP.

cross-linked as in Fig. 2. The second sucrose gradient centrifugation in Triton before analysis by SDS-polyacrylamide gel electrophoresis is important to remove the fraction of dimers formed in 'T' and a possible fraction of monomers remaining in 'C' in case of incomplete cross-linking.

Fig. 4 shows the two-dimensional SDS-polyacrylamide gel electrophoresis analysis of monomeric (A) and dimeric (B) cytochrome oxidase, which were cross-linked with DSP. By comparing Fig. 4A and B it can be seen that, while most of the spots appear in both of them, there is one very clear spot that is seen only in the cross-linked dimer. This spot (encircled, Fig. 4B) most probably arises from a cross-link between two subunits VIb since no other spots are observed on the same vertical line. Some faint spots appearing nearly on the same vertical line appear also in the cross-linked monomer, and are thus probably intra-monomeric cross-links. This interpretation is sup-

* The faster sedimenting band if two were observed.

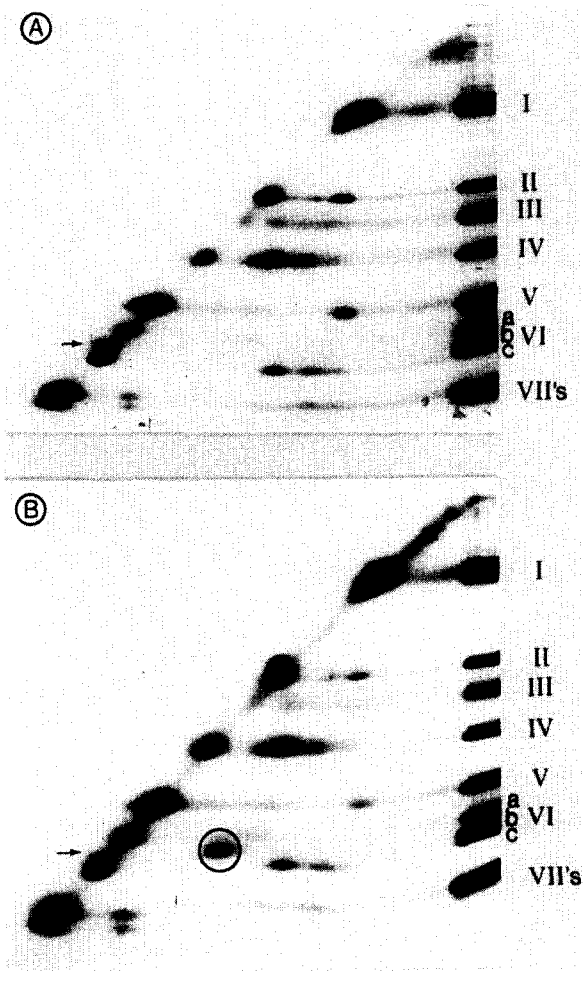

Fig. 4. Two-dimensional SDS-polyacrylamide gel electrophoresis of monomeric (A) and dimeric (B) cytochrome oxidase cross-linked with DSP. Cross linking and SDS-polyacrylamide gel electrophoresis are described in Materials and Methods. Monomers for the first dimension were from the slowly sedimenting band in Fig. 2'T' and dimers from the band shown in Fig. 2'C'. Control enzyme was also applied to the second gels on the right-hand side to facilitate identification of subunits.

ported by the observation that the spot of subunit VIb on the diagonal (marked by a small arrow) in the cross-linked dimer (Fig. 4B) is much reduced in comparison to that in the monomer (Fig. 4A).

Discussion

We have recently suggested that the slow sedimentation of cytochrome oxidase in a Triton-containing sucrose gradient is mainly due to monomerization of the enzyme [3]. This was recently supported by independent observations that Triton dissociates cytochrome oxidase dimers into monomers [12]. Bovine heart cytochrome oxidase was reported previously to exist as a dimer in Triton solution [13–15]. This could be due to reasons discussed by Robinson and Talbert [12] or to overestimation of the haem/protein ratio in Ref. 14.

Covalently cross-linked dimers should not be monomerized by the Triton centrifugation. This treatment can thus be used after cross-linking to separate monomers from dimers. In this way the difference in sedimentation of both species can be compared, and the fractions of monomers and dimers assayed.

Cross-linking of two monomers requires close contact between primary amines in the case of DSP and DST [17]. If there are forces of attraction between monomers in a given solution, or if the dimeric structure is favoured thermodynamically, then dimerization can occur and might be affected by the cross-linker, but may not necessarily be complete.

The results shown in Fig. 2'T' suggest that some redimerization occurred with DSP after primary monomerization. However, the structure was different from that of native dimers since cross-linking with the shorter DST failed (Fig. 3).

It is of interest to identify the subunits that may participate in cross-linking of the two monomers in the native dimer. This can be done by 'difference cross-linking map', dimer minus monomer, as in Fig. 4. This approach to study the organization of the dimer can be extended by the use of different types of reagent which can be shown to bind differently to monomeric and dimeric enzymes.

When a link is identified between two copies of the same subunit (as in Fig. 4), it must be intermonomeric since nearly all subunits of monomeric cytochrome oxidase are present in a single copy [18,19]. This supports the finding that subunit VIb is involved in such a cross-link.

This observation suggests that in the dimer subunits VIb of each monomeric unit are exposed on the enzyme surface, and face each other. This is an interesting finding for two reasons. Subunit

VIb is one of the small subunits that are removed by all the different treatments reported up to now to remove subunit III [3,20–22]. All these treatments were also suggested to monomerize cytochrome oxidase [3]. Thus, subunit VIb may well be involved in stabilizing the dimeric enzyme, by itself or together with subunit VIa (Buse's subunit VIb, Ref. 23) which is also removed by the same treatments.

The fact that two subunits VIb are cross-linked in the dimer suggests a 'face-to-face' organization of the monomers, where identical structures of two asymmetric units interact with one another. This supports a model suggested for dimeric membranous cytochrome oxidase, which was derived from analysis of two-dimensional crystals [24].

Subunit VIb is most likely identical with a hydrophilic cytoplasmically coded polypeptide having the N-terminal Ac-Ala-Glu-Asp [23].

Finally, it should be emphasized that the cross-linking results themselves do not necessarily mean that subunit VIb is essential for the native dimeric structure. Nor do they exclude such a function for other subunits, which may not have primary amines suitably positioned for cross-linking.

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