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CROSS-LINKING STUDIES ON A CYTOCHROME c - CYTOCHROME c OXIDASE COMPLEX

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SUMMARY

A cytochrome c - cytochrome c oxidase complex containing 0.8-1.0 moles of cytochrome c per mole of cytochrome c oxidase (heme a + a_3) was isolated as described by Ferguson-Miller, S., Brautigan, D.L., and Margoliash E., J. Biol. Chem. 251, 1104 (1976). This complex was reacted with dithiobissuccinimidyl propionate, an 11 Å bridging bifunctional reagent, and the cross-linked products obtained were analyzed by two dimensional gel electrophoresis. Cytochrome c was cross-linked to subunit II of cytochrome c oxidase. Other cross-linked products were formed involving different subunits of cytochrome c oxidase. These included I+V, II+V, III+V, V+VII, IV+VI and IV+VII. Experiments are also described using N,N'-bis(3-syccinimidyloxycarbonylpropyl) tartarate. The major product formed with this 18 Å bridging bifunctional reagent was a pair containing II+VI.

INTRODUCTION

Cytochrome c oxidase is a multipeptide complex containing hemes and copper atoms. The enzyme catalyzes the oxidation of cytochrome c by molecular oxygen and conserves the energy liberated in this redox reaction for the synthesis of ATP [for review see 1-3].

We have been using several different cleavable bifunctional reagents to study the arrangement of subunits in the cytochrome c oxidase complex [4]. The cross-linking approach can also be used to determine the binding site for cytochrome c on the enzyme. Ferguson-Miller et al. [5] have described conditions under which a tight complex between cytochrome c and cytochrome c oxidase is formed (Kd 10^{-7} M), and in which cytochrome c is presumably bound at its functionally important "active site". Here we describe cross-linking of this cytochrome c - cytochrome c oxidase complex with dithiobissuccinimidyl propionate (DSP), a reagent with reactive groups 11 Å apart and bridged by a

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readily cleavable disulfide bond. Experiments are also reported in which a newly synthesized vic-glycol-bridged cross linker, N,N'-bis(3-succinimidyl-oxycarbonylpropyl) tartaramide has been used to determine near neighbor subunits in cytochrome c oxidase.

MATERIALS AND METHODS

Beef heart mitochondria were prepared by the method of Crane et al. [6]. Cytochrome c oxidase was isolated as described by Capaldi and Hayashi [7]. Preparations contained between 9.3-11.7 nmoles heme a/mg protein as determined by the pyridine hemochromagen difference spectral method of Williams [8]. Molecular activities were measured spectrophotometrically at 25°C as described by Vanneste et al. [9] and using an assay medium containing cytochrome c (25 μM), 0.5% Tween 80, 0.05 M potassium phosphate (pH 7.0). Values between 130-250 μmole cytochrome c oxidized/sec/μmole heme a_3 were obtained for several different enzyme preparations.

The cytochrome c - cytochrome c oxidase complex was prepared essentially as described by Ferguson-Miller et al. [5] but in a different buffer system. Cytochrome c oxidase (3-5 mg) was dialyzed against 25 mM HEPES, 0.25% Tween 20, 0.05% cholate (pH 7.8) at 4°C to remove residual ammonium sulfate. A five-fold molar excess of cytochrome c (Type VI from Sigma) was added and the mixture (final volume 0.25 ml) was applied to a column of Sephadex G100 (18 x 1 cm) and eluted in the same buffer used in the dialysis step. The cytochrome c - cytochrome c oxidase complex eluted in the void volume, well separated from the excess cytochrome c. The concentration of each component in the complex was determined spectrally. The protein concentration was adjusted to 1 mg/ml and cross-linking was initiated by adding 0.1 to 1.0 mg/ml cross linker in $(CH_3)_2SO$. The reaction was allowed to proceed 10-30 min before quenching with 50 μl of a 1 M ammonium acetate solution. Also, 10 μl of a 10% solution of NEM was added to prevent disulfide exchange during analysis of the cross-linked products.

SDS polyacrylamide gel electrophoresis in one and two dimensional systems was performed using the general procedures of Weber and Osborn [10] and Swank and Munkres [11] but with the modifications described before [4]. In some experiments a composite slab gel was used consisting of a lower layer of 20% acrylamide (plus 0.7% bisacrylamide) and an upper layer of 15% acrylamide (plus 0.5% bisacrylamide). Staining and destaining of gels was performed as described previously [4].

RESULTS

A tight complex (Kd = 10^{-7} M), containing 0.8-1.0 moles of cytochrome c per mole of cytochrome aa_3 (2 hemes), was isolated by the general procedure of Ferguson-Miller et al. [5]. Cross linking of this cytochrome c - cytochrome c oxidase complex was performed with DSP at different concentrations and for different incubation times. Figure 1 shows the polypeptide profile of a sample cross-linked with 0.2 mg DSP/mg protein for 10 min and then run on a 12% gel in the Weber-Osborn [10] buffer conditions but without β mercaptoethanol present.

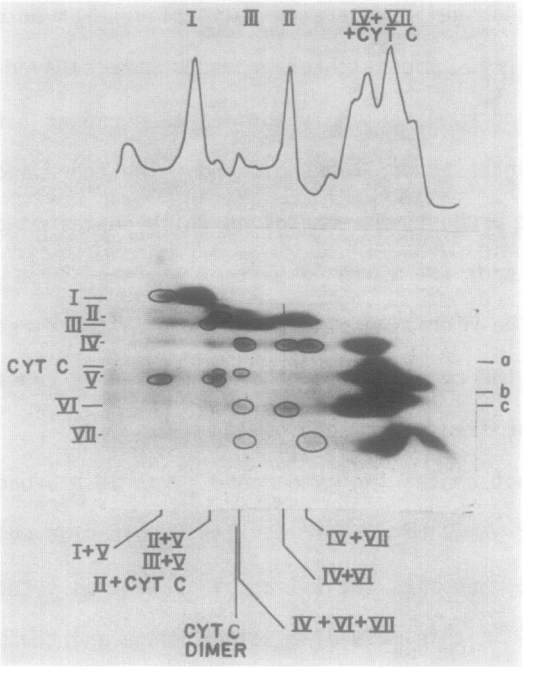

Figure 1 Two dimensional SDS polyacrylamide gel electrophoresis of the cytochrome c - cytochrome c oxidase complex to resolve the cross-linked products formed by reaction with DSP. The upper gel trace shows the polypeptide profile fo the cross-linked sample on a 12% gel in the Weber-Osborn buffer conditions but without β mercaptoethanol present. The slab gel shows the resolution of cross-linked products after they have been cleaved with β mercaptoethanol. Most of the untreated subunits of cytochrome c oxidase and any free cytochrome c runs on or close to a diagonal across the gel. Subunit III however runs off the diagonal (see Ref. 12). Also seen on the slab are several polypeptides present in small amount in cytochrome c oxidase preparations and these are labelled a, b, and c. Our recent studies indicate that there are multiple copies of VII in the enzyme (Ludwig, B. and Capaldi, R.A., manuscript in preparation). The migration of VII on this two dimensional gel suggests that this band may contain different polypeptides of similar size. Components involved in cross-linked products are off and below the diagonal. Those which could be related to one another are circled. There are several off-diagonal spots for which a partner or partners could not be clearly resolved.

The major bands seen were unreacted subunits of cytochrome c oxidase and free cytochrome c. Cross-linked products were present but in many instances they ran as shoulders to these bands or they were hidden under the bands themselves.

Identification and analysis of cross-linked products was achieved by electrophoresing polypeptides out of an unstained tube gel (the duplicate of that stained in Figure 1), through a layer of agarose containing β mercapto-

ethanol, and into a slab gel. Unreacted polypeptides ran on or close to a diagonal in the slab gel. Cross-linked products were cleaved as they passed through the agarose and component polypeptides then ran as a function of their monomer molecular weights to positions off and below the diagonal (Figure 1).

Two cross-linked products were resolved which contained cytochrome c. One was a very faint spot for which no partner was seen on a vertical directly above or below it. The migration of this product in the first dimension was identical to that of the cytochrome c dimer generated by cross-linking high concentrations of purified cytochrome c with DSP.

The second product containing cytochrome c ran as a broad band in the first dimension, just ahead of subunit I, with a molecular weight in the range of 37000-35000. Subunits II, III and V as well as cytochrome c were seen off the diagonal in this molecular weight range and these must be involved in several different cross-linked products all of similar size. Cross-linked products involving II+V and III+V (aggregate molecular weights 36500 and 33400 respectively) have been resolved before [4]. Cytochrome c was seen as a fairly dark spot under II. This subunit II-cytochrome c pair has an aggregate molecular weight of 33100. Other cross-linked products seen included IV+VI, which was identified in an earlier study [4], and I+V and IV+VII, which have not been resolved clearly before. All of the pairs just listed, with the exception of those involving cytochrome c, were also seen in the control (done without cytochrome c present).

Recently we have synthesized two novel vic-glycol bridged cross-linkers, DST and DSPT, the former spanning a maximal distance of 6 Å and the latter 18 Å between reactive groups. These have proved particularly useful in our studies of the ubiquinol cytochrome c reductase segment of the respiratory chain (Smith, R.J., Capaldi, R.A., Muchmore, D. and Dahlquist, F.W., manuscript in preparation). Both DST and DSPT were tested for their ability to cross-link cytochrome c to cytochrome c oxidase, but neither generated greater amounts of the cytochrome c containing products than did DSP. However, as shown by the two dimensional gel in Figure 2, reaction of cytochrome c oxidase with DSPT

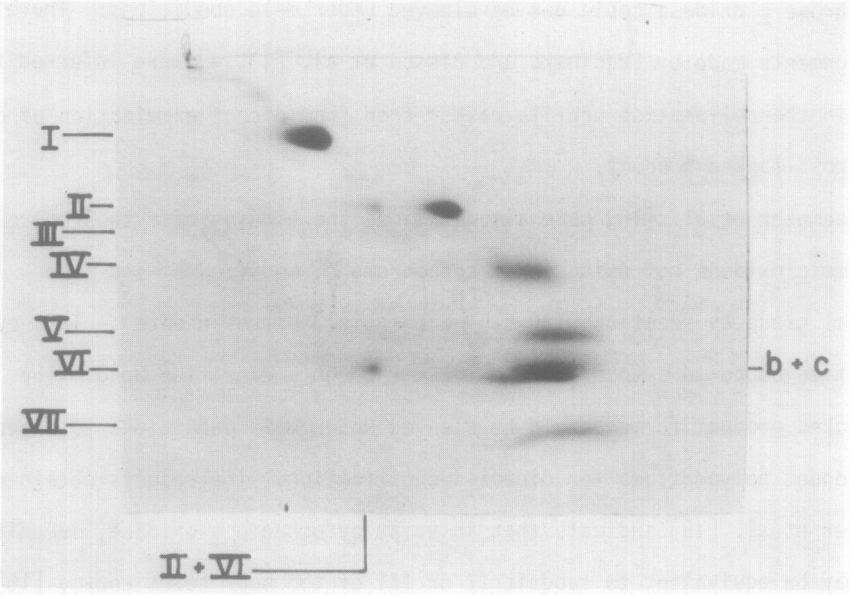

Figure 2 Two dimensional SDS polyacrylamide gel electrophoresis of cytochrome c oxidase cross-linked with 0.25 mg DSPT/mg enzyme for 30 min at room temperature. Cross-linked products were separated on a 12% gel in the Weber-Osborn gel conditions. The bifunctional reagent was cleaved by soaking the gel for 2 hr in several changes of a solution containing 0.015 M sodium periodate, 0.1% SDS 0.01 M sodium phosphate (pH 7.0). Electrophoresis in the second dimension was in the Swank-Munkres buffer system.

generated a cross-linked product containing II and VI which has not been seen before in studies with either the 6 Å or 11 Å cross linkers.

RESULTS

The question of which subunits of cytochrome c oxidase are involved in cytochrome c binding has been examined in several recent studies. Both Erecinska [13] and Bisson et al. [14] have synthesized a photoaffinity-labelled cytochrome c and used this to tag the binding site for cytochrome c on cytochrome c oxidase from heart mitochondria. According to Erecinska [13] the binding site for this cytochrome c derivative is on the lower molecular weight subunits (probably V and VII in our terminology). In contrast, Bisson et al. [14] concluded that the binding site for cytochrome c was on subunit II. The linkage between the photoaffinity-labelled cytochrome c and subunit(s)

of cytochrome c oxidase could not be cleaved under mild conditions. Therefore the assignments made by Erecinska and Bisson et al. [13,14] were inferred from changes in the polypeptide profile rather than from direct examination of components in the cross-linked product.

Birchmeier et al. [15] have also examined the binding of cytochrome c to cytochrome c oxidase but using yeast cytochrome c, modified at its free sulfhydral group by reaction with 5,5'-dithiobis(2-nitrobenzoate). This cytochrome c derivative bound to cytochrome c oxidase through a disulfide bond. The cross-linked product formed could be cleaved under mild conditions to regenerate the component polypeptides for direct identification. The results obtained by Birchmeier et al. [15] indicate that in yeast cytochrome c oxidase, subunit III (which may be equivalent to subunit II or III of the beef heart enzyme [16]) is at or close to the binding site for cytochrome c.

In our experiments unmodified cytochrome c was bound to cytochrome c oxidase and the interaction between the two was stabilized by DSP. This cross-linker reacts with lysine residues of which there are several on the cytochrome c oxidase - binding face of cytochrome c [17]. The cross-linked product obtained had an apparent molecular weight of 37000-35000. This is similar in in size to the product obtained with photoaffinity-labelled cytochrome c (36000 [13]; 36100 [14]) and with 5,5'-dithiobis(2-nitrobenzoate) modified cytochrome c (38000 [15]). It contained cytochrome c in association with subunit II. Our results then are in agreement with the findings of Bisson et al. [14] and place subunit II within 11 Å of cytochrome c in the cytochrome c - cytochrome c oxidase complex.

Several other cross-linked products were identified in this study. These along with our previously published cross-linking results are summarized in Figure 3. The cross-linking data shown are consistent with the other available information on the arrangement of subunits in cytochrome c oxidase. For example the studies of Eytan et al. [18] and our own experiments (Ludwig, B. and Capaldi, R.A., manuscript in preparation), in which [^{35}S] DABS was used to label

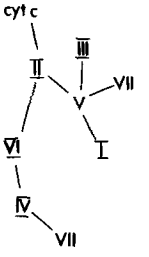

Figure 3 A schematic drawing summarizing the cross-linking results otained to date for beef heart cytochrome c oxidase.

polypeptides exposed at the different sides of the mitochondrial inner membrane, place II, III and V exclusively on the outer (intracristal) surface. Therefore these components are close to one another and subunit II is on the correct side of the membrane for interaction with cytochrome c in mitochondria.

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