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THE CYTOCHROME c BINDING SITE ON CYTOCHROME c OXIDASE

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SUMMARY

Cytochrome c was chemically coupled to cytochrome c oxidase using the reagent 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) which couples amine groups to carboxyl residues. The products of this reaction were analyzed on 2.5-27% polyacrylamide gradient gels electrophoretically. Since cytochrome c binds to cytochrome oxidase electrostatically in an attraction between certain of its lysine residues and carboxyl residues on the oxidase surface, EDC is an especially appropriate reagent probe for binding-subunit studies. Coupling of polylysine to cytochrome oxidase using EDC was also performed, and the products of this reaction indicate that polylysine, an inhibitor of the cytochrome c reaction with oxidase, binds to the same oxidase subunit as does cytochrome c, subunit IV in the gel system used.

INTRODUCTION

Three recent studies have attempted to identify the subunit of beef heart cytochrome oxidase which interacts with cytochrome c. Bisson et al. (1) and Erecinska et al. (2) have used arylazido-cytochrome c as a photoaffinity label for oxidase. In one case it was concluded that subunit II of oxidase was the binding site (1), and in the other case that one of the four smaller subunits (2) was the binding site. A curious feature of these experiments is that Bisson et al. (1) showed that the arylazido-cytochrome c derivative labelled at lysine 13 does not bind correctly to oxidase and forms an essentially inactive complex, so that reasonable doubt is possible that the resulting oxidase subunit identified by this technique is the natural binding site. A study by Briggs and Capaldi (3) employed the cross-linker dithiobissuccinimidyl propionate, which links amine functions to amine functions, and applied this reagent to a pre-formed oxidase-cytochrome c complex (4). This method gave a variety of products, including cytochrome c

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linked to subunits II, and the results suggest that neighboring subunits of the cytochrome c binding site may be more likely to be labelled than the site itself. Experiments using yeast cytochrome c labelled with 5,5'-dithiobis (2-nitrobenzoate) at its free sulfhydryl to react with yeast cytochrome oxidase suffer from this same disadvantage (5) in position of the lable.

We have attempted to couple directly the positively charged lysine residues on cytochrome c with the negatively charged carboxyl residues of its binding site (6, 7), using the water soluble carbodiimide 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), which links amine to carboxyl functions (8). Furthermore, polylysine was coupled to cytochrome oxidase with EDC in an attempt to identify the oxidase subunit with the greatest number of exposed carboxyl residues.

MATERIALS AND METHODS

Beef heart cytochrome oxidase was prepared according to the methods of Yonetani (9) and Sun et al. (10) for comparison of detergent effects on the coupling reaction. Both purified oxidase preparations contained 11-12 moles heme a per mg protein (11). Absorption spectra and electron paramagnetic resonance spectra were both recorded as indicators of purity.

The reaction system consisted of 10^{-5} M cytochrome oxidase in the presence of 10^{-4} M EDC and 3 X 10^{-5} M cytochrome c in one case and approximately 10^{-5} M polylysine (M.W. 24,000) in the other, at pH 6.0 and 20 C. The progress of the reaction was monitored (8) with a pH reference electrode to indicate completion (2 to 10 min.).

The electrophoresis system employed pore gradient slab gels, 2.5-27% in acrylamide at 0.5% bisacrylamide crosslinking, available commercially from Isolab, Inc., Akron, Ohio. Both sodium dodecyl sulfate and cetyltrimethylammonium bromide were employed as solubilizing detergents, according to the method of Williams and Gratzer (12). The subunits and their molecular weights in this gel system (13) are independent of detergent concentration for both sodium dodecyl sulfate and cetyltrimethylammonium bromide (14), a feature which eliminates migration anomalies among the smaller oxidase subunits (15). Electrophoresis also employed 1% β-mercaptoethanol and phosphate buffer (PH 7.1), typically running 0.2 mg protein in a given slab gel channel. Staining with Coomassie Blue R250 and destaining followed conventional procedures (16).

Cytochrome c (Type VI) and polylysine (M.W. 24,000) were purchased from Sigma Biochemical.

RESULTS

The results of two cross-linking experiments and reference experiments with marker proteins are summarized in the schematic diagram of Fig. 1. Both cytochrome c and polylysine are found to couple to subunit IV of oxidase when

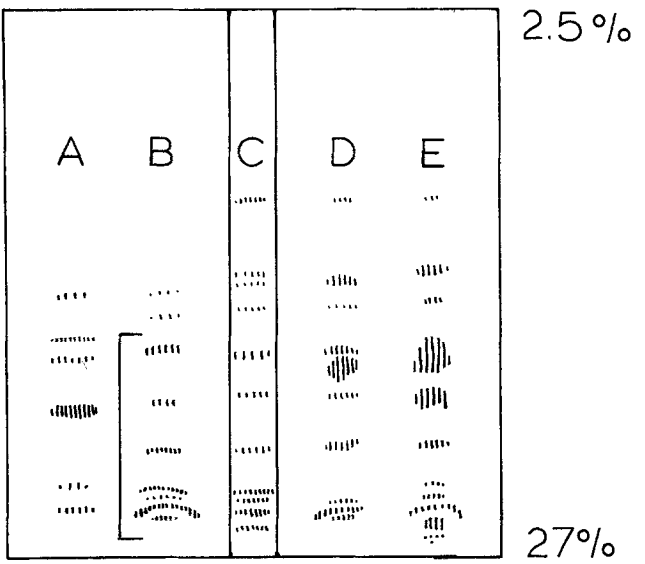

Figure 1. Gradient (2.5-27%) slab gel electrophoresis of cytochrome oxidase. Channel A, molecular weight marker proteins in 1% cetyltrimethylammonium bromide, 1% β-mercaptoethanol, phosphate buffer at pH 7.1; in descending order: serum albumin, M.W. 68,000; ovalbumin, M.W. 45,000; carbonic anhydrase, M.W. 32,000; polylysine, M.W. 24,000; β-lactoglobulin, M.W. 18,000; lysozyme, M.W. 14,500. Channel B, cytochrome oxidase (12) in conditions of channel A, seven oxidase subunits indicated by a bracket; curved band of subunit VI characteristic of cetyltrimethylammonium bromide gels. Channel C, cytochrome oxidase (12), conditions as in A except 1% sodium dodecyl sulfate in place of cetyltrimethylammonium bromide. Channel D, cytochrome oxidase linked to cytochrome c with EDC, new band at M.W. 30,000, electrophoresis as in A. Channel E, cytochrome oxidase linked to polylysine (M.W. 24,000), electrophoresis as in A. Unreacted polylysine and a new band at 35-43,000 M.W. appear. (Presence of multiple crosslinks possible in this product.)

EDC is the coupling reagent. Few additional products are found, probably because EDC is too small to effect extensive inter-subunit crosslinking in oxidase. The coupling results are substantially the same whether the deoxycholate Yonetani preparation (9) or the Triton X-100 preparation (Sun et al.) (10) is used, except that in the former case a certain amount of reaction product containing cytochrome c and polylysine linked to deoxycholate carboxyls may be present.

The implications of these results with respect to oxidase subunit structure (16, 17, 18) may be discussed in the light of recent kinetic studies which

observed the reaction of mammalian oxidase with a variety of c-type cytochromes (19). These studies demonstrated that lysines 13, 27, 72/73, and 86/87 are necessary for effective oxidase binding. There is no reason to expect that reagents, such as photoaffinity labels, which alter the charge of these lysines will correctly identify the oxidase subunits, and the fact that modified cytochrome c binds poorly emphasizes this caution. Likewise, the use of reagents which specifically attack only amine functions is unlikely to discriminate the cytochrome c-binding subunit of oxidase, the chief characteristic of which is a patch of negative charge contributed by glutamate and/or aspartate. The EDC oxidase-cytochrome c complex, formed with the specific aim of maintaining charge interactions intact, produces a subunit IV - cytochrome c complex. It is clear that a variety of experiments using reagents specific for different amino acid residues would be helpful in establishing a topography of cytochrome oxidase. It would also be of value to repeat some earlier work (16) on oxidase membrane orientation, as a review of the subunit molecular weights for subunits III, IV, V and VI (Eytan, (16)) and amino acid compositions for the smaller subunits (Yu, (18) and Briggs, (20)) show that these authors are in considerable disagreement about the labelling of the four smallest subunits of oxidase. The result in this paper would appear to conflict with the results of Eytan et al. (16), but other studies (15, 18, 20) show that the location of subunit IV on the matrix vs. cytoplasmic side of the mitochondrial membrane has not been conclusively established.

In conclusion, cytochrome c is found to link through EDC to a subunit of cytochrome oxidase which appears as subunit IV in gradient electrophoresis. Further characterization of this reaction product and others by isoelectric focussing is in progress.

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