LOCALIZATION OF Cu AND HEME a ON CYTOCHROME c OXIDASE POLYPEPTIDES*

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SUMMARY

After mild dissociation of cytochrome c oxidase protomers, and polyacrylamide gel electrophoresis, copper was found predominantly in polypeptides of Bands V (m.w. 12,100) and VII (m.w. 3,400), and heme a predominantly in polypeptides of Bands I (m.w. 35,300) and II (m.w. 21,000). Some copper was found in Band II - III, and heme a in Band V.

INTRODUCTION

Although the protomeric polypeptides of mammalian cytochrome c oxidases have been separated and partially characterized by several investigators (1-10), the localization of copper and heme a prosthetic groups among the polypeptides is still uncertain. Tzagaloff and MacLennan separated a polypeptide, m.w. 25,000, containing about two atoms of copper per molecule, by gel filtration of succinylated oxidase (1). Tanaka and his coworkers purified a similar polypeptide, m.w. about 22,000, by dissociation with 5% SDS. It contained two atoms of copper (6); and Yu and his coworkers reported the isolation by pyridine treatment of a subunit (m.w. 11,600) containing 0.44 moles of Fe per mole of polypeptide (18). Another

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Abbreviations: SDS, sodium dodecylsulfate; TEMED, N,N,N'N'-tetramethylethylenediamine.

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heme a binding polypeptide m.w. 40,000, was reported by these investigators.

We recently developed a sensitive test for the detection of copper-containing polypeptides on polyacrylamide gels (11), and have now applied it, and two methods for the localization of heme a, to polypeptides of beef heart cytochrome c oxidase dissociated and electrophoresed under mild conditions. The identities of the polypeptides were checked by a second electrophoresis under strongly dissociating conditions.

METHODS AND MATERIALS

Materials. Beef heart cytochrome c oxidase, prepared by the method of Downer et al. (7), and having the same properties, was generously provided by Dr. R. Capaldi, University of Oregon. Acrylamide, bisacrylamide, sodium dodecylsulfate (SDS), and N,N,N'N'-tetramethylethylenediamine (TEMED) were purchased from Biorad; tetramethylbenzidine, benzidine, and bathocuproine sulfonate were purchased from Sigma. All other chemicals utilized in this work were the best grade commercially available.

Methods. Gel electrophoresis was carried out with the Ortec system (Ortec, Inc., TN), utilizing gel slabs, 7 x 10 cm, for simultaneous electrophoresis of 8 or 12 samples. Tris-acetate, 50 mM, pH 8.0, was used as reservoir buffer, and 0.1 M Tris-sulfate, pH 8.0 for the gels; both contained 0.1% w/v SDS. For one dimensional electrophoresis, the cap, well, and slab gels contained 0.1% SDS. For electrophoresis in a second dimension, the gels contained 6 M urea and 12% polyacrylamide. Sample treatment - Cytochrome c oxidase in 0.1 M sodium phosphate buffer, pH 7.4, was mixed with SDS in 50 mM sodium phosphate buffer, pH 7.5, to desired concentration ratios, and incubated at room temperature for varying times as given, usually 2 hours. The sample was then mixed with an equal volume of 40% sucrose in 50 mM Tris-sulfate, pH 8.0, loaded on the gel, capped, and electrophoresed for 30 min at 120 V and 80 pulses per second, then 160 V and 100 pps. Development was tracked with bromophenol blue, and was usually complete at 3.5 hours. For development in a second dimension, the unstained track cut from the first dimension slab was equilibrated with appropriate solutions, e.g., 3% SDS, 2% mercaptoethanol in 10 mM Tris-acetate, pH 8.0 for 30 min, then heated at 100°C for 10 min. The treated gel track was then laid at the top of a second gel slab, fixed in place with capping gel, and developed at 300 V and 200 pps. Detection of copper, heme a and protein - Copper was detected on polyacrylamide gels by the quenching of fluorescence of bathocuproine sulfonate by Cu^{1+}. The test is sensitive to 5 μM free or protein-bound copper and is not interfered with by 500 μM hemeprotein, Fe^{3+} ion, Fe^{2+} ion, Co^{2+}, or Mn^{2+} (11). Heme a was located visually on the gels by its yellow-green color, and by test reagents for peroxidative activity, either 3,3', 5,5'-tetramethylbenzidine (14), or benzidine. Protein was located by its fluorescence (Mineralite model R51, 17 watt, UV Products, Los Angeles, CA), or by staining with 0.05% Coomassie blue in 25% 2-propanol, 10% acetic acid for 16 hours, then destaining with 10% acetic acid until good constrast between stain and background was obtained. All copper and heme a bands were marked on the

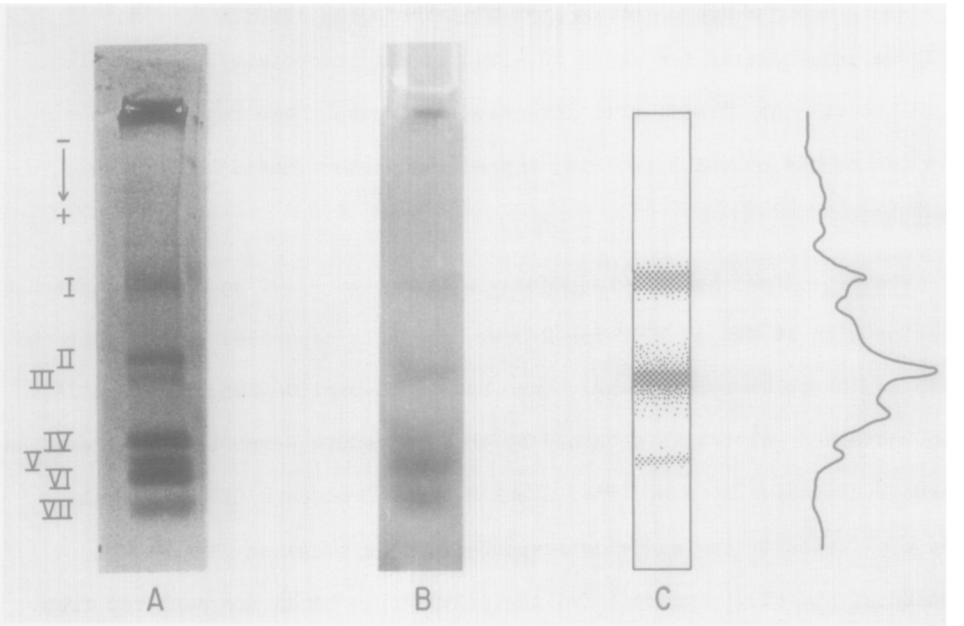

Figure 1. Cytochrome c oxidase incubated for 2 hrs at room temperature in 1% SDS and electrophoresed as described in Methods. Subunits are numbered according to the sequence of Capaldi et al. (9).
A. 25 µg enzyme stained for protein with Coomassie blue.
B. 200 µg protein stained with bathocuproine sulfonate for copper, as described previously (11).
C. Localization of yellow-green heme color after electrophoresis of 150 µg protein (drawing shows band positions and relative intensities); Transidyne densitometric scan after peroxidative staining with benzidine, displayed on a vertical axis to the right.

gels with incisions which were then correlated with protein staining. The heme-stained and protein-stained bands were recorded with a Transidyne densitometer (Transidyne Corp., Ann Arbor, MI); the fluorescence-quenched copper-stained bands were photographed with Polaroid Land film #p55 during irradiation with a Mineralite R51 lamp. The photograph, taken through a Kodak Wratten 1A gelatin filter, was exposed for 1 min at F4.5.

RESULTS

After exposure to mild conditions of dissociation (0.1% - 1% SDS, 0 - 2 hrs incubation, 20 - 22°C) a fraction of the beef heart cytochrome c oxidase being electrophoresed moved from the sample slot into the gel track. The smaller polypeptides were labilized most readily. As the stringency of dissociation conditions increased, the proportion of high

molecular weight polypeptides in the gel track also increased. In this study we investigated the range 50 - 200 µg enzyme protein/20 µl sample volume containing 1% SDS, most intensively because these concentrations gave gel tracks on which protein, copper, and heme a could be readily detected.

Protein. Beef heart cytochrome c oxidase, 50 - 100 µg sample, after incubation with 1% SDS at 20°C for 2 hrs, partially separated upon electrophoresis on 10% polyacrylamide gel, into seven polypeptide bands (Figure 1A). When unstained gel tracks obtained by this procedure were electrophoresed in a second dimension by a modified Swank-Munkres procedure (13), the relative mobilities of the bands corresponded to the sequence observed by Capaldi et al. (9). Accordingly, the polypeptide bands are numbered from the origin in the first dimension, and we assign to them the molecular weights determined by Capaldi et al. (9) When concentrations of cytochrome c oxidase higher than 200 µg/20 µl sample were electrophoresed, the general appearance of the one-dimensional electrophoretic patterns remained similar to that in Figure 1A, but band overlaps and changes in relative intensities occurred.

Copper. When 200 µg cytochrome c oxidase/20 µl sample was incubated with 1% SDS for 2 hrs at 20°C, then electrophoresed and exposed to bathocuproine sulfonate reagent, Bands V and VII displayed strong, approximately equal intensity of fluorescence quenching. Band II - III (overlap) gave a positive test about 50% of the intensity of Bands V or VII. Bands IV and I showed traces of copper. No copper has been observed in Band VI (Figure 1B). When protein levels of 300 µg or more were electrophoresed, or when less than 1% SDS was employed in the incubation mixture, there was an apparent shift in the location of the copper, which was then found in an area of the gel corresponding to the Band IV region, with a concomitant loss of copper in Bands V and VII. Upon second-dimensional

electrophoresis, however, no Band V or VII protein was detected in the region of Band IV.

Heme. When 200 µg cytochrome c oxidase/20 µl sample was exposed to 1% SDS for 2 hrs at 20°C and then electrophoresed, yellow-green heme bands could be seen in the regions of Bands III, I, and V, in descending order of intensity. Figure 1C shows that peroxidative activity was observed on the gels in regions which did not coincide with protein peak maxima, e.g., just ahead of Bands II and IV. At high concentrations of protein per sample, or low SDS concentrations, the heme of Band V also shifted in position relative to protein, to the region of Band IV.

DISCUSSION

Under the conditions of our experiments, copper and heme were each bound to several subunits. At present we are unable to say conclusively which of these copper and heme containing subunits are responsible for the native function of the oxidase, because under some conditions of dissociation of the protomers of cytochrome c oxidase, the native polypeptides can be denatured, and their functionally bound copper and heme a mobilized. These conditions have not been defined.

Since cytochrome c oxidase contains at least four types of copper namely, EPR characteristic copper, EPR undetectable copper, denatured EPR detectable copper (16), and adventitious copper, some of the multiplicity of copper-containing polypeptides may be explained on this basis. Under the relatively mild conditions of protomer dissociation employed in our experiments, copper remained bound predominantly to two of the farthest migrating bands, V and VII. This leads us to believe that polypeptides V and VII are candidates for functional components of cytochrome c oxidase. The smaller amounts of copper detected at other positions is uncertain and may arise from adventitious and denatured sources (17).

Heme a was detected in Band I, confirming the observation of heme a

peptide from cytochrome c oxidase by Yu et al. (18). A smaller but significant amount of heme was detected in Band V, possibly corresponding to the heme a binding subunit, molecular weight 11,600, isolated by Yu et al. (18). In contrast to previous reports, however, we find the largest amount of heme a in subunit III. The finding of three heme-containing subunits in cytochrome c oxidase requires further explanation. It is also interesting to point out that Band V was the only one observed by us to contain both copper and heme. The weak peroxidatic activity near subunits II and IV is of questionable significance because no heme color was observed at those positions, and because the peroxidase test is non-specific (cf 15).

The final proof of the identities of the copper and heme a bearing subunits must wait upon their isolation in reconstitutable states; and our observations must be tested with cytochrome c oxidase prepared by methods other than that of Downer et al.(7).

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