THE SUBUNIT STRUCTURE OF BEEF HEART CYTOCHROME OXIDASE

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SUMMARY

Beef heart cytochrome oxidase was studied by SDS-polyacrylamide gel electrophoresis. It was found to consist of one polypeptide of 37,000 Daltons, one of 19,000, two of 14,000, and four of 10,000. Various steps were taken to demonstrate that none of the bands were due to lipid, and that proteolysis did not occur during SDS incubation.

Cytochrome oxidase (EC: 1.9.3.1), the terminal member of the mitochondrial electron transport chain, is a membrane bound protein, which is soluble only in the presence of detergents. The possibilities of aggregation and of interference from detergent micelles has resulted in a rather confusing picture of its molecular weight and subunit structure. Okunuki and collaborators (1,2) reported a sedimentation coefficient of 22S and a molecular weight of 530,000. The minimum molecular weight based on heme a and amino acid analysis was calculated to be 130,000 of which 93,000 was protein. By light scattering, Tzagoloff et. al. (3) determined a molecular weight of about 290,000 or 230,000 after subtraction of the effect of lipid. Love et. al. (4) found a sedimentation coefficient of 11S and a molecular weight of 190,000. Incubation at high pH resulted in dissociation of this dimer into monomers of 6S or 100,000 Daltons.

Since sodium dodecyl sulfate (SDS) is used in this work, it is of interest to consider the previous use of this denaturant with cytochrome oxidase in molecular weight studies. By ultracentrifugation Criddle and Bock (5) found a predominance

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of material of about 72,000 Daltons with some higher molecular weight material. Okunuki (2,6) found that incubation of cytochrome oxidase in a ratio of 1:1 SDS to protein (w./w.) gave a more than twofold increase in activity and a change in sedimentation coefficient from 22 S to 17 S. Substantially higher amounts destroyed activity and resulted in a 6S species. Love et. al. reported that treatment with SDS resulted in dissociation of his 11S enzyme into 9S and 5S subunits. In no case were species smaller than 5 S or 70,000 Daltons reported. However, it is likely that in all cases, these experiments were done with an insufficient amount of SDS to fully dissociate the enzyme into subunits. Reynolds and Tanford (7) found for several proteins that 1.4 g. of SDS was bound per g. of protein to give the fully denatured form of protein. In the work of Okunuki, the amount of SDS was insufficient. The other workers do not report the protein concentrations used in their incubations. Thus we undertook this study to investigate the number and size of the polypeptides which make up cytochrome oxidase.

MATERIALS AND METHODS

Cytochrome oxidase was prepared from beef heart mitochondria by the method of Yonetani (8) incorporating the modifications of Lemberg (9). This involves precipitating the mitochondria at pH 5.6, extraction with sodium cholate and repeated ammonium sulfate fractionations during which the cholate is replaced by Tween 80 (polyoxyethylene (20) sorbitan monooleate). The dithionite reduced enzyme had the following absorbance ratios: $A_{280}:A_{445} = 2.2$, $A_{424}:A_{445} = 0.48$, $A_{605}:A_{553} = 2.3$. The activity was determined by the procedure described by Yonetani (10). The K_m for ferrocytochrome c was 13 ± 2μ M., and the turnover number at infinite cytochrome c was 3,000 moles of ferrocytochrome c oxidized/min./mole of heme a. Then the enzyme was passed through a Sephadex G-100 column and chromatographed on diethylaminoethyl (DEAE) cellulose as shown in Figure 1 and Table 1. The proteins used as standards, phenyl methane sulfonyl fluoride (PMSF), and the proteolytic enzyme substrates were obtained from Sigma and used without further purification.

Reagents and methods for SDS polyacrylamide gel electrophoresis were as

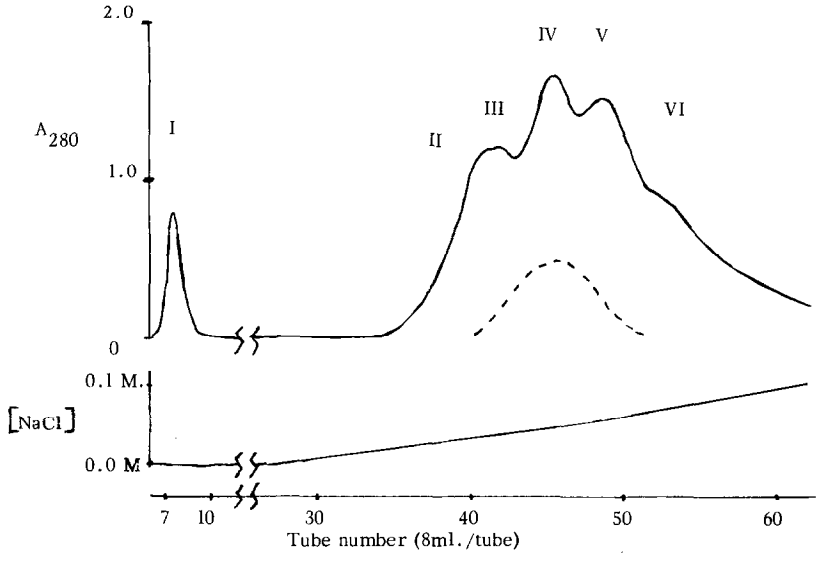

Figure 1. Elution of cytochrome oxidase from a 2.5 x 25 cm. DEAE-cellulose column (DE 32, Whatman). Eluant is 0.01 M. sodium phosphate buffer pH 7.0 containing 0.5% Tween 80 and NaCl as indicated. Absorbance at 280 nm. was determined with a Cary 11 spectrometer subtracting absorbance due to the detergent. The concentration of NaCl was determined by conductance. No additional protein peaks appeared on increasing the NaCl concentration up to 1 M. The dotted peak shows the result of rechromatography of peak IV.

TABLE 1

Fractions off DEAE column

Peak	A_{280}/A_{418} (oxidized form)	Turnover number at 17 μM. cyt c
Before	2.9	2200
I	40	1700
II	2.7	2800
III	2.8	2100
IV	2.8	2200
V	2.9	2200
VI	2.9	2100

described by Shapiro (11) incorporating the modifications of Weber and Osborn (12). Gels were scanned with a Gilford linear scanning attachment to a Beckmann DU spectrometer using a 5 mm. path length, 10 cm. long quartz cuvette. In order to make a quantitative estimate of the amount of protein in each band, the gels were scanned at 550 nm., the absorption maximum of the Coomassie brilliant

blue stain, and the resulting traces graphically integrated. Previous work (13) indicates that such a procedure is reproducible for native proteins to better than ± 10%, although there is variation of as much as 30% for comparing different proteins. For SDS treated serum albumin, ovalbumin, and cytochrome c run at different concentrations and scanned at 550 nm. we found reproducibility of about ± 15% for a particular protein and variation between different proteins of about 25%. There is a deviation from Beer's law for all three proteins at 550 nm. and for cytochrome c at 410 nm., so that large peaks are underestimated by as much as 30% compared to very small peaks. The results of the integration were divided by the nominal molecular weight to get the relative number of molecules of each polypeptide. These numbers were related to the amount of heme a by running a known amount of cytochrome oxidase with a known amount of either serum albumin or phosphorylase on the same gel. During these experiments with "doped" samples, it was found that staining in 50% CH_3OH with 10% CH_3COOH allowed about one third of the cytochrome oxidase to leach out, compared to serum albumin or phosphorylase. This problem was prevented by staining in 50% CH_3OH with 5% CCl_3COOH.

RESULTS AND DISCUSSION

The elution pattern of cytochrome oxidase from the DEAE cellulose column indicates at least four distinguishable species. However except for the leading edge of the peak (II), we found no differences in activity or heme content. Also polyacylamide gels run on peaks III, IV, V, and VI gave identical SDS gel patterns. Possible sources of the heterogeneity are different states of aggregation or different amounts of bound lipid.

The mobilities, that is, the distance a protein migrates in a gel divided by the distance bromophenol blue migrates, for various proteins fall on a smooth curve when plotted against the log of molecular weight. When cytochrome oxidase is run on the same type of gel (Figure 2), there are four major bands with mobilities 0.39, 0.62, 0.74, and 0.84 corresponding to polypeptide molecular weights of 37,000, 19,000, 14,000, and 10,000. There are minor bands of variable intensity with mobilities between 0.1 and 0.3, these bands predominating if the protein is incubated

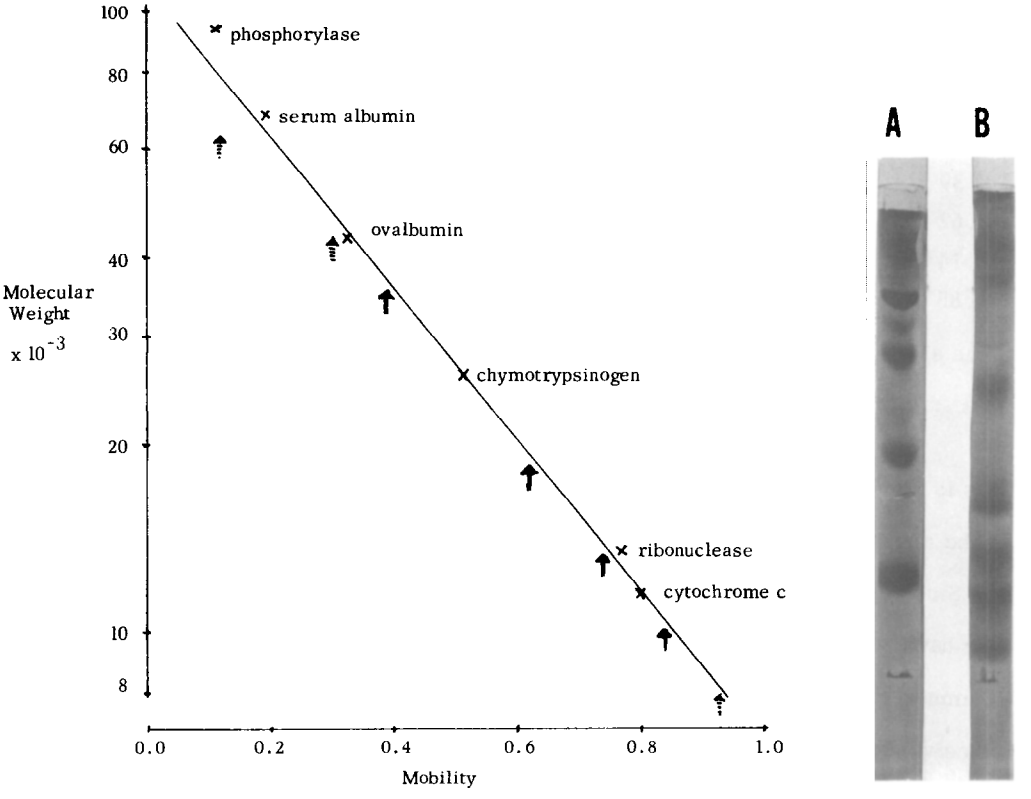

Figure 2. SDS gel electrophoresis pattern. Samples were run on 6 mm i.d. and 10 cm long 10% acrylamide gels with normal amount of crosslinker. The narrow line at the bottom of each gel marks the position of the bromophenol blue band. Gel A: standard proteins, serum albumin, ovalbumin, chymotrypsinogen, cytochrome c. Gel B: cytochrome oxidase before chromatography on DEAE cellulose. On the calibration plot, standard proteins are x, the major cytochrome oxidase bands are solid arrows. The cytochrome oxidase bands which appear only at low SDS (mobilities 0.12 and 0.30) and the band which is removed by DEAE cellulose (mobility 0.93) are dotted arrows.

in the presence of an insufficient amount of SDS. There is also a band at 0.93 which is removed from the enzyme by the DEAE cellulose column. Estimating the molecular weight for the 0.84 band requires extrapolation into a region where the size of proteins in SDS is rather insensitive to molecular weight, so the 10,000 molecular weight is less certain than the others (14). An estimate of the number of each polypeptide per heme a is given in Table 2. It is interesting to consider how the total weight of the polypeptides compares to the previously reported molecular weights. As Table 2 indicates, we find a total of 124,000 Daltons of protein per heme a.

TABLE 2

Molar ratios of cytochrome oxidase peptides to heme a.
Determined as described in the text.

Mobility	Molecular weight	number/heme a	Total weight
0.39	37,000	0.8	37,000
0.62	19,000	0.9	19,000
0.74	14,000	1.7	28,000
0.85	10,000	4.2	40,000
heme a		1	124,000

This is somewhat higher than the reported minimum molecular weight of 93,000 or the monomer of 100,000.

Since cytochrome oxidase is a "sticky" lipoprotein, an extraneous contaminant may have been carried through the purification, however our addition of the DEAE column to the usual $(NH_4)_2SO_4$ fractionations makes this less likely. Another conceivable source of an extraneous band is the lipid. It was found that lecithin gave a low level of staining from mobility 0.33 to beyond 1.0, but that the intensity of this stain was negligible compared to the observed cytochrome oxidase bands. In addition, unstained gels were scanned at 280 nm. and some gels were cut up and protein eluted and determined by the method of Lowry (15). These methods are very inferior to the Coomassie blue method in sensitivity and in the high blanks they give but they qualitatively confirmed the results of the 550 nm. scans.

The large number of different polypeptides and their small size suggests that they may have resulted from the limited specific proteolysis of a smaller number of chains. Such cleavage could occur during isolation of the active enzyme or during denaturation by SDS. To check the first possibility, the enzyme was treated with PMSF during the cholate extraction step, but no effect was found. The second possibility is similar to the situation recently found for hexokinase (16) where proteolysis occurred during SDS denaturation. This degradation was prevented by pretreatment with PMSF, or by heating the SDS solution to 100° C. before adding protein. Use of these treatments for cytochrome oxidase had little effect on the gel

pattern. In addition, native cytochrome oxidase was tested for proteolytic enzyme activity at pH7.0, using N-acetyl L tyrosine ethyl ester, N-benzoyl L arginine ethyl ester, and N-benzoyl DL alanine methyl ester, substrates for chymotrypsin, trypsin, and elastase respectively. No activity was detected under conditions where 1 of these enzymes per 1,000 heme a would have been detected. Also, a proteolytic enzyme acting during denaturation should chew up added serum albumin or phosphorylase. But no such effect was seen when cytochrome oxidase was doped with these proteins.

Our gel pattern is not the same as that reported by Chuang and Crane (17). We have no explanation for this. Also we found that treatment of the enzyme according to their procedure did not separate the protein into fractions containing different polypeptides but only denatured half the enzyme and formed a precipitate we could not dissolve in SDS.

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