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$b \cdot c_1$ COMPLEX FROM BEEF HEART: HYDRODYNAMIC PROPERTIES OF THE COMPLEX PREPARED BY A REFINED HYDROXYAPATITE CHROMATOGRAPHY IN TRITON X-100

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SUMMARY

The homogeneity and the molecular weight of the $b \cdot c_1$ complex prepared in Triton X-100 by hydroxyapatite chromatography were studied by ultracentrifugal measurements. The detergent binding was determined using [^{3}H]Triton X-100.

1. The isolation procedure is modified as compared to that described recently (von Jagow, G., Engel, W. D., Riccio, P. and Schägger, H. (1976) in Genetics and Biogenesis of Chloroplasts and Mitochondria (Bücher, Th., et al., eds.), pp. 267–272 Elsevier, Amsterdam; Riccio, P., Schägger, H., Engel, W. D. and von Jagow, G. (1977) Biochim. Biophys. Acta 459, 250–262). The resulting preparation is devoid of the iron sulfur protein and contains more heme *b* (8.6 as compared to 8.0 μmol/g protein).

2. The sedimentation coefficient was determined to be $s_{20,w} = 13.03$–13.09.

3. Equilibrium runs observed at 415 nm and 280 nm exhibit straight curves (log *c* vs. r^2) indicating that the preparation is homogenous. After correction for bound Triton X-100, molecular weights of 400 000–440 000 were calculated from the curves obtained at the respective wavelengths. From the heme contents a minimum molecular weight of about 200 000 is calculated. It is therefore concluded that the complex is present as a dimer.

4. The average polarity of the whole complex as calculated from the amino acid analysis is relatively high (45 %).

5. In accordance with the high polarity the amount of bound detergent is relatively low, it amounts to 0.2 g Triton X-100/g protein. The amount of Triton bound to 1 mol of b-c_1-dimer corresponds to the molecular weight of the Triton micelle.

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INTRODUCTION

The apparent molecular weight of the $b \cdot c_1$ complex prepared according to Hatefi and Rieske [1, 2] was studied in 1965 by light scattering and ultracentrifugal measurements [3]. In these studies the complex was dispersed by taurocholate or taurodesoxycholate. Homogenous Schlieren boundaries indicated that the preparation was homogenous. The sedimentation runs gave a value of $s_{20,w} = 10.2$. Although the diffusion coefficient, the partial specific volume of the complex, and the amount of the specifically bound detergent were not determined, it was assumed that the sedimentation coefficient is consistent with a spherical molecule of a molecular weight in the region of 250 000 [3, 4]. It was concluded [4] that this preparation contains the $b \cdot c_1$ complex in a monomeric form. In the meantime no further centrifugation studies on this $b \cdot c_1$ complex were published.

In the present paper we present hydrodynamic studies in conjunction with the determination of the detergent binding performed to test the purity and the apparent molecular weight of the new preparation in which the complex is dispersed by Triton X-100 after isolation by hydroxyapatite chromatography [6, 7]. This preparation differs from the "Hatefi-Rieske" preparation, which was isolated in bile salts by ammonium sulfate fractionation, mainly by its lack of the iron sulfur protein, lack of the phospholipids and of ubiquinone, and by its complete enzymatic inactivity [7]. The heme b and c_1 contents of both preparations are about the same.

MATERIALS AND METHODS

Although the hydroxyapatite was always prepared according to the same procedure [5], it revealed a variability in its binding strength. A few preparations had remarkably lower binding capability. When such batches were used, the $b \cdot c_1$ complex started eluting already during application of the first buffer. Therefore, the phosphate concentration of the first elution buffer had to be decreased in parallel with an increase of the NaCl concentration (cf. Methods). With this modification it is possible to apply 2.5 times as much protein to the column as could be applied in the previously described procedure [6, 7].

Instead of submitochondrial particles [6, 7], mitochondria preextracted with Triton X-100 were taken, in order to decrease the preparative effort and to increase the preparative yield. EDTA was omitted from all buffers; the amount of antimycin for loading the mitochondria was decreased. The $b \cdot c_1$ complex can either be eluted with citrate, as it was described in the previous publication, or with a phosphate buffer (cf. Methods). All preparative steps were performed at 0 °C.

Purification of the $b \cdot c_1$ complex

(*i*) *Loading of the mitochondria with carboxyatractylate and with antimycin.* The mitochondria were loaded with carboxyatractylate (2 nmol/mg protein) (cf. refs. 22,7) directly after the preparation of the mitochondria. Then they were stored in liquid nitrogen at a protein concentration of 50–60 mg/ml. After thawing the mitochondria were charged with antimycin (2 nmol/mg protein) in 20 mM MOPS, pH 7.2 at a protein concentration of about 15 mg/ml.

(*ii*) *Preextraction of cytochrome c and matrix proteins from the mitochondria*

loaded with carboxyatractylate and antimycin. The preextraction was performed at a protein concentration of 15 mg/ml in a medium containing 1 % w/w Triton X-100, 20 mM MOPS, 40 mM $(NH_4)_2SO_4$, pH 7.2. After incubation for 10 min, the preextracted mitochondria were centrifuged at 123 000 $\times g$ for 1 h. During this preparation step, 30 % of the protein is lost, but the total amounts of the $b \cdot c_1$ complex and cytochrome a, a_3 remain in the pellet.

(*iii*) *Extraction of membrane proteins from the preextracted mitochondria.* The pellet was suspended for further extraction at a protein concentration of 15 mg/ml in a medium containing 20 mM MOPS, 200 mM $(NH_4)_2SO_4$, pH 7.2. Then Triton X-100 to a final concentration of 5.5 % w/w was added. After incubation for 10 min the solubilized proteins were removed by centrifugation at 123 000 $\times g$ for 40 min. The supernatant was applied to the hydroxyapatite column. Approximately 95 % of the $b \cdot c_1$ complex was solubilized by this procedure.

(*iv*) *Column chromatography.* The chromatography was performed at 4 °C. The column (30 $\times$ 1.5 cm) was packed with 50 ml hydroxyapatite which has to be degassed after suspending in 10 mM sodium phosphate, pH 6.8. For the equilibration of the columns, 2 column volumes of a buffer containing 0.5 % Triton X-100 and 85 mM sodium phosphate, pH 7.2 were used. *Step 1.* About 70 % of the whole amount of proteins and the total amount of cytochromes a, a_3 and c, as well as about 20 % of the b type cytochromes are eluted by a buffer containing 0.5 % Triton X-100, 250 mM NaCl, 85 mM sodium phosphate, pH 7.2. A buffer volume of 5 column volumes was applied. *Step* 2. The salt and detergent concentration were decreased by a buffer containing 0.05 % Triton X-100, 50 mM NaCl, 50 mM MOPS, pH 7.2. A buffer volume of half of the column volume was applied. *Step* 3. (*a*) elution by citrate: The $b \cdot c_1$ complex was released from the column by a buffer containing 0.05 % Triton X-100, 12 mM sodium citrate, 50 mM NaCl, 50 mM MOPS, pH 7.2. It is delivered from the column after a buffer volume of 3 column volumes. (*b*) elution by phosphate: The $b \cdot c_1$ complex was released from the column by a buffer containing 0.05 % Triton X-100 and 150 mM sodium phosphate, pH 7.2. It is delivered from the column after applying a buffer volume of one column volume.

Both elution procedures give a complex with a final concentration up to 30 μM (3.5 mg protein/ml). The final yield amounts to 55 % when referred to the b content of the mitochondria; the enrichment is 18-fold.

Ultracentrifugal studies

Analytical ultracentrifugation was performed with a Beckman Spinco Model E instrument equipped with multiplex unit and Schlieren and absorption optics with photoelectric scanner. For higher scanning precision, a 10-inch recorder as used for the Beckman DB-photometer was adapted to the scanner. From the scanner tracings, the apparent sedimentation coefficient was calculated as described by Schachman and Edelstein [8]. The partial specific volume of the $b \cdot c_1$ complex evaluated from the amino acid composition of the enzyme according to Cohn and Edsall [11] is 0.737 ml/g.

In a multicomponent system (protein, detergent and buffer) the interaction of the detergent Triton X-100 with the protein has to be considered. Therefore, the molecular weight of the $b \cdot c_1$ complex was corrected for the bound detergent by the use of a calculated buoyant density factor as outlined by Tanford [12].

Amino acid analysis

Amino acid composition of the $b \cdot c_1$ complex was determined after acid hydrolysis with 6 N HCl according to Spackman et al. [13] and with 3 N *p*-toluene sulfonic acid according to Lin and Chang [14] at 110 °C for 20 h. For stabilization of tryptophan during hydrolysis indole was added [14]. Besides for tryptophan, tyrosine and serine, where higher yields with *p*-toluene sulfonic acid were obtained, both hydrolysis procedures yielded identical results. Cysteine was determined after performic acid oxidation according to Hirs [15]. The individual amino acids were quantitatively evaluated using a Beckman Multichrom amino acid analyzer.

Phospholipids, ubiquinone, heme, nonheme iron and protein determinations

These determinations were made as described in the previous publication [7].

Triton X-100 determination

Triton X-100 was determined by its specific radioactivity. [^{3}H]Triton X-100 was a generous gift from the Rohm and Haas Company, Philadelphia.

RESULTS

Characteristics of the isolated $b \cdot c_1$ complex

The new isolation procedure was improved (cf. Methods) in order to cleave completely the iron sulfur protein from the multisubunit complex. This was desirable for obtaining a homogenous population of complexes. In the recently published method [7] not more than 3 column volumes of the first buffer for eluting foreign protein could be applied, otherwise the $b \cdot c_1$ complex was also eluted by this buffer.

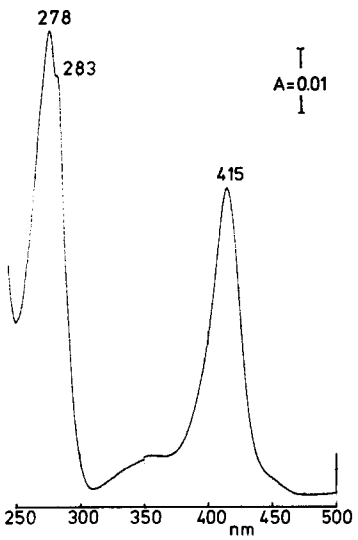

Fig. 1. Main absolute absorbance signals of the $b \cdot c_1$ complex in the visible and ultraviolet region when it is prepared in Triton X-100. The spectrum was performed in a Cary 118 spectrophotometer. The complex was dispersed in a buffer containing 0.025 % Triton X-100, 50 mM NaCl and 20 mM MOPS, pH 7.2. The reference cuvette contained the same buffer.

With the new method it was possible to apply 5 column volumes of the first buffer without elution of the complex. In this manner the iron sulfur protein is eluted by the first buffer and completely separated from the complex. At the same time the specific heme *b* content is increased by 8 %.

The preparation displays in its oxidized state two main absorbance bands (cf. Fig. 1); one in the visible region with a maximum at 415 nm and a second one in the ultraviolet region with a maximum at 278 nm with a shoulder on its long wave length flank at 283 nm. The signal at 415 nm is caused by the heme b chromophore. By the heme signal the $b \cdot c_1$ complex, and heme containing subunits, which may be cleaved during preparation, can be detected. Dissociated subunits or impurities lacking this "marker" can only be detected at 280 nm, although this signal has a more complex character. Obviously, the ultraviolet signals of the isolated complex are composed of the absorption of proteins and that of Triton. It can be calculated using absorbance coefficients of $\varepsilon_{\text{prot.}} = 1.6\ \text{g}^{-1} \cdot \text{l}$, and $\varepsilon_{\text{Trit.}} = 2.2\ \text{g}^{-1} \cdot \text{l}$, and referring to the measured Triton binding of 0.2 g/g protein, that the ultraviolet band is composed of about 80 % of the protein signal and of about 20 % of the Triton signal. Therefore the 280 nm absorption can be taken as a signal for the Triton solubilized protein in analytical ultracentrifugation.

The detergent binding of the complex

The Triton binding of the complex was determined by two different kinds of experiments. Firstly, by a low scale preparation in [^{3}H]Triton X-100 and, secondly, by equilibration studies performed with the complex bound on a hydroxyapatite column. Both kinds of experiments gave the same results.

Fig. 2A shows the elution pattern of the low scale preparation. It is similar to the published pattern of a normal scale preparation [7]. A column with 4 ml hydroxyapatite was used. The first buffer eluted about 70 % of the applied protein. The drastically varying curve of Triton concentration reflects the different detergent

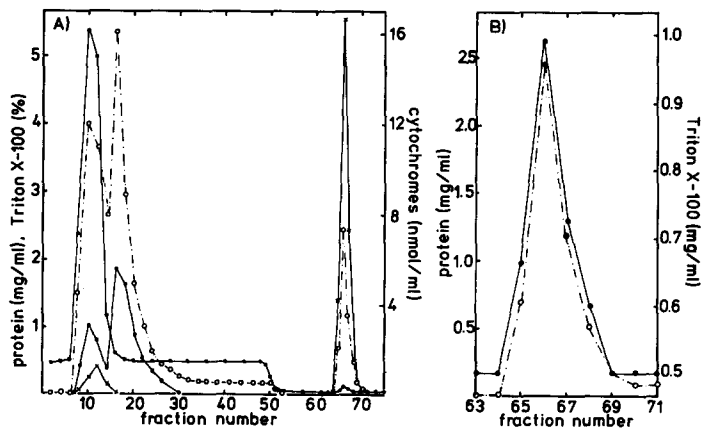

Fig. 2. Eluate profile of the low scale preparation of the $b \cdot c_1$ complex in [^{3}H]Triton X-100. The heme and protein concentrations were determined as described [7]. The Triton X-100 concentration was determined by aid of the specific radioactivity of the [^{3}H]Triton X-100. ○—○, Protein; ●—●, Triton X-100; × — ×, cytochrome *b*; o—o, cytochrome *a*, a_3.

concentrations used in the various buffers. As shown by the ^3H activity in the eluted fractions, the solubilization buffer contained 5.0 % Triton, the first elution buffer 0.5 % and finally the wash and second elution buffers 0.05 % Triton. After elution of the main part of protein and all of cytochrome *a*, a_3, the second buffer was applied as "wash" buffer. The $b \cdot c_1$ complex was eluted by the third buffer containing 12 mM citrate.

Fig. 2B shows the pattern of the eluted complex on a different scale. Only protein and detergent concentrations are plotted. The constant ratio of bound Triton/$b \cdot c_1$ complex becomes obvious. A Triton binding of 0.2 ± 0.01 g/g protein was calculated from 5 chromatographic runs.

From equilibration studies the same value for Triton binding was obtained (0.2 ± 0.02 g Triton X-100/g protein). In these experiments 4 mg purified complex were applied and bound to a column with a volume of 2 ml hydroxyapatite. The complex was equilibrated with 0.05 % Triton X-100 for 7 h. Then equilibration with [^3H]Triton X-100 for 5.5, 7.5 and 16 h was performed in a recycling buffer system. After equilibration the recycling system was opened and the complex eluted from the column by adding solid sodium citrate to a final concentration of 12 mM (not shown).

In addition to the value of detergent binding, the equilibration studies reveal a complete Triton X-100 exchange in less than 5.5 h. An exact value for the exchange

TABLE I

AMINO ACID COMPOSITION OF THE $b \cdot c_1$ COMPLEX OF BEEF HEART PREPARED IN TRITON X-100 AND LACKING THE IRON SULFUR PROTEIN

The amino acid analysis was performed as described in Methods.

Amino acid	mol %	residues	
Nonpolar			
Alanine	9.50	171	(171.0)
Valine	5.32	96	(95.8)
Leucine	9.93	179	(178.7)
Isoleucine	3.65	66	(65.7)
Proline	8.52	153	(153.4)
Methionine	1.47	27	(26.5)
Phenylalanine	4.10	74	(73.8)
Tryptophan	1.00	18	(18.0)
Glycine	6.90	124	(124.2)
Cysteine	0.71	13	(12.8)
Tyrosine	3.77	68	(67.9)
Polar			
Aspartate	8.40	151	(151.2)
Glutamate	10.23	184	(184.1)
Lysine	6.85	123	(123.3)
Arginine	5.17	93	(93.0)
Histidine	2.87	52	(51.7)
Serine	6.26	113	(112.7)
Threonine	5.36	97	(96.5)
Polarity		–	–
total residues	45.14	1802	–

time cannot be obtained in these experiments, since an elution time of 5 h is necessary.

The amino acid analysis

The analysis was performed to determine the partial specific volume and the polarity of the multisubunit protein. Table I gives the complete amino acid analysis of the $b \cdot c_1$ complex of beef heart when it is prepared in Triton X-100 in the presence of antimycin. Although this preparation lacks the iron sulfur protein, its amino acid composition is similar to that of the $b \cdot c_1$ complex from *Saccharomyces carlsbergensis* prepared in cholate as described by Katan et al. [16]. In both species the most abundant nonpolar amino acids are leucine and alanine, the proline content of the beef heart preparation seems to be somewhat higher. The most frequent polar amino acids are in both cases glutamic acid/glutamine and aspartic acid/asparagine. The polarity of both preparations is unusually high for a membrane protein complex; it amounts to 45 %. This property is in good agreement with the relatively low detergent binding.

The molecular weight of the monomer of the complex from which the iron sulfur protein is cleaved amounts to about 200 000 as will be described below. Assuming a total of about 1800 amino acid residues, the calculation from the measured "mol % for cysteine" gives a total of about 13 residues. A tentative assignment of the cysteinyl residues to the different polypeptides can be performed. Since the cytochrome c_1 subunit contains at least 2 cysteinyl residues, for the binding of heme only 11 can be attributed to the 5 remaining subunits. The estimated number of cysteinyl residues per molecule of cytochrome *b* of *Neurospora crassa* is 5 using the amino acid composition and molecular weight published by Weiss [17]. Since there are two cytochrome b molecules in the $b \cdot c_1$ complex, 12 of the 13 cysteinyl residues are accounted for and one might expect that the core proteins and the smaller subunits are relatively devoid of this amino acid.

The molecular weight and state of aggregation of the $b \cdot c_1$ complex

Fig. 3 shows a plot of a sedimentation velocity run, recorded in (A) at 415 nm

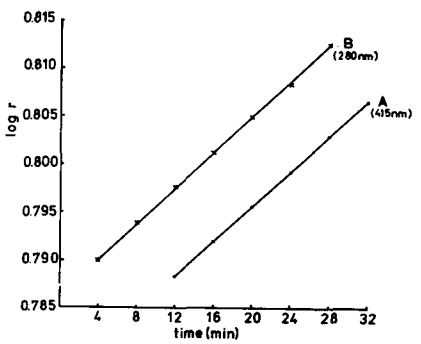

Fig. 3. Sedimentation velocity runs of the $b \cdot c_1$ complex. The logarithm of the distance of the gradient from the center of rotation is plotted versus the time. Rotorspeed: 48 000 rev./min, protein concentration 0.5 mg/ml in 20 mM MOPS, pH 7.5. (A) Sedimentation was followed at 415 nm, Triton concn. 0.05 %, temperature 22.4 °C; (B) Sedimentation was followed at 280 nm, Triton concn. 0.025 %, temperature 21.7 °C.

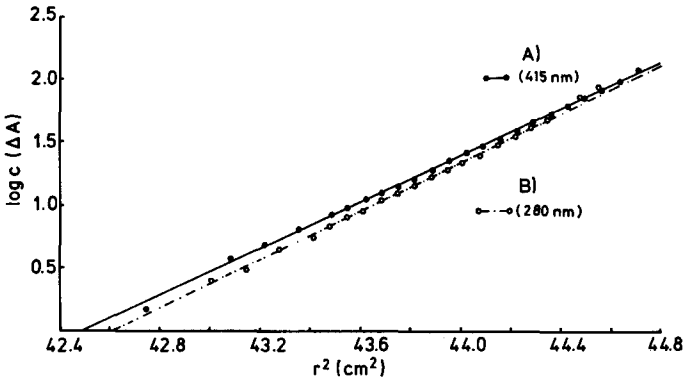

Fig. 4 Sedimentation equilibrium runs of the $b \cdot c_1$-complex in Triton X-100. The logarithm of recorder deflection in mm (proportional to the absorption) is plotted versus the square of the distance to the center of rotation, r^2 (cm^2). The rotor speed was 9000 rev./min, the protein concn. about 0.2 mg/ml. After 20 h of centrifugation the equilibrium was reached. (A) Light of 415 nm wavelength was used, temperature 22.3 °C; (B) light of 280 nm wavelength was used, temperature 22 °C.

and in (B) at 280 nm. In the first case, the complex was dispersed in 0.05 % Triton, in the second, in 0.025 % Triton, in order to lower the Triton absorbance. Both runs give straight lines in the plot log r vs. time. From both curves a value of $s_{20,w} =$ 13.0–13.1 is calculated. Although sedimentation velocity runs are not as convincing as sedimentation equilibrium runs, the linearity of these curves indicates that the preparation is essentially homogeneous.

Fig. 4A and B give curves log c vs. r^2 of sedimentation equilibrium runs observed at the two wavelengths, respectively. The runs were performed with detergent concentrations as used in the sedimentation velocity runs. The longest centrifugation time was usually 23 h. The analyses of the curves obtained at 415 nm after correction for bound Triton (cf. Methods) give a molecular weight of 400 000 (cf. Table II). The measuring points of each run could be fitted with only minor deviations by a straight line.

The measurements performed at 280 nm do not give a straight line over the whole range. In the region near the bottom of the cell the points deviate slightly from the ideal values. The relatively low detergent concentration (0.025 % Triton X-100), used in these runs for optical reasons, seems to be at the lower limit of detergent con-

TABLE II

HYDRODYNAMIC PARAMETERS OF THE $b \cdot c_1$ COMPLEX OF BEEF HEART PREPARED IN TRITON X-100 AND LACKING THE IRON SULFUR PROTEIN

The ultracentrifugal studies and the measurement and correction for bound detergent were performed as described in Methods.

Sedimentation coefficient ($s_{20,w}$) followed at		Molecular weight from sedimentation equilibrium followed at	
415 nm	280 nm	415 nm	280 nm
13.03	13.09	400 000 ± 10 000	440 000 ± 15 000

centration required for the dispersion of the $b \cdot c_1$ complex. In this case the system appears not to be in a completely homogenous state because of a small amount of aggregation. It may be for this reason that the molecular weight determined at 280 nm of 440 000 differs slightly from that calculated from the 415 nm runs (cf. Table II). Nonetheless, the straight lines which were obtained in the sedimentation equilibrium runs again indicate the high homogeneity of the preparation.

DISCUSSION

The sedimentation equilibrium runs give an apparent molecular weight of about 400 000–440 000 after correction for bound detergent. But, the minimum molecular weight as calculated from the heme b content, and assuming two molecules of cytochrome *b* per molecule of complex, amounts to about 220 000. Therefore, the conclusion can be drawn that the $b \cdot c_1$ complex, when it is prepared in Triton X-100 is present as a dimer.

The question arises whether the dimer reflects the natural physiological state of the $b \cdot c_1$ complex, or whether the dimer is formed during isolation. Previously another protein from the mitochondrial membrane, the ADP, ATP carrier, was isolated as a dimer in the same Triton-hydroxyapatite system [22] (Hackenberg, H., unpublished). In this case the existence of a dimeric form in the native state is indicated by one binding site per two subunits. Obviously Triton can preserve the quaternary structure of protein complexes. Therefore, it appears probable that the dimer of the $b \cdot c_1$ complex is the native association state, carrying two cytochromes c, and four cytochromes *b*. As mentioned in the introduction, the monomer possibly is present when the complex is dispersed in the ionic detergent taurocholate, whereas the non-ionic detergent Triton preserves the dimeric state. A similar situation exists with complex IV, which was found to be a monomer in the presence of cholate [3], and a dimer in the presence of Triton X-100 [18–20, 23].

The described $b \cdot c_1$ complex binds only 0.2 g Triton X-100/g protein, which corresponds to about one detergent micelle of molecular weight 90 000 [12] per one b-c_1 dimer. Even if it is assumed that the dimer has a spherical shape with a minimal calculated surface area of $2.984 \cdot 10^4$ Å^2, only part of the $b \cdot c_1$ complex can be covered by the bound Triton molecules.

The apparent molecular weight (200 000–220 000 for the monomer) is somewhat below the previously published value (250 000 for the monomer). This molecular weight can be accounted for by assuming that in analogy to complex IV the isolated $b \cdot c_1$ complex is composed of only one of each polypeptide subunit: The present complex lacks the iron sulfur protein of Rieske [24] and is composed of the two core proteins (molecular weights of 47 000 and 44 000) [7], two molecules of cytochrome *b* with a molecular weight each of 27 000 (own results, unpublished), one molecule of cytochrome c_1 (29 000) [21] and finally two small proteins with molecular weights of 11 000 and about 10 000 [7] respectively. The sum of the molecular weights of these subunits gives a total of 195 000, which is not so far from the value obtained in our sedimentation equilibrium studies for the monomer.

It may be concluded that the $b \cdot c_1$ complex is not the product of any specific isolation procedure, but a native multienzyme system, existent in the mitochondrial membrane. The $b \cdot c_1$ complexes prepared either in bile salts by salt fractionation

[1, 2] or in Triton X-100 by adsorption chromatography [6, 7] are composed of the same protein subunits, although the latter preparation has lost one subunit, the iron sulfur protein, due to its chromatographic isolation on hydroxyapatite in the presence of antimycin.

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