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bc_1-COMPLEX FROM BEEF HEART

ONE-STEP PURIFICATION BY HYDROXYAPATITE CHROMATOGRAPHY IN TRITON X-100, POLYPEPTIDE PATTERN AND RESPIRATORY CHAIN CHARACTERISTICS

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SUMMARY

A new simple method for the purification of the bc_1-complex has been developed. The polypeptide composition of the complex was analysed by dodecyl sulfate-polyacrylamide gel electrophoresis. The content of chain components and phospholipids was determined. The *b*-type cytochromes were further characterized by their absorbance spectra and midpoint potentials.

(1) Starting from a Triton X-100 extract of submitochondrial particles supplemented with antimycin, the bc_1-complex is purified by adsorption chromatography on hydroxyapatite with citrate as specific eluant.

(2) The complex splits in dodecyl sulfate into five main polypeptides with apparent molecular weights of 47, 44, 31, 11 and < 10 kdalton.

(3) The purified complex has a heme-*b* content of 8.0 μmol/g protein and a cytochrome c_1 content of 3.8 μmol/g protein.

(4) The cytochromes show the typical absorbance spectra of cytochromes *b*-562 and *b*-565 and are present in approximately equal amounts with midpoint potentials of $E_{m7} = +100$ mV and $E_{m7} = +10$ mV respectively. Carbon monoxide does not bind to the cytochromes.

(5) The nonheme iron protein content of the complex is diminished to 0.6 μmol/g protein.

(6) The use of the nonionic surfactant Triton X-100 leads to a complete loss of lipids and ubiquinone of the bc_1-complex.

(7) The complex contains no succinate dehydrogenase as indicated by the absence of the 69 kdalton subunit in the dodecyl sulfate gel electrophoresis. In addition, it lacks an ubiquinone cytochrome *c* reductase activity and other electron

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Abbreviations: TMPD, tetramethyl-*p*-phenylenediamine; MOPS, morpholinopropane sulfonate.

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transferring activities. This may be inferred from an inhibition by antimycin and depletion of ubiquinone and phospholipids.

The highly purified and relative stable complex can be prepared giving 50 % yield and may be suitable for protein chemistry studies.

INTRODUCTION

The aim of this study was to purify cytochrome *b* by a simple method. H. Weiss [1] has recently published a new preparation method for the isolation of this cytochrome. This method is complicated by the necessity to prepare a special chromatographic material. In addition, the low yield of the isolation procedure and an uncharacteristic absorbance spectrum and midpoint potential do not allow a definitive decision as to whether one or both of the *b*-type cytochromes are purified.

Although the development of our method for the preparation of cytochrome *b* is not yet finished, it seems to be worthwhile to publish the first step of the new procedure because it leads to a bc_1-complex which possesses an unexpectedly high heme content and has an unexpectedly low content of phospholipids and ubiquinone.

In the literature, the preparations of the complex pursue a common scheme consisting of a series of ammonium sulfate precipitations under dispersion of the protein by bile salts. The original method developed by Hatefi [2] and Rieske [3] was slightly modified in the course of about 15 years. All recently published preparation procedures [4–6] include 4 to 5 cycles of ammonium sulfate precipitations.

Rieske [7] warns against applying chromatography to the complex because 'the enzyme is inactivated rapidly by adsorption on surfaces (passage of the enzyme through a chromatographic column)'. This may be valid with respect to the electron transferring capability, since chromatography depletes the complex of phospholipids and ubiquinone, entities which may be obligatory for catalytic activity. These phospholipid-ubiquinone depleted complexes may be valuable for protein chemistry, immunochemistry and as starting material for cytochrome *b* preparations.

This study deals with the preparation of an antimycin loaded complex by means of only one chromatographic step under dispersion of the proteins by Triton X-100.

METHODS

Preparation of submitochondrial particles: beef heart mitochondria were prepared as described elsewhere [8]. The submitochondrial particles were prepared by sonication of the mitochondria (10 mg/ml) for 15×7 s at 3 °C in a medium containing 100 mM NaP_i, 1 mM EDTA and 1 mM $MgCl_2$, pH 7.2. The suspension was centrifuged for 10 min at 10 000 rev/min in a Sorvall SS 34 rotor, and the resulting supernatant was centrifuged for 60 min at 40 000 rev/min in a Spinco 42.1 rotor. The particles were washed once (with the same medium), and centrifugation was repeated. The resulting pellet can be stored at −20 °C for a few weeks.

Purification of the bc_1-complex

(a) Extraction of membrane proteins of the submitochondrial particles by

Triton X-100: the pellet was suspended for extraction at a protein concentration of 35 mg/ml in a medium containing 10 mM MOPS, 0.1 M NaCl and 1 mM EDTA, pH 7.4. Then, antimycin (5 nmol/mg protein) and carboxyatractylate (2 nmol/mg protein) were added. To 1 vol. of this suspension, 1.2 vols. of 8 % Triton X-100, 1 M NaCl, 10 mM MOPS, 1 mM EDTA, pH 7.4, were added. Approx. 85 % of the cytochrome *b* was solubilized under these conditions (cf. Table I). The unsolubilized proteins were sedimented by centrifugation for 45 min at 40 000 rev/min in a Spinco 40.2 rotor. The supernatant was applied on the hydroxyapatite column.

(b) Column chromatography on hydroxyapatite: hydroxyapatite was prepared according to Tiselius [9]. A column (30 × 1.5 cm) was packed with 50 ml hydroxyapatite. The gel was equilibrated with a buffer containing 110 mM NaP_i, 90 mM NaCl, 1 mM EDTA and 0.5 % Triton X-100, pH 7.4. A concentration of approx. 4 mg protein/ml gel was applied on the column. The flow rate was maintained at 30 ml/h. The proteins were eluted stepwise from the column by four different buffer solutions as will be described under results. All steps were performed at 4 °C.

Dodecyl sulfate-polyacrylamide gel electrophoresis

The slab gels consisted of 8 % acrylamide monomer and 0.3 % *N,N'*-methylenebisacrylamide. The gel dimensions were 3 × 80 × 160 mm; the running distance was 110 mm. The samples were run in a buffer containing 0.1 M Tris · HCl, 0.5 % sodium dodecyl sulfate, pH 8.0. The samples were prepared for electrophoresis by heating at 50 °C for 20 min in the presence of 2 % dodecyl sulfate and 2 mM dithioerythrol. The gels were stained overnight in 0.25 % Coomassie Brilliant Blue in methanol/water/acetic acid (5 : 5 : 1, by vol.) and destained in 10 % acetic acid.

Spectrophotometric measurements: light absorption spectra were performed with a Cary 118 spectrophotometer at room temperature and low temperature spectra were obtained with a split beam spectrophotometer designed by Klingenberg according to Chance [10]. The used difference extinction coefficients (reduced — oxidized) were $\varepsilon_{563-577nm} = 28.5\ mM^{-1}\ cm^{-1}$ for cytochrome *b* [11] and $\varepsilon_{553-531nm} = 19.1\ mM^{-1}\ cm^{-1}$ for cytochrome c_1. The value for cytochrome c_1 was calculated from the coefficient at 554 nm and the spectrum measured by Green et al. [12].

Pyridine hemochromogen determination

The heme *b* and the heme c_1 contents of the complex were determined as described by Basford et al. [13].

The content of nonheme iron protein was calculated from acidlabile sulfide determinations, which were performed following the method of Fogo and Popowsky [14] in the modification of King and Morris [15]. A ratio of 2 : 1 for acid-labile sulfide to non-heme iron protein was assumed [16].

Redox titration experiments

The redox titrations were performed according to Wilson and Dutton [17] with a double beam spectrophotometer designed by M. Klingenberg in a special redox titration chamber designed by G. v. Jagow (unpublished observation). The midpoint potentials of the *b*-type cytochromes were measured at 563 — 575 nm. The titration curve was analyzed by a computer program, assuming the presence of two one-electron transferring cytochromes.

Ubiquinone extraction and determination

Ubiquinone was extracted and determined as described by A. Kröger and M. Klingenberg [18].

Phospholipid detection by thin layer chromatography

3 mg of protein were extracted with 2 ml chloroform/methanol (2 : 1, v/v) and the water phase reextracted once with 1 ml of this mixture. The combined organic phases were washed with 0.02 % $MgCl_2$ and then the solvent was evaporated. The residue was dissolved in 400 µl chloroform/methanol (2 : 1, v/v) and 100 µl were applied on a plate in a band of 1 cm width. One-dimensional thin layer chromatography was performed with a solvent containing chloroform/methanol/water (65 : 25 : 4, by vol.). The phospholipids were detected by spraying the plates with 0.33 % Mo, 0.66 % MoO_3 in 8 M H_2SO_4.

Protein was determined by a modified biuret [19] or a modified Lowry method [20]. Bovine serum albumin was used as a standard.

The Triton X-100 content of the preparation was determined according to Garewal [21].

Duroquinone-cytochrome *c* reductase activity was measured in a reaction medium containing 250 mM sucrose, 20 mM Tris · HCl, 1 mM $MgCl_2$, pH 6.5 and 25 µM DQH_2 prepared according to H. Aquila [22]. The bc_1-complex was preincubated at varying concentrations with the quinone at 15 °C for 2 min, and reaction was started by addition of 50 µM cytochrome *c*. The reference signal of the double beam spectrophotometer was adjusted to automatic balance.

QH_2-2 cytochrome *c* reductase and succinate cytochrome *c* reductase activities were determined in the same manner, using 50 µM QH_2-2 and 5 mM succinate, respectively, as electron donors.

RESULTS

Purification of the bc_1-complex

Column chromatography was performed with the same material used by Riccio et al. [23] for isolation of the adenine nucleotide carrier. In this case, the translocase represented the main part of the pass through of the hydroxyapatite column when the submitochondrial particles were saturated with carboxyatractylate before chromatography. As already shown by these authors, a variety of further proteins can be eluted subsequently by a linear phosphate gradient.

Preliminary experiments revealed that it is not possible to prepare the complex by this method without special precautions, because the protoheme of cytochrome *b* dissociates from the apoprotein during chromatography. This could be recognized on the column by a bleaching of the red-brown bc_1-fraction during the run. Saturation of the complex with antimycin before chromatography overcomes this difficulty. Rieske has found that the complex is protected against dissociation of cytochrome *b* under these conditions [24], even in a buffer of high ionic strength. Obviously, cytochrome *b* looses its heme more easily during chromatography, when it is split from the complex.

When the elution was performed with a linear phosphate gradient, the complex was released from the gel at a sodium phosphate concentration of approximately 140 mM. The purification was insufficient at this salt concentration since most of the

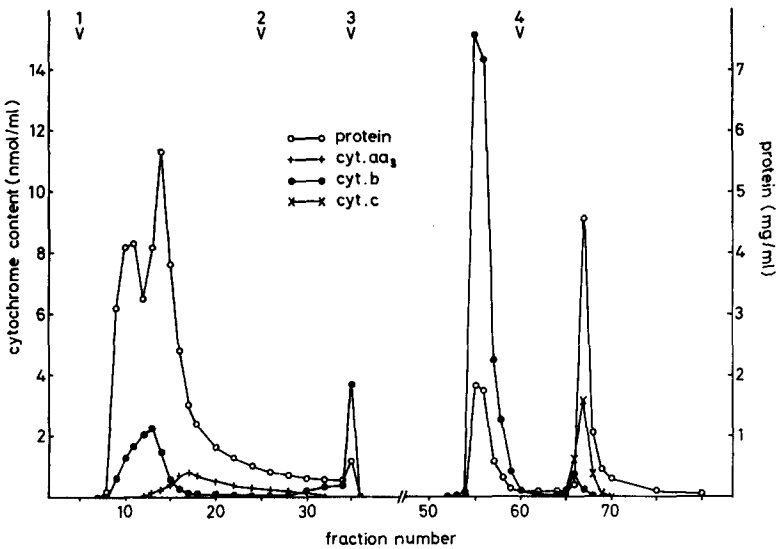

Fig. 1. Eluate profile of a Triton X-100 extract of submitochondrial particles after hydroxyapatite chromatography under application of a stepwise elution of the proteins. For technical details, see Methods and Results. Arrows indicate the buffer changes. The fraction volume was 5.4 ml.

other proteins were coeluted. Accordingly, the heme *b* content of the best fractions was only 4–5 μmol/g protein.

The hydroxyapatite chromatography became efficient with the introduction of a stepwise elution and the application of citrate as an appropriate eluant for the complex. Citrate has the effect of eluting the complex specifically even at concentrations as low as 8 mM.

The chromatography is performed at pH 7.4. Fig. 1 shows an eluate profile in which the proteins are delivered by a series of three specific buffers. The first elution buffer contains 110 mM NaP_i, 90 mM NaCl, 1 mM EDTA and 0.5 % Triton X-100. When this buffer is used, more than 70 % of the total protein is eluted. The first protein peak may represent the adenine nucleotide carrier which accounts for about 20 % of the inner membrane proteins [25].

The application of the first buffer must be stopped when the main part of the cytochrome oxidase is eluted, otherwise the bc_1-complex will be eluted with this buffer.

A wash-buffer is applied afterwards. It contains 10 mM NaP_i ,1 mM EDTA and 0.05 % Triton X-100. This step is necessary in order to decrease the salt and detergent concentrations before elution of the complex.

The complex can be separated by means of a second elution buffer containing 8 mM citrate, 10 mM NaP_i, 1 mM EDTA and 0.05 % Triton X-100. Under these conditions, the complex forms a red-brown disc which migrates through the column. The complex is eluted 4 h after application of the citrate buffer, the entire chromatography lasting for 9 h.

The remaining proteins can be eluted quantitatively by means of a third elution buffer containing 200 mM citrate, 10 mM NaP_i, 1 mM EDTA and 0.5 % Triton X-100. They account for about 20 % of total protein.

TABLE I

ENRICHMENT AND YIELD OF HEME *b* DURING PREPARATION

The heme content was calculated from difference absorbance spectra. The protein contents were determined by a modified Biuret method[a] or by a modified Lowry method[b] [19, 20]. smp, submitochondrial particles.

Fraction	Total protein (mg)	Total heme *b* (nmol)	Heme *b* per protein (nmol/mg)	Purification (fold)	Yield (%)
Submitochondrial particles (smp)	354[a]	350	1.0	1	100
Triton extract of smp	220[a]	306	1.4	1.4	87
Hydroxyapatite chromatography	22[b]	180	8.2	8.2	51

As can be seen in Fig. 1, a small part of cytochrome *b* is already eluted before elution of the main part of the complex (the first *b*-peak). However, the absorbance maximum of the cytochrome *b* is at 560 instead of 562 nm.

Cytochrome aa_3 is released from the column immediately after elution of the first *b*-peak. It comprises the total heme *a* extracted under the selected solubilization conditions and does not exceed 15 % of the cytochrome aa_3 of the particles.

The small amount of cytochrome *c* which remained in the particles after sonication is then liberated from the column by the third elution buffer.

As can be seen in Fig. 1 and Table I, the three best fractions contain, in a volume of 16 ml, 51 % of the applied heme *b*, enriched by a factor of more than 8. The complex is relatively pure and stable. After storage at 4 °C for two weeks, more than 80 % of heme *b* is recovered.

The isolated complex contains 0.3 g bound Triton X-100/g protein (total Triton content of the column eluate minus Triton content of the elution buffer).

The described isolation yields the bc_1-complex in a low salt and detergent concentration. It can be applied to further procedures, such as ion exchange chromatography, without preceding dialysis. No other common difficulties, such as disturbance of protein bands on polyacrylamide gels, occur.

The entire chromatographic procedure is relatively simple, reproducible and effective.

Polypeptide composition

The dodecyl sulfate-polyacrylamide gel electrophoresis shows five main bands (Fig. 2). Three polypeptides with molecular weights of 50, 47, and 44 kdalton are revealed in the high molecular weight region, but the band of 50 kdalton, which corresponds to one major subunit of the F_1-ATPase, is present in low concentrations only. One polypeptide with 31 kdalton is present in the region of middle molecular weights. Two polypeptides with 11 and less than 10 kdalton are detected in the low molecular weight region.

A comparison of the pherograms obtained by the various cholate preparations

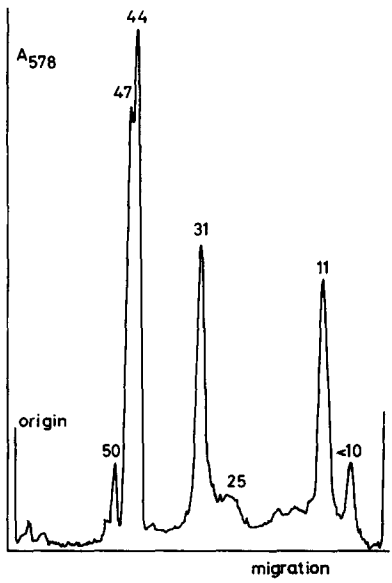

Fig. 2. Polypeptide pattern of the bc_1-complex. Dodecyl sulfate-polyacrylamide gel electrophoresis and densitometry were performed as described under Methods.

[4–6, 26, 27] shows differences in the polypeptide pattern and molecular weights. Some preparations contain succinate dehydrogenase as indicated by a 69 kdalton band [5, 6, 27]. With the exception of the yeast complex [4], and perhaps that of Yu et al. [26], all preparations are contaminated in varying amounts by one or two major subunits of the F_1-ATPase [5, 6, 27]. The 'core proteins' give the highest bands with molecular weights between 53 and 40 kdalton. In the region of the middle molecular weights (40–20 kdalton), 1 to 4 polypeptides with different molecular weights and

TABLE II

CONTENTS OF THE CHAIN COMPONENTS OF THE bc_1-COMPLEX

Heme contents were calculated from difference absorbance spectra of cytochromes and pyridine hemochromogenes. Ubiquinone and nonheme iron protein were determined as described in Methods. The results are mean values of three preparations and are given with standard deviation. *n*, number of determinations.

Component	Content (μmol/g protein)	*n*	Molecular ratio
Cytochrome *b*			
Difference absorbance spectra	8.1 $\pm$ 0.4	6	
Pyridine hemochromogen	8.0 $\pm$ 0.4	8	$\equiv$ 2
Cytochrome c_1			
Pyridine hemochromogen	3.8 $\pm$ 0.2	6	0.95
Nonheme iron protein	0.6 $\pm$ 0.2	11	0.15
Ubiquinone	$<$ 0.1		$<$ 0.02

contributions are found. The number of the polypeptides with low molecular weights varies between two and three.

The Triton X-100 complex consists of only five polypeptides, excluding the low contribution of the F_1-ATPase subunit. The main area of the pherogram is comprised of the two highest bands referred to as the 'core proteins'. The 31 kdalton band should represent the cytochromes *b* and c_1 [1, 28]. These cytochromes should account for about 40 % of the total protein of the bc_1-complex as calculated with the heme contents given in Table II and the respective molecular weights. This result is inconsistent with the small area of the 31 kdalton band. It remains uncertain whether this is due to a various staining index (Weiss, H., personal communication) or whether part of cytochrome *b* does not enter the gel (Groot and Katan, personal communication, and ref. 29). The contribution of the 11 kdalton band in the pherogram of the Triton X-100 complex is unexpectedly high. However, it can not yet be excluded that it is a composite band.

Contents of chain components

The contents of chain components are shown in Table II. The complex contains about 8 μmol cytochrome *b* per g protein. There is a good agreement between the calculations from light absorbance spectra and pyridine hemochromogen determination. The cytochrome c_1 content amounts to 3.8 μmol/g protein, determined by the pyridine hemochromogen method.

The direct determination of the nonheme iron content is disturbed by the presence of EDTA in the preparation. Therefore, the acid-labile sulfide was determined. The nonheme iron protein content was calculated to be 0.6 μmol/g protein. When compared with the nonheme iron contents of the complexes prepared by cholate/ammoniumsulfate, it amounts to only 15 % of the normal content. Obviously, the main part of the iron sulfur protein is split from the complex by the high ionic strength in the presence of antimycin [24] during solubilization. This result is also supported by the polypeptide pattern (Fig. 2) which lacks a distinct band at 25 kdalton.

TABLE III

COURSE OF UBIQUINONE DEPLETION OF THE bc_1-COMPLEX DURING THE VARIOUS PREPARATION STEPS

The ubiquinone and cytochrome *b* contents were determined as described in Methods. The cytochrome *b* content in μmol/g protein was 0.9 in submitochondrial particles, 1.3 in the Triton X-100 extract and 8 in the bc_1-complex. smp, submitochondrial particles.

Fraction	Ubiquinone content	
	(μmol/g protein)	(μmol/μmol *b*)
Submitochondrial particles (smp)	8.0	9.4
Triton extract of smp	6.3	4.8
bc_1-complex after chromatography	< 0.1	< 0.01

The ubiquinone contents were determined by the reliable extraction method of Kröger and Klingenberg [18]. As demonstrated in Table III, the ubiquinone present in the bc_1-complex after Triton X-100 extraction is one-half the amount of that present before solubilization. After chromatography, the complex appears to be completely depleted of ubiquinone. The lower limit of detection under the conditions used was 0.1 μmol/g protein.

Thin layer chromatography (cf. Methods) shows only traces of phospholipids when 3 mg of complex protein were extracted. The same procedure performed with 3 mg protein of submitochondrial particles shows three intensively stained phospholipid bands.

Light absorbance spectra and redox potentials

The absorbance spectra of the Triton X-100 complex resemble those of the cholate complex [5, 6]. The difference spectrum (dithionite reduced — ferricyanide oxidized) has a maximum at 563 nm and a shoulder at 556 nm (Fig. 3A). A better resolution of the cytochromes *b* and c_1 is obtained at low temperature (Fig. 3B). There the *b* cytochromes have a maximum at 559 nm and cytochrome c_1 has a maximum at 552 nm. The molar 2 : 1 stoichiometry of the cytochromes in the complex becomes more apparent in such a spectrum due to diminished overlapping of the single absorbance bands. However, the signals of the two *b*-type cytochromes also keep interfering at — 196 °C.

As will be shown by the following spectra, the *b*-cytochromes can only be demonstrated separately by aid of a series of special electron donor additions which do exert appropriate redox potentials (E^h) on the system.

Cytochrome c_1 can be reduced exclusively by addition of ascorbate (Fig. 4A). It has a symmetrical band with a maximum at 552 nm. Then cytochrome *b*-562 can be

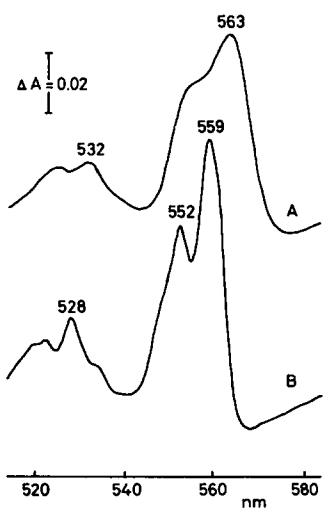

Fig. 3. Difference absorbance spectra of the bc_1-complex at 25 °C and −196 °C. The cytochromes were reduced by dithionite in the measuring side and present in oxidized state in the reference side. The complex (0.6 mg/ml) was measured in 0.5 M sucrose, 10 mM KP_i, pH 7.2 (A) at room temperature with $d = 5$ mm and (B) at low temperature with $d = 1$ mm.

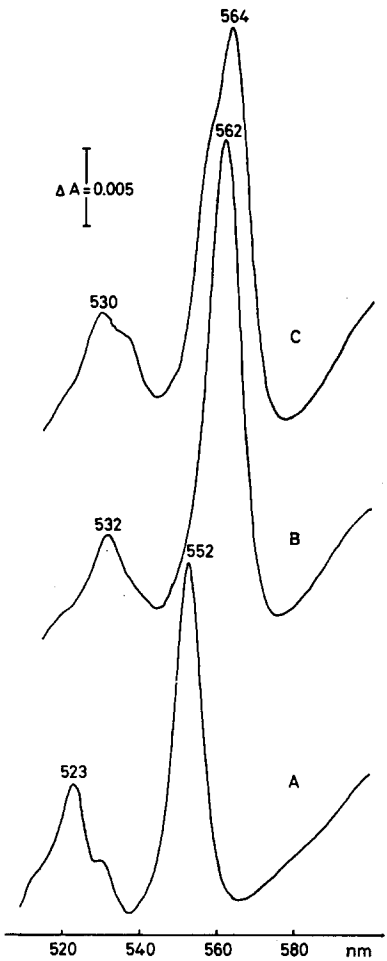

Fig. 4. Difference absorbance spectra of cytochrome c_1, *b*-562 and *b*-565 of the bc_1-complex at room temperature. (A) spectrum of cytochrome c_1; (B) spectrum of cytochrome *b*-562 and (C) spectrum of cytochrome *b*-565. Cytochrome c_1 was reduced by addition of 5 mM ascorbate; cytochrome *b*-562 is demonstrated by a difference spectrum: ascorbate, 50 μM TMPD minus ascorbate; and cytochrome *b*-565 by a difference spectrum dithionite minus ascorbate, TMPD. Protein content: 0.3 mg/ml; $d = 10$ mm.

shown after turning the cuvettes in the light beams and the addition of ascorbate plus TMPD to the measuring sample (Fig. 4B). It displays a symmetrical band with a maximum at 562 nm. Finally, cytochrome *b*-565 can be demonstrated by addition of dithionite to the measuring side when the reference side is reduced by ascorbate plus TMPD. The spectrum has a band with a maximum at 564 nm which carries a shoulder on the short wavelength flank.

Low temperature spectra, however, allow a more definite discrimination between the two *b* cytochromes (Fig. 5). In this case, cytochrome *b*-562 has a symmetrical band with a maximum at 559 nm, whereas the spectrum of cytochrome *b*-565 is now double banded with maxima at 561 and 553 nm.

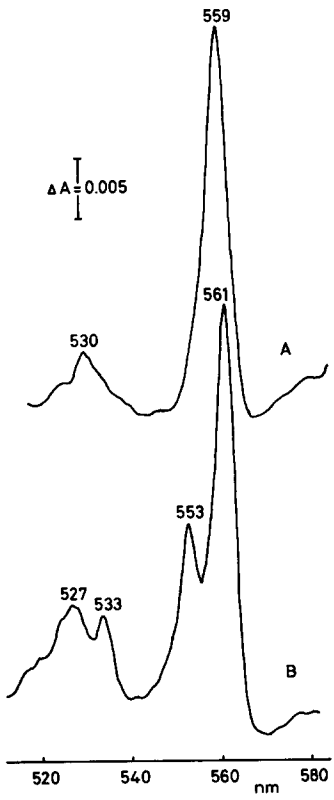

Fig. 5. Low temperature difference absorbance spectra of cytochrome *b*-562 and *b*-565 of the bc_1-complex. (A) spectrum of cytochrome *b*-562; (B) spectrum of cytochrome *b*-565. The spectra were obtained and performed as described in legends to Figs. 3 and 4.

The dithionite-reduced cytochrome *b* spectrum was not diminished by addition of carbon monoxide.

In the Triton X-100 complex a reduction of the cytochromes was not possible, whether QH_2-2, QH_2-3 or by succinate.

Fig. 6 gives the redox titration curve of the *b* cytochromes with its best computer-fit and analysis as performed at pH 7.0. The relative contribution of cytochrome *b*-562 to the curve is 0.55 and that of cytochrome *b*-565 is 0.45. The midpoint potentials are $E_{m\,7} = +100$ mV for cytochrome *b*-562 and $E_{m\,7} = +10$ mV for cytochrome *b*-565. The titrations show that the values of the midpotentials of the *b* cytochromes of the isolated complex are shifted in more positive direction by about 50 mV when compared to those of submitochondrial particles [30]. Similar midpoint potentials for cytochrome *b* (-10 mV and $+95$ mV) were found in a succinate-cytochrome *c* reductase preparation by Erecinska et al. [31, 32], but only after addition of a mixture of lysolecithin/lecithin to this preparation. Before addition of these phospholipids, the midpoint potentials of the two *b*-type cytochromes were more negative, namely -44 and $+55$ mV. In their preparation, the solubilization of the proteins was made possible by the unusual combination of a nonionic and an anionic amphiphile

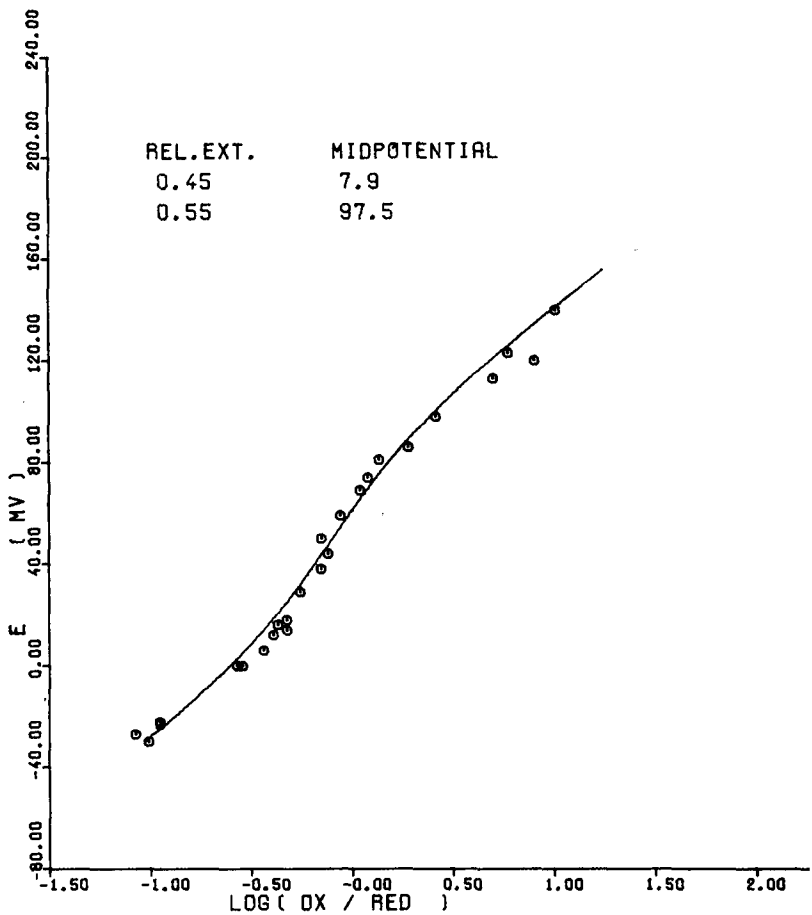

Fig. 6. The redox titration curve of the *b*-type cytochromes of the bc_1-complex. The redox titration was performed and analysed as described in Methods.

(Triton X-100 and deoxycholate). This preparation still contains phospholipids and ubiquinone in contrast to our Triton preparation.

DISCUSSION

From a functional point of view, the bc_1-complex described here has the disadvantage that the electron flow from the cytochromes *b* to cytochrome c_1 is blocked, since it is prepared in the presence of antimycin. Recent studies (unpublished) have shown that the complex, when prepared without antimycin, shows a very low rate of QH_2-3 cytochrome *c* reductase activity, which can be completely inhibited by antimycin. The reason for this low activity may be that this complex, as the antimycin complex, lacks phospholipids and ubiquinone.

Reconstitution studies with phospholipids and ubiquinone should elucidate whether one or both of these entities are indispensable components for electron flow from cytochrome *b* to cytochrome c_1.

The lack of a prominent 25 kdalton band in the polypeptide pattern and the low nonheme iron content indicate that most of the Rieske iron sulfur protein is split from the bc_1-complex. Nevertheless, this complex may be a valuable material for the study of the other polypeptide subunits, particularly when used as starting material for the preparation of cytochrome b or c_1.

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