Vol. 95, No. 1, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 16, 1980 Pages 90-96

ISOLATION OF BOVINE CYTOCHROME $\underline{c}_1$ AS A SINGLE NON-DENATURED SUBUNIT USING GEL FILTRATION OR HIGH PRESSURE LIQUID CHROMATOGRAPHY IN DEOXYCHOLATE[1]

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Received May 22,1980

SUMMARY: The non-denatured cytochrome $\underline{c}_1$ subunit of bovine ubiquinone-cytochrome $\underline{c}$ reductase was isolated using either gel filtration or high pressure liquid chromatography in 1% deoxycholate. The preparation was a single band on polyacrylamide gel electrophoresis in dodecyl sulfate, had a heme content of 31 nmol heme/mg protein, had an absorbance ratio A_{417}/A_{278} = 2.65, a visible spectrum with maxima at 553, 530, 523.5, 417, 317, and 277 nm for the reduced protein, and an amino acid analysis identical to that previously reported for the isolated denatured protein. The Stokes' radius of this non-denatured deoxycholate solubilized protein was 34Å, indicating that the protein either is a dimer in deoxycholate, is asymmetric, or binds large amounts of detergent.

Cytochrome $\underline{c}_1$ (cyt. $\underline{c}_1$)[3] has previously been isolated from bovine cytochrome $\underline{bc}_1$, i.e., ubiquinone-cytochrome $\underline{c}$ reductase as either: 1) a single denatured polypeptide in dodecyl sulfate (1,2); or 2) a non-denatured two polypeptide complex of cyt. $\underline{c}_1$ and subunit VI (2-4). From the studies that have been performed on cyt. $\underline{c}_1$ when it is part of cytochrome $\underline{bc}_1$ (2,5-7) or in the non-denatured two subunit complex (2,3,5,7), this subunit is thought to be partially buried in the cytochrome $\underline{bc}_1$ complex, probably having an amphiphilic type structure with both hydrophilic and hydrophobic areas on its surface. However, to date bovine cyt. $\underline{c}_1$ has not been isolated as a single polypeptide without first denaturing the protein, thus, direct studies of its structure have been impossible. As a first step in studying the possible hydrophilic-hydrophobic domain structure of cyt. $\underline{c}_1$, we have developed a

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[1]This investigation was supported by a research grant from the National Institutes of Health (GM 24795).

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[3]Abbreviation used: (cyt. $\underline{c}_1$) cytochrome $\underline{c}_1$.

0006-291X/80/130090-07$01.00/0

relatively simple procedure for isolating a monodisperse, non-denatured, cyt. $\underline{c}_1$ subunit that is not contaminated by the other subunits of the cytochrome $\underline{bc}_1$.

Experimental Procedure

Materials: Sodium deoxycholate, sodium cholate, acrylamide and bis-acrylamide were all recrystallized before use. Other chemicals were A.C.S. reagent grade.

Assay Procedures: Protein concentration was measured by the biuret method (8), or the Lowry method (9), the latter was used in determining heme content, using bovine serum albumin as a standard. Heme content was determined using $\varepsilon_{417} = 1.43 \times 10^5$ (3). Absorbance was measured using a Zeiss PM 6K spectrophotometer; spectra were obtained using a Cary Model 14 spectrophotometer. Amino acid analysis was performed according to Spackman et al., (10) using a Durrum 500 analyzer. Hydrolysis times of 22,45, and 67 hr. were used for determination of thr, ser, ile, and val; cys was determined as cysteic acid after performic acid oxidation. Polyacrylamide gel electrophoresis was performed using a stacking gel system (15% acrylamide in the running gel) in 0.1% dodecyl sulfate (11) with 2 M urea in the running gel. Gels were stained and destained according to Fairbanks, et al., (12) and scanned at 590 nm using a Helena Laboratories Quick Scan R and D densitometer.

Isolation of Cytochrome $\underline{bc}_1$ (Complex III): Cytochrome $\underline{bc}_1$ was isolated from beef heart mitochondria by a modification of the method of Reiske (13). The initial red pellet (S_1) was resuspended in 0.066 M sucrose, 0.05 M Tris-Cl, 1 mM histidine pH 8.0 to 10 mg/ml and solubilized by 1 mg deoxycholate/mg protein, rather than 0.5 mg/mg protein. Reiske's procedure was then followed to the stage where "crude enzyme" is obtained, i.e., prior to ammoniun sulfate precipitation from a cholate solution. This preparation contained the eight polypeptides normally isolated with complex III (1) with a small amount of contamination from high molecular weight proteins (Figure 1A).

Isolation of Partially Purified Cytochrome $\underline{c}_1$: Cyt. $\underline{c}_1$ was extracted from cytochrome $\underline{bc}_1$ by the method of Trumpower and Katki (2) with the following modifications: 1) complex III was reduced by dithionite at pH 8.0 in 0.05 M Tris-Cl, 0.066 M sucrose, 1 mM histidine; 2) the reduced sample was made 1.2% cholate and 1.5 M guanidinium chloride to dissociate cyt. $\underline{c}_1$ from the complex 3) after dilution with 0.5 volumes of buffer, the solution was centrifuged 5 min. at 12,800 x g; 4) the supernatant was dialyzed overnight rather than 4 hrs. at 5°. The partially purified cyt. $\underline{c}_1$ obtained after precipitation of the 42% ammonium sulfate supernatant was dissolved in a small amount of 0.02 M Tris-Cl, 0.09 M NaCl, 0.01 mM EDTA pH 8.1 buffer and dialyzed 3 hrs. against this same buffer. This preparation had an absorbance ratio, A_{417}/A_{278}, of 1.3-1.5, a heme content of 20-22 nmol heme/mg protein and contained predominantly subunit IV with smaller amounts of subunits VI and VIII. (Figure 1B).

Results and Discussion

Isolation of the non-denatured cyt. $\underline{c}_1$ subunit was accomplished by two alterations of a method previously used to prepare the two subunit complex of cyt. $\underline{c}_1$ and subunit VI (2): 1) increasing the amount of cholate added to the

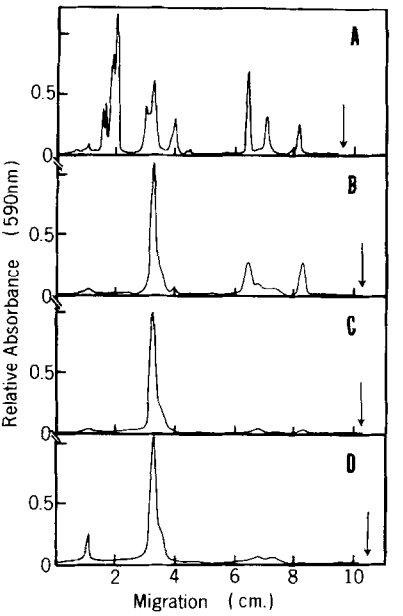

Figure 1. Densitometric tracings of dodecyl sulfate polyacrylamide slab gels. The arrow in each panel indicates the position of the tracking dye. A. 13 μg of cytochrome $\underline{bc}_1$. B. 3 μg of partially purified cytochrome $\underline{c}_1$. C. 3 μg of purified cytochrome $\underline{c}_1$ obtained after gel filtration on Sephadex G-200. D. 3 μg of purified cytochrome $\underline{c}_1$ obtained after HPLC. The slight shoulder present on the right side of the cytochrome $\underline{c}_1$ peaks in panels B, C, and D is caused by the anomalous behavior of the cytochrome $\underline{c}_1$ in dodecyl sulfate (2). The amount of cytochrome $\underline{c}_1$ that migrated at this faster position was a function of the amount of dodecyl sulfate used to denature the protein; the scans are of protein that was denatured in 2.5% dodecyl sulfate; with 1% dodecyl sulfate, 30-40% of the protein migrated at this position.

cytochrome $\underline{bc}_1$ complex from 0.7% to 1.2% during the extraction of cyt. $\underline{c}_1$ by cholate and 1.5 M guanidine; and 2) separation of the low molecular weight proteins from cyt. $\underline{c}_1$ by molecular sieve chromatography in a large excess of deoxycholate. The first alteration resulted in a more complete extraction of cyt. $\underline{c}_1$, but did result in some extraction of subunits VI and VIII (Figure 1B). The advantage of this preparation over the partially purified preparations of Trumpower and Katki was that the contaminating proteins were not tightly associated with cyt. $\underline{c}_1$ and they could be removed by molecular sieve chromatography.

Separation of the low molecular weight contaminants without denaturation of cyt. $\underline{c}_1$ was achieved by dissolving the partially purified preparation in 5% deoxycholate at pH 8.1 followed by high pressure liquid chromatography

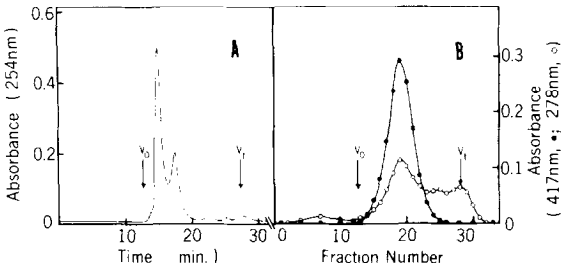

Figure 2. Purification of cytochrome $\underline{c}_1$ by molecular sieve chromatography in 0.02 M Tris-Cl pH 8.1 buffer containing 0.09 M NaCl, 0.1 mM EDTA and 1% deoxycholate. The arrows at V_o and V_t mark the excluded and total volumes of each column. Two high performance liquid chromatography columns were used in series: a 0.4 x 30 cm I-300 column followed by a 0.78 x 30 cm. I-125 column, both obtained from Waters Associates, Inc. A 0.2 mL sample was applied in 5% deoxycholate after equilibration of the columns at 23° with pH 8.1 buffer containing 1% deoxycholate. The operating pressure was 1000 psi at the flow rate of 0.5 mL/min. Elution of protein was detected using a 254 column monitor. 0.5 mL fractions were also collected; the peak fraction eluting at 14.6 min. was cytochrome $\underline{c}_1$, the peak eluting at 17.2 min. was the low molecular contaminants. B. A Sephadex G-200 column (0.7 x 44 cm) was equilibrated with pH 8.1 buffer containing 1% deoxycholate at 5°. A 0.2 mL sample containing 5% deoxycholate was applied and 0.67 ml fractions were collected, 3 fractions/hr. Fractions 18-20 contained cytochrome $\underline{c}_1$, having a A_{417}/A_{278} = 2.60 and were pooled; fractions 24-30 were predominantly the low molecular weight contaminants.

(HPLC) using molecular sieve columns, or by gel filtration using a Sephadex G-200 column, each equilibrated with 1% deoxycholate. Either chromatography method gave approximately the same result (see Figure 2). The high concentration of detergent in the sample and columns was found to be necessary to disperse the aggregated cyt. $\underline{c}_1$. (With lower detergent concentrations, the protein eluted at earlier positions, indicating aggregation). The HPLC method was extremely rapid, taking only 20 min. rather than 10 hours for the Sephadex G-200 column. The only disadvantage of the HPLC method was the nearness of the cyt. $\underline{c}_1$ peak (14.6 min.) to the excluded volume of this particular column (12.4 min.), preventing the complete removal of the high molecular weight contaminants. However, the rapidity of the method was extremely useful, since it allowed the preparation of highly purified cyt. $\underline{c}_1$ from cytochrome $\underline{bc}_1$ within 24 hrs., or from partially purified cyt. $\underline{c}_1$ within 15 min. Although slower, the Sephadex G-200 column yielded slightly more purified protein, having an absorbance ratio A_{417}/A_{278} = 2.60-2.65 rather than 2.50-2.55 with the HPLC preparation. The only problem encountered in

isolating cyt. $\underline{c}_1$ by either procedure was the instability of the protein in 1% deoxycholate at room temperature; the absorbance was found to decrease 1-2%/hr. under these conditions, which caused some difficulty in obtaining good spectra and accurate analysis of the heme content. However, immediate determination of the heme content of the protein eluted from the Sephadex G-200 column gave 31 nmol heme/mg protein.

The cyt. $\underline{c}_1$ subunit prepared by either chromatography method has the purity of the denatured protein previously isolated in dodecyl sulfate (1,2) yet retains the native heme environment that has previously been found only with the two subunit complex of cyt. $\underline{c}_1$ and subunit VI (3). The basis for these conclusions are: 1) The deoxycholate solubilized cyt. $\underline{c}_1$ is at least 95% pure as judged by polyacrylamide gel electrophoresis in dodecyl sulfate (Figure 1C and 1D); 2) the amino acid composition of either preparation is nearly identical with the reported analyses of cyt. $\underline{c}_1$ isolated after denaturation in dodecyl sulfate (Table I); 3) the visible spectrum of reduced cyt. $\underline{c}_1$ is virtually identical to the spectrum obtained by Yu *et al.* (3) for their two subunit complex (Figure 3); and 4) the heme content is very close to the theoretical value of 32 nmol heme/mg protein based upon the molecular weight of 31,000 for cyt. $\underline{c}_1$.

From the elution position from either the HPLC or Sephadex column in 1% deoxycholate, we estimate that the deoxycholate solubilized cyt. $\underline{c}_1$ has a Stokes' radius of 34Å $\pm$ 10%. This would correspond to a molecular weight of 65,000 for a globular, water soluble protein, therefore, the purified cyt. $\underline{c}_1$ is either: 1) a dimer with very little bound detergent (this is unlikely since the protein elutes in the excluded volume of either column in decreased detergent concentrations); 2) a monomer with very large amounts of bound deoxycholate, i.e., 1 g deoxycholate/g protein (this is unlikely for a protein of this size); or 3) an asymmetric protein with some bound detergent indicating the possibility of a hydrophilic cytochrome $\underline{c}$ binding domain and a hydrophobic detergent/membrane binding domain. Investigation of the possible asymmetry,

Table I. Amino Acid Composition of the Cytochrome c_1 Polypeptide

Amino Acid	Non-Denatured Protein Isolated in Deoxycholate		Denatured Protein Isolated in Dodecyl Sulfate	
	G-200[a]	HPLC[b]	Trumpower & Katki ((1975)[c]	Bell & Capaldi (1976)[d]
Asx	22.0	23.6	23.2	23.3
Thr	9.9	10.1	9.2	10.2
Ser	16.8	15.9	15.8	17.3
Glx	32.6	31.7	27.9	28.3
Pro	20.0	19.3	24.8	24.9
Gly	17.7	20.2	19.4	21.7
Ala	18.4	19.6	21.9	22.8
Val	17.7	16.9	16.6	17.0
Met	7.7	6.0	8.5	7.3
Ile[e]	4.8	5.2	5.0	7.9
Leu[e]	28.3	28.3	28.3	28.3
Tyr	13.3	10.6	15.0	7.1
Phe	10.4	9.0	9.9	9.7
His	10.2	9.7	9.6	6.8
Lys	13.9	14.0	12.9	14.4
Arg	18.3	17.7	16.7	14.9
Cys[f]	4.3	6.0	4.3	——

[a]The sample was obtained by pooling the peak fractions from a G-200 column in 1% deoxycholate (Figure 2B).

[b]The sample was obtained by pooling the peak 0.5 mL from the HPLC column in 1% deoxycholate (Figure 2A).

[c]A hydrolysis time course was performed.

[d]Sample was hydrolyzed 24 hours, no time course performed.

[e]All analysis normalized to 28.3 leucine.

[f]Determined as cysteic acid.

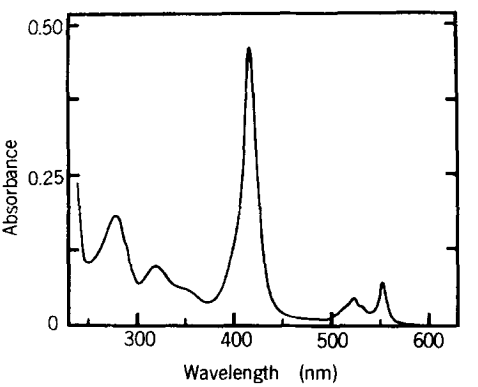

Figure 3. Ultraviolet-visible spectrum of reduced cytochrome c_1 obtained after gel filtration on Sephadex G-200 in 1% deoxycholate. Maxima were observed at 553, 530, 523.5, 417, 317, and 277 nm. A similar spectrum was also obtained for cytochrome c_1 prepared by HPLC.

dimerization, detergent binding and overall structure of this purified cyt. c_1 subunits is currently under investigation.

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