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THE INDISPENSIBILITY OF A MITOCHONDRIAL 15K PROTEIN FOR THE FORMATION OF THE CYTOCHROME c_1-CYTOCHROME c COMPLEX

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Summary

The cytochrome c_1-cytochrome c complex is formed when c reacts with c_1 prepared by either of two methods reported (King, T.E. (1978) Methods of Enzymol. 53, 181). By the sodium dodecyl sulfate-polyacrylamide gel electrophoresis technique, the c_1 thus prepared shows a heme-containing subunit of about 29,000 together with a smaller unit of 15,000. Recent modification (König *et al.* (1980) Biochim. Biophys. Acta *621*, 283) of our mercaptoethanol method yields "one band c_1" but this preparation does not react with c to form the c_1-c complex. Addition of a protein fraction of 15,000 (15K) isolated from succinate-cytochrome c reductase produces the complex. The 15K protein is a function of the formation of the complex judging from the results of titration with the c and "one band c_1" system.

Introduction: The discovery of the cytochrome c_1-cytochrome c complex (1,2) quite naturally has led to the conclusion that the complex may be an intermediate in the reaction of these two cytochromes, and the reductase domain of c is at the binding site of c_1 of the b-c_1 complex (*e.g.* 3-6 and references cited therein). The original c_1-c complex is prepared from cytochrome c and the cytochrome c_1 preparations (7), the latter possessing two bands on polyacrylamide gel electrophoresis (PAGE) in sodium dodecyl sulfate (SDS) (abbreviated as "two band c_1"). These two bands show molecular weights of a heme-containing subunit of approximately 29,000 (or 27,874 by complete amino acid sequence determination (8)) and a subunit without heme of 15,000 (15K). Recently the preparation of cytochrome c_1 with only the heme-containing band (abbreviated as "one band c_1") has been reported* (9) by modifications of one of our original methods (7).

*That the ratio of the smaller heme-not-containing subunit to the heme-containing subunit was not constant was first observed 3 or 4 years ago by Dr. F. C. Yong then in this laboratory and now at the New Mexico University (cf. 8). However, no enzymically active cytochrome c_1 completely in one band form was ever obtained.

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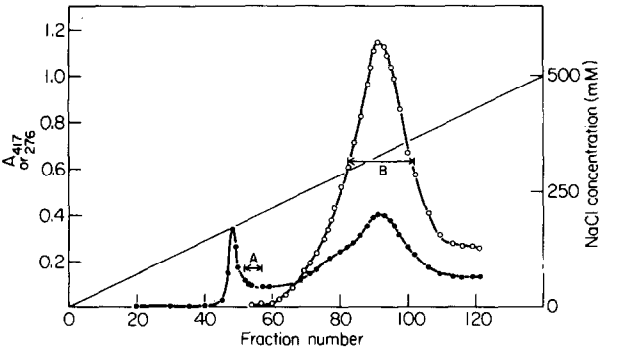

Fig. 1. Gradient elution pattern from a DEAE cellulose column of the solubilized cytochrome c_1 from succinate-cytochrome c reductase. The column (2.8 x 40 cm) was equilibrated with 50 mM potassium phosphate buffer, pH 7.4, containing 1% cholate and 0.1% β-mercaptoethanol and washed with 10 to 12 bed volumes of the same buffer. Elution was made with a linear gradient from 0.0 to 0.5 M NaCl in phosphate-cholate-β-mercaptoethanol buffer. The flow rate was 12 ml/hr and each fraction was collected for 24 min. The filled circles represent absorbance at 276 nm; open circles indicate absorbance at 417 nm. Thus the ratio of A_{417}/A_{276} may be calculated in all fractions. The absorbance was recorded in a cell with 0.2 cm light path. A is the pooled fractions used in experiment as a 15K protein after purification (see the text), and B is the pooled fractions used for subsequent Ultrogel AcA 44 column chromatography.

We have found the cytochrome c_1-c complex is not formed by a reaction of "one band c_1" and c. Upon addition of a fraction of protein with molecular weight of 15,000 (15K), the complex is then produced.

Experimental: "Two band c_1" was prepared by two methods previously reported (7) as well as obtained as described below. König *et al.* (9) have reported that "one band c_1" is prepared from a modification of our β-mercaptoethanol method (7) by addition of 0.5% deoxycholate to the solubilizing mixture.

After repeated failure to reproduce the method of König *et al.* (9), we modified the procedure and obtained the gradient elution pattern from a DEAE cellulose column as shown in Fig. 1. The fractions pooled from No. 82 to 102 (labeled as B in Fig. 1) were further purified by chromatography on an Ultrogel AcA 44 column. The elution pattern is depicted in Fig. 2. The yield of "one band c_1" (combined fractions labeled C of Fig. 2) was low, less than 10%. Pooled fractions A and B (cf. Fig. 2) showed 2 bands of about 29,000 and 15,000, respectively, on SDS-PAGE columns. Fraction A contained more 15,000 subunit than Fraction B, although the ratio was still not exactly 1:1. This result was in agreement with our unpublished observation.* The Keilin-Hartree preparation of beef heart made by *grinding* rather than by Waring blendering (10) was used. The succinate-cytochrome c reductase was prepared by the heart muscle preparation as previously reported (11) with modifications. The c_1 content of the reductase used was 2.2 nmol/mg or higher.

A 15K protein fraction was collected as shown by A in Fig. 1 and the pooled fraction was passed through a Sephadex G-25 column. At more than 30 μg protein, this fraction showed a single band at 15,000 on an 0.6 x 8.0 cm SDS-PAGE column. The fraction labelled C in Fig. 2 as "one band c_1" was used in

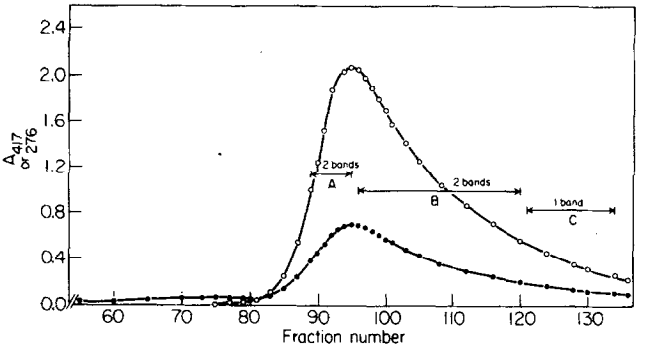

Fig. 2. Gel permeation chromatogram on Ultrogel AcA 44 column. The column (2.8 x 95 cm) was equilibrated with 50 mM potassium phosphate buffer, pH 7.4, containing 1% cholate and 0.1% β-mercaptoethanol. The flow rate was 4.4 ml/hr and each fraction was collected for 24 min. The filled circles represent absorbance at 276 nm: the open circles indicate absorbance at 417 nm. All absorbance was recorded in a cell with a 0.2 cm light path. Pooled fractions of A and B contain different ratios of heme to 15K protein as shown by SDS-PAGE. Fraction C showed only one heme-containing band at 31 nmol/mg protein on SDS-PAGE columns.

the experiment. Similar, almost identical, results were obtained from other batches of preparations using different samples of succinate-cytochrome c reductase. However, the 15K protein did not always remain in the same position at A as shown in Fig. 1; therefore the SDS-PAGE test must be performed each time for practically every fraction.

SDS-PAGE was conducted as reported (12). Circular dichroic (CD) spectra were obtained in a Cary Spectropolarimeter 6000-6001 as previously described (2).

Results and Discussion: One characteristic of the cytochrome c_1-c complex prepared from c and "two band c_1" is the dramatic increase of the induced Cotton effect in the Soret region with more than 3 times molar ellipticity as reported previously (2). The result was confirmed once again in present experiments using the cytochrome c_1 samples prepared according to original methods (7). Either method described (7) gave remarkable increase of the Cotton effect with a maximum of 416-417 nm. However, no such increase of the Cotton effect was observed when "one band c_1" was used. On the other hand, as summarized in Fig. 3, when "two band c_1" (Fraction A of Fig. 2) prepared from the same batch of "one band c_1" (Fraction C) was used, a great increase of the Soret Cotton effect was noticed. It was practically the same as the "two band c_1" prepared by the original methods (7).

The lack of the increase of the Cotton effect was due to the fact that

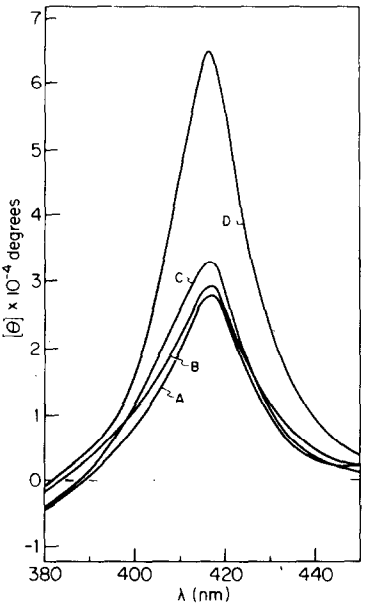

Fig. 3. Circular dichroic spectra of the cytochrome c_1-cytochrome c complex, and unmixed cytochrome c_1 plus cytochrome c. The systems were in 10 mM phosphate buffer, pH 7.4. Cytochrome concentrations in the complex and in the free forms (*i.e.*, unmixed in two separate cuvettes and placed in the sample compartment of spectropolarimeter in series) were 9 μM (Curves A and C) or 7 μM (Curves B and D) each for c_1 and c. Curve A is spectrum of unmixed c and "one band c_1"; B is spectrum of unmixed c and "two band c_1"; C is spectrum of mixed c and "one band c_1": and D is spectrum of mixed c and "two band c_1." Notice the formation of the c_1-c complex in Curve D and the dramatic increase of the Cotton effect at 416-417 nm compared to Curves B and C.

"one band c_1" did not form the cytochrome c_1-cytochrome c complex. Figure 4 summarizes these results. Two distinct peaks are seen (curve A of Fig. 4, namely cytochromes c_1 and c) when a reaction mixture of equal molar amount of c and "one band c_1" was subjected to gel permeation chromatography with Sephacryl S-200, in contrast to "two band c_1" which gave only one peak (curve B). However, when an excess of 15K protein fraction was added to the system with "one band c_1," a large increase in the peak corresponding to the c_1-c complex was observed (curve C). The fact that a small peak of cytochrome c still remained indicates the 15K protein may not be pure; even in excess it does not appear to be in a molar equivalent to c_1 or c in the system. That the first peak was the cytochrome c_1-c complex was confirmed by the results showing two peaks on a gel permeation chromatograph after decomposition of

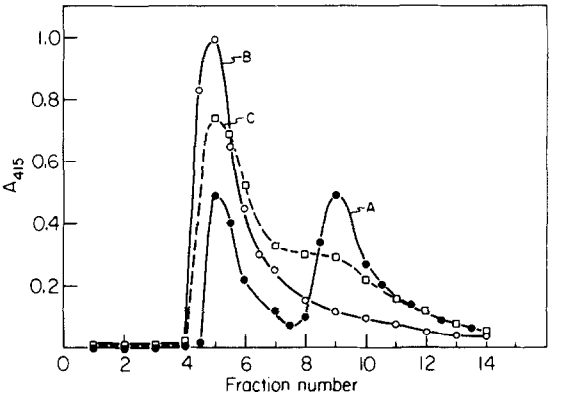

Fig. 4. Elution pattern of the cytochrome c_1-cytochrome c complex formation. Curve A represents a mixture of equimolar concentration of "one band c_1" and c on Sephacryl S-200 column (1.0 x 12 cm). Curve B is the same as A but "two band c_1" from the same preparation in which "one band c_1" was isolated. Curve C indicates addition of 15K at 4 times the concentration of cytochrome c_1 to system A. The systems originally contained 17 nmol each of c_1 and c in 0.4 ml of 10 mM potassium phosphate buffer, pH 7.4. The elution was made with 10 mM potassium phosphate buffer at 4°C. The flow rate was 0.2 ml/min and each fraction was collected for 5 min.

the complex in media of high ionic strength (>0.15 M KCl).

Another line of evidence strongly attesting to the indispensibility of the 15K protein for formation of the c_1-c complex is illustrated in Fig. 5. The degree of complex formation was a function of the amount of the 15K protein fraction added as reflected from the increase of molar ellipticity of the Soret Cotton effect. The 15K protein itself was optically inactive and did not affect CD spectra measured for either c_1 or c. Although the titration did not reach a plateau, the molar amount required was more than the equivalent of c_1 or c content again indicating that the 15K protein was not pure. That the 15K band consisted of multiple subunits or proteins was already concluded previously (13): only one protein or subunit with this molecular weight, however, was needed in the formation of the c_1-c complex.

The "heme-containing subunit" isolated from "two band c_1" (8) is already denatured and is carbon monoxide reactive. It is not unexpected that such subunit would not be able to form the c_1-c complex as experimentally observed (unpublished results from this laboratory). On the other hand, the "one band

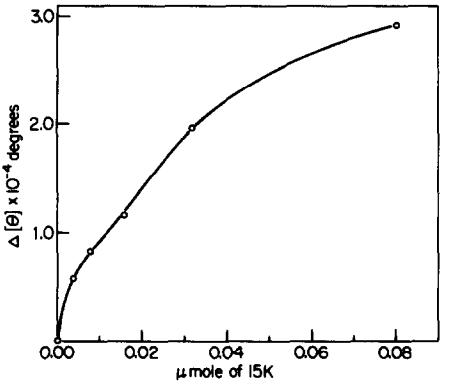

Fig. 5. The increase of the molar ellipticity of the cytochrome c_1-cytochrome c complex as a function of the amount of mitochondrial 15K protein. The system contained 3.7 μM each of "one band c_1" and c in 5.2 ml. 15K fraction was the pooled fraction A of Fig. 1 after further purification; the molarity was simply calculated based on 15K as the molecular weight. The molar ellipticity was calculated on the basis of heme at λ = 416.

c_1" was found to be enzymically active and completely inactive to carbon monoxide. The formation of the c_1-c complex is very sensitive to conformational change of the cytochromes, for example, the oxidation of only two methionine sulfur to sulfoxide in cytochrome c completely abolishes the complex formation (2). Nevertheless, we might mention that the 15K protein is not likely to be sandwiched between cytochromes c_1 and c. Indeed, most probably 15K links on the substrate side (cf. 14) of "one band c_1" and induces c_1 conformation in such a way that the c_1-c complex can be formed.

An intriguing question is whether this 15K protein possesses an electron transfer property. The evidence thus far indicates that it does not possess a prosthetic group. However, these results do not definitively rule out its electron transport activity. Indeed our current working hypothesis is that this 15K protein may be the second ubiquinone-bound protein acting in the b-c_1 region or so-called QP-C. Ubiquinone can be dissociated under these conditions of solubilization and purification of cytochrome c_1. The QP-C which we have reported (cf. 15) possesses a much higher molecular weight. The formation of a stable Q radical of more than 50% of the Q content from QP-C (16,17) in EPR spectrometry with g = 2.0046 is demonstrated under conditions

(*i.e.*, without a membrane) that do not show "sideness." The 15K protein might be the second QP-C from which Q has dissociated. Thus, the second QP-C is either Q_o or Q_i in the protonmotive Q cycle (18,19 and also see 14) denoting the particular ubiquinone acting on the protoplasmic (o) or matrix (i) sides of the mitochondrial inner membrane respectively. Significant results have accumulated (*e.g.* 14 and references cited therein) to support the general correctness of the Q cycle, but one QP-C is very unlikely to serve both as Q_o and Q_i in the Q cycle, which would require rapid relocation in the phospholipid bilayer over a distance approaching 80 Å.

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