Preparation and Properties of Cardiac Cytochrome c_1†

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ABSTRACT: A method for the large-scale isolation of beef heart mitochondrial cytochrome c_1 in high purity was developed. This method gave higher yield of "one-band" cytochrome c_1 than previously reported [Kim, C. H., & King, T. E. (1981) *Biochem. Biophys. Res. Commun. 102*, 607–614]. In addition, the present method was effective in the preparation of "two-band" cytochrome c_1 which was used to prepare the hinge protein according to the principle of sequential resolution [Kim, C. H., & King, T. E. (1983) *J. Biol. Chem. 258*, 13543–13551]. The isolation of one-band and two-band cytochrome c_1 by this procedure could be completed within 3 or 4 days starting with succinate–cytochrome *c* reductase. One-band cytochrome c_1 showed a molecular weight of 44 000 by sedimentation equilibrium and 29 000 by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The disparities in these data from the actual value of 27 924 by amino acid sequence analysis, as previously reported [Wakabayashi, S., Matsubara, H., Kim, C. H., & King, T. E. (1982) *J. Biol. Chem. 257*, 9335–9344], are most probably due to the formation of detergent or detergent–phosphate complex. A comparison of some properties of one-band cytochrome c_1 with those of two-band cytochrome c_1 clearly showed significant differences between the two preparations. These results suggest the hypothesis that one of the possible roles of the hinge protein in the mitochondrial respiratory chain is to stabilize the conformation of cytochrome c_1.

Cytochrome c_1 was independently discovered by Keilin and Hartree in Cambridge (Keilin, 1966) and Yakushiji and Okunuki in Japan (Yakusiji & Okunuki, 1940). However, the preparation of pure and active cytochrome c_1 was not successful until King and co-workers reported a method in 1972 (King, 1978; Yu et al., 1972), and, more recently, van Gelder and co-workers reported another method in 1980 (König et al., 1980). However, the sample prepared even by the latter method was still heavily contaminated with the glutamic acid rich small protein known as the "hinge protein" [cf. Wakabayashi et al. (1982a) and Kim & King (1983)]. The evidence was the amino acid composition of this preparation. Our cytochrome c_1 preparation reported in 1972 was actually cytochrome c_1 with the hinge protein in a 1 to 1 molar ratio, which we called "two-band" c_1, and similarly, the cytochrome c_1 preparation free of the hinge protein was called the "one-band" c_1 (Kim & King, 1981, 1983).

In our preliminary report (Kim & King, 1981), the yield of the preparation of one-band cytochrome c_1 free of the hinge protein was very low (<10%). During the last few years, we have substantially improved the yield of cytochrome c_1 (ca. 25%). Moreover, we have found that the yield of one-band c_1 was closely related to that of two-band c_1.

This paper reports an improved method in a relatively large scale of cytochrome c_1 isolation. Many properties of one-band cytochrome c_1 were studied, and a comparison in the behavior between the pure one-band and two-band cytochrome c_1 is also presented.

Experimental Procedures

Materials. DEAE-cellulose (DE-52, Whatman), Ultrogel AcA 44 (LKB), Sephacryl S-200, Sephadex G-75 and G-25 (Pharmacia), and horse cytochrome *c* type III (Sigma) were purchased commercially. Sodium dodecyl sulfate (Fisher), cholic acid (Sigma), and deoxycholic acid (Sigma) were recrystallized in aqueous ethanol with the aid of active carbon. Acrylamide obtained from a commercial source was recrystallized in chloroform. Emasol 1130 was a gift from Kao Soap Co. (Tokyo). All other chemicals were commercially obtained in the highest grade available.

The Keilin–Hartree preparation (HMP)[1] of beef heart was obtained as previously reported (King, 1967). The succinate–cytochrome *c* reductase (Takemori & King, 1964) was prepared from the heart muscle preparation with the following modifications. The precipitate of crude succinate cytochrome *c* reductase was dissolved in 0.1 M phosphate buffer, pH 7.4, containing 0.5% cholate with a volume of one-sixth of the original HMP and then kept overnight (~16 h). The turbid red-brown solution was then centrifuged at 20 000 rpm for 1 h in a Beckman Model J-21B to remove the insoluble dark brown precipitate. The resulting clear red supernatant was brought to 33% ammonium sulfate saturation. Following 20-min incubation with stirring, the solution was centrifuged at 13 000 rpm for 30 min, and the supernatant was brought to 48% ammonium sulfate saturation. This was incubated and centrifuged as above, and the resulting precipitate was dissolved in 50 mM phosphate buffer, pH 7.4. The reductase thus obtained contained cytochrome c_1 of 2.0 nmol/mg of protein or higher.

All the procedures for the purification described were performed at 0–4 °C unless otherwise indicated.

Spectrophotometric Measurements. All measurements were performed at room temperature (about 23 °C) except otherwise stated. Absorption spectra were recorded by using a Cary spectrophotometer, Model 14 or 16. The concentration of cytochrome c_1 was determined spectrophotometrically, by

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[1] Abbreviations: HMP, Keilin–Hartree heart muscle preparation; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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using an extinction coefficient of 17.5 mM^{-1} for $A_{red}^{552.5} - A_{red}^{540}$ (Yu et al., 1972). Absorption spectra at low temperature (−180 °C) were recorded in a Cary Model 14 spectrophotometer equipped with a scattered transmission accessory, an RCA 06217 photomultiplier, and a Sylvania DWY quartzline lamp. The sample was placed in a cuvette of approximately 2-mm optical path and was cooled by liquid nitrogen. The temperature was directly recorded on a Leeds–Northrop Speedomax W strip-chart recorder with a calibrated copper–constantan thermocouple; one end of the thermocouple was inserted in the sample. The flow of nitrogen gas was constantly blown onto the surface of cuvette to avoid any ice buildup. The setting was checked with crystalline cytochrome *c* and gave the same result as reported (Estabrook, 1961). Circular dichroic spectra were recorded in a Cary spectropolarimeter 6000–6001 as previously described (Kim & King, 1981, 1983; Kaminsky et al., 1975) and expressed in degrees centimeter squared per decimole. Photoreduction was performed according to a previous report (Yu et al., 1975) using 9.8 μM ferricytochrome c_1 in 50 mM phosphate buffer with a 750-W projector lamp. The reduction was measured by scanning the sample in the spectrophotometer at predetermined time intervals.

Polyacrylamide gel electrophoresis in sodium dodecyl sulfate was performed according to the methods of Weber and Osborn (1969) and Swank and Munkres (1971). In the former method, 10% gel plus 0.3% cross-linker and in the latter 12.5% and 0.44% were used, respectively. Sedimentation equilibrium was conducted in a Beckman Model E analytical ultracentrifuge. The experiment was made at 20 °C with the scanner wavelength set at 417 nm. It was kindly performed by Dr. R. MacColl in the New York State Health Laboratory. Molecular weight was calculated as usual (Kim & King, 1983). Isoelectric focusing was carried out in a sucrose density gradient using 1% ampholine, pH range 3–6. The details of the method were described before (Kim & King, 1983), and redox potential measurements were conducted in a conventional manner using ascorbate as a reductant and Wurster's blue (Mustafa et al., 1968) as a mediator between cytochrome c_1 and the platinum electrode (Chiang, 1976); 7 μM one-band cytochrome c_1 in 5 mL of 50 mM phosphate buffer, pH 7.4, was titrated with 1 mM sodium ascorbate at 23 °C. Wurster's blue was present at 20 μM, and reduction was measured at 417–434 nm. Measurement was performed in a closed vessel under a continuous flow of argon.

Enzymatic Activity. Enzymatic oxidation and reduction of cytochrome c_1, i.e., as electron donor or acceptor, were estimated as previously reported (Yu et al., 1972); oxidation was conducted in 1 mL of reaction mixture of 16.6 μg of the heart muscle preparation, 0.75 μM cytochrome *c*, and the various concentrations of reduced one-band c_1 in 0.1 M phosphate buffer, pH 7.4. Reduction was conducted in 1 mL of reaction mixture of 20 mM succinate, 1.5 mM cyanide, 0.75 μM cytochrome *c*, 17 μg of the heart muscle preparation, and the various concentrations of oxidized one-band c_1. Reaction was initiated by addition of the heart muscle preparation or succinate in the case of reduction.

RESULTS

Purification

The method reported here was based on the method by König et al. (1980) with modifications. This method was improved since our previous brief report (Kim & King, 1981) so that pure one-band cytochrome c_1 completely free of the hinge protein could be prepared with a reasonable yield.

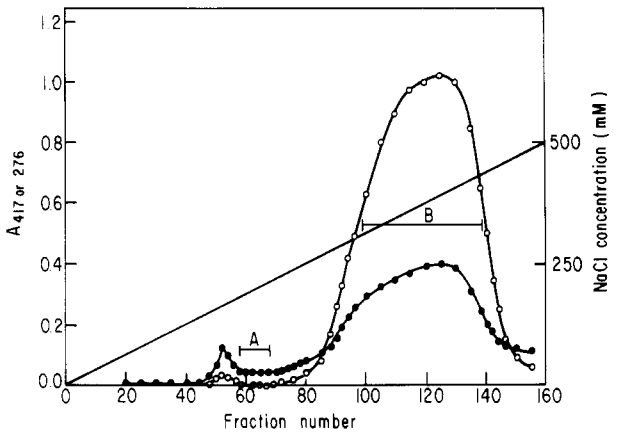

FIGURE 1: DEAE-cellulose column chromatography. The repeatedly washed c_1-adsorbed DEAE-cellulose (80 mL) was packed on a cushion of DEAE-cellulose column (2.6 × 10 cm) equilibrated with 50 mM potassium phosphate buffer containing 1% cholate and 0.1% β-mercaptoethanol. It was further washed with 2 bed volumes of the buffer. The linear gradient of 0.0–0.5 M NaCl in the same buffer was used to elute the cytochrome c_1. The total volume of gradient buffer was 3.2 times the bed volume. The flow rate was 12.6 mL/h with each fraction of 4.2 mL. The closed circles represent A_{276}; open circles indicate A_{417}. The absorbance was recorded in a cell with a 0.2-cm light path. "A" was mainly the hinge protein with the impurities, and "B" was used for subsequent Ultrogel AcA 44 column chromatography.

Succinate–cytochrome *c* reductase with a purity above 2.0 nmol of c_1/mg, 3.7 g, was adjusted to a protein concentration of 11–13 mg/mL in 50 mM potassium phosphate buffer, pH 7.4, containing 1.5% potassium cholate, 0.5% potassium deoxycholate, 8% of ammonium sulfate saturation, and 15% β-mercaptoethanol with the addition of appropriate stock solutions. This was incubated for 1 h with slow stirring at 4 °C. The extracted cytochrome c_1 from the reductase was separated from the cytochrome *b* by centrifugation at 20 000 rpm in a Beckman Model J-21B for 20 min. The supernatant was quickly passed through a Sephadex G-25 ("medium" size particle) column (5 × 60 cm) which had been equilibrated with 50 mM potassium phosphate buffer, pH 7.4, mainly for desalting. The slightly turbid cytochrome c_1 fraction at the void volume was centrifuged as before for 20 min. The clear supernatant was immediately adjusted to 1% potassium cholate and adsorbed, in a beaker, to 80 mL of DEAE-cellulose equilibrated with 50 mM potassium phosphate buffer, pH 7.4, containing 1% potassium cholate and 0.1% β-mercaptoethanol. The c_1-adsorbed DEAE-cellulose was washed on a 9-cm diameter sintered glass funnel, but not sucked to dryness, with the above buffer until the washing showed no absorbance at 280 nm. More than 2 L of buffer was usually required. The washed c_1-adsorbed DEAE-cellulose was packed on the top of a DEAE-cellulose column (2.6 × 10 cm) as a cushion equilibrated with the same buffer. The whole column (2.6 × 25 cm) was then washed again with 2 bed volumes of the buffer. The elution was performed with a linear gradient of 0.0–0.5 M NaCl in the 50 mM potassium phosphate buffer, pH 7.4, containing 1% potassium cholate and 0.1% β-mercaptoethanol.

Fractions containing cytochrome c_1 (labeled "B" in Figure 1) were pooled and concentrated on an Amicon ultrafiltration cell, using a PM 30 Diaflo ultrafiltration membrane, to a protein concentration of 14–16 mg/mL. The concentrated c_1 fraction was applied on an Ultrogel AcA 44 column of 2.6 × 44 cm, equilibrated with 50 mM potassium phosphate buffer, pH 7.4, containing 1% potassium cholate and 0.1% β-mercaptoethanol. The elution profile is shown in Figure 2, and

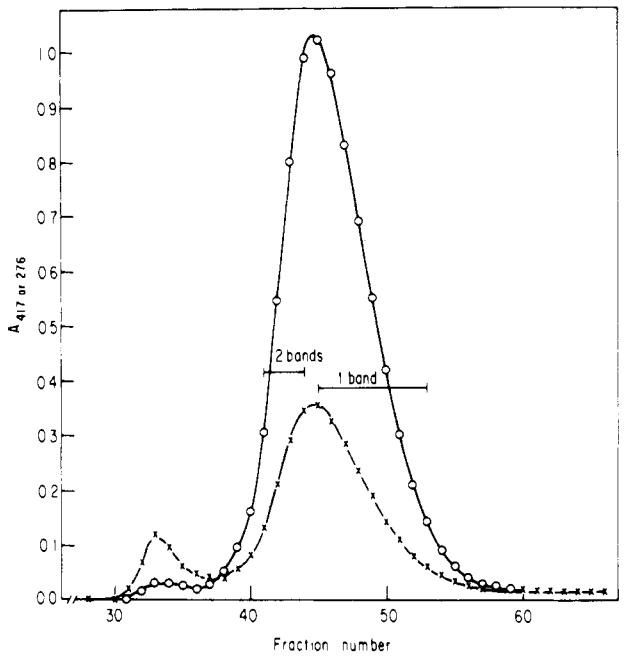

FIGURE 2: Ultrogel AcA 44 column chromatography. The cytochrome c_1 preparation obtained from DEAE-cellulose chromatography was concentrated to 16 mg/mL and then applied to an Ultrogel AcA 44 column (2.6 × 44 cm). The elution was performed with 50 mM potassium phosphate buffer, pH 7.4, containing 1% cholate and 0.1% β-mercaptoethanol. The flow rate was 6 mL/h with each fraction of 2 mL. (×) Absorbance at 276 nm; (○) absorbance at 417 nm. All absorbance was recorded in a cell with a 0.2-cm light path. The first peak was impure cytochrome c_1 with high molecular weight species as revealed by SDS–PAGE. The fractions labeled "2 bands" and "1 band" are purified cytochrome c_1 preparations.

Table I: Purity and Yield of One-Band Cytochrome c_1 from Succinate–Cytochrome c Reductase by the Improved Method

purification step	A_{417}/A_{276}	purity (nmol/mg)	cytochrome c_1 (μmol)	yield (%)
succinate–cytochrome c reductase		2.0–2.2	7.4	100
Sephadex G-25 column	1.1		4.8	65
DEAE-cellulose batchwise washing	2.5		3.4	45
Ultrogel AcA 44 chromatography				
two-band c_1	2.4–2.7	25–27	1.1	15
one-band c_1	2.9–3.0	31–32	1.9	25
total c_1				40

the purification steps are summarized in Table I. It was found that the yield of one-band cytochrome c_1 was increased by further washing of c_1-adsorbed DEAE-cellulose and the yield of two-band c_1 was decreased concomitantly. This fact suggested that the heme-containing polypeptide and the hinge protein of two-band c_1 were associated with each other around neutral pH and the latter could be removed by washing. A decrease in the washing step of c_1-adsorbed DEAE-cellulose increased the yield of two-band cytochrome c_1. In other words, the hinge protein which was tightly associated with the cytochrome c_1 was very slowly washed away from the c_1 fraction on a DEAE-cellulose column. However, sufficient washing on the sintered glass funnel was required in order to remove other impurities as much as possible and to obtain even pure two-band c_1. Nevertheless, the yield of one-band c_1, at a purity of 32 nmol/mg of protein, was usually not more than 25%, and that of two-band c_1, with a purity of 25–27 nmol of heme/mg of protein, could reach as high as 40%. In general, the total yield of these two species of c_1 was found always to be approximately 40%. It was observed that if the sample for Ultrogel AcA 44 chromatography were concentrated too high (>18 mg/mL), the yield of one-band c_1 decreased, probably because some aggregation took place.

It may be mentioned that there are two methods for the preparation of two-band c_1. The one reported in 1972 [Yu et al., 1972; also cf. King (1978)] is referred to as the original method. The other is the one described in this paper.

Properties

General Properties of Purified Cytochrome c_1. The purified one-band c_1 thus obtained was found completely free of the hinge protein. Two-band c_1 which showed a purity higher than 25 nmol of heme/mg of protein consisted of only two band on SDS-PAGE (Kim & King, 1983); one was M_r 29 000, and the other was M_r 11 000. The amino acid composition of one-band c_1 was in agreement with that from the sequence analysis (Wakabayashi et al., 1980, 1982b). Both two-band c_1 and one-band c_1 were found to be enzymically active. They did not react with carbon monoxide at all. However, one-band c_1 did not form the c_1–c complex with cytochrome c (Kim & King, 1981, 1983), while two-band c_1 did form the complex.

Molecular Weight. An analysis of SDS–PAGE revealed the molecular weights of one-band c_1 and the heme peptide of two-band c_1 to be 29 000. From the sedimentation equilibrium experiments, the molecular weight of one-band cytochrome c_1 was found to be 44 000 when the sample at 2 μM in 50 mM potassium phosphate buffer, pH 7.4, containing 1% cholate was run at 30 000 rpm for 20 h. The molecular weight obtained was higher than that obtained by the sequence analysis (Wakabayashi et al., 1980, 1982), which was apparently due to micelle and/or complex formation with detergent. Gel filtration chromatography on Sephacryl S-200 or Ultrogel AcA 44 of one-band and two-band c_1 in 50 mM phosphate buffer, pH 7.4, without detergent showed a molecular mass of ~240 000 for both one-band and two-band c_1, indicating one-band c_1 to be an octamer and two-band c_1 to be an hexamer in the absence of detergent. These highly polymerized cytochromes c_1, however, were easily depolymerized after dialysis in the presence of detergent. In contrast, the two-band c_1 prepared by the original method was not depolymerized by detergent [cf. King (1983)].

Isoelectric Point. Isoelectric focusing of two-band c_1 was performed in a sucrose density gradient using a pH gradient range of 3–6 and showed the pI value for the hinge protein to be 3.9 as reported (Kim & King, 1983) and the pI for c_1 (heme peptide) to be 5.8, although the pI value for c_1 by this method is approximate because of its precipitation at such low pH. Nevertheless, the observed value seemed to be close enough to the actual value, since even after 6 h at 500 V, the position of the red sharp band was not changed. It may be mentioned that the calculated value of pI should be higher since it contained 36 basic amino acids (12 Lys, 9 His, and 15 Arg) and only 29 acidic amino acids. The discrepancy is evidently due to the formation of c_1–detergent or c_1–phosphate–detergent complexes in which perhaps the basic residues are buried in the interior.

Absorption Spectra. Both preparations of two-band c_1 and one-band c_1 in the oxidized and reduced forms showed the same absorption spectra (cf. Figure 3A). One-band cytochrome c_1 in the oxidized form also showed a broad peak at 690 nm as that in two-band c_1, and this broad band was abolished after reduction.

At −180 °C, the reduced cytochrome c_1 exhibited a colorful spectrum as shown in Figure 3B–D, and essentially (see

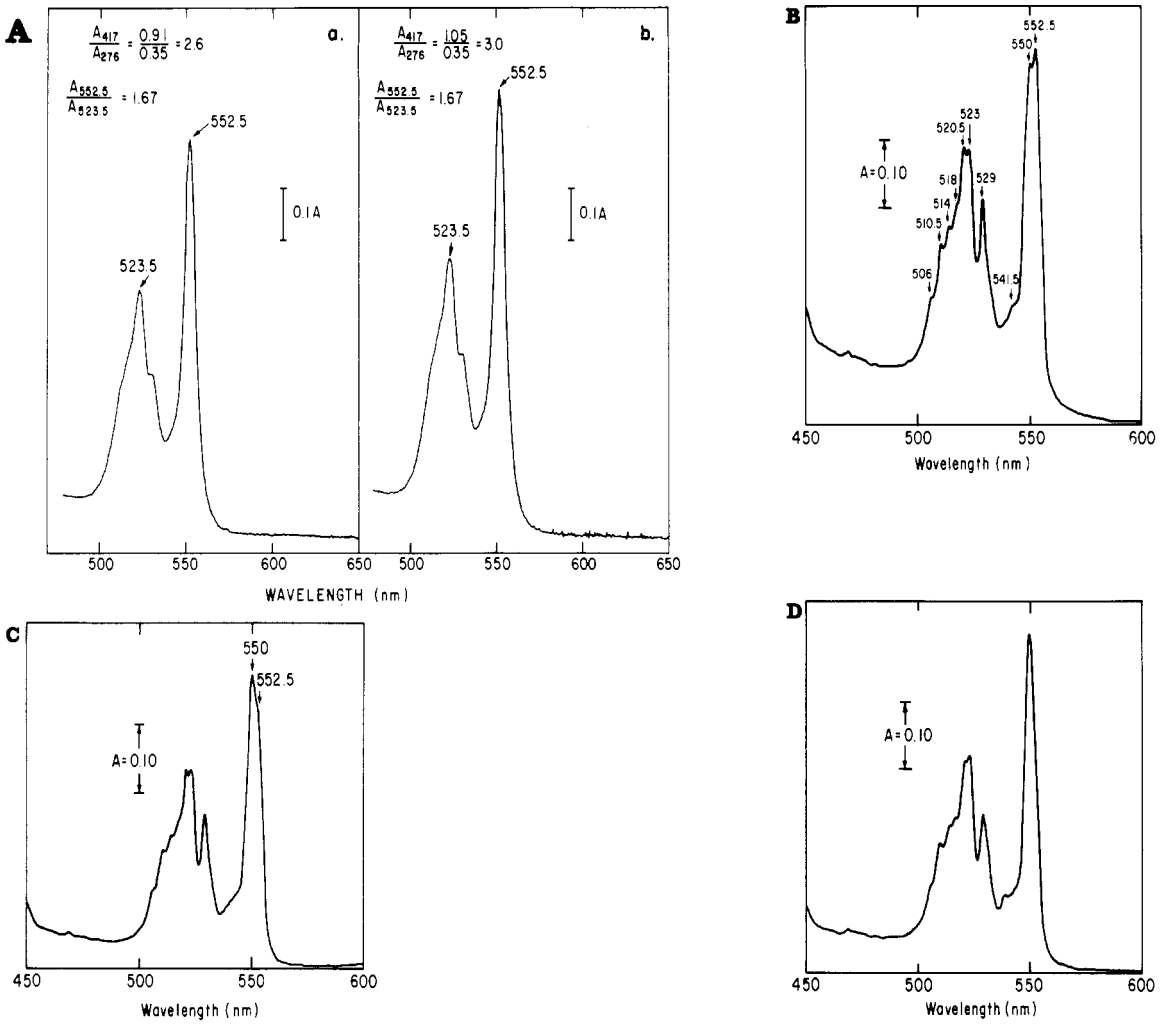

FIGURE 3: (A) Absorption spectra of one-band and two-band cytochrome c_1. (a) Two-band c_1 and (b) one-band c_1 in 50 mM potassium phosphate buffer, pH 7.4, containing 1% cholate and 0.1% β-mercaptoethanol. (B–D) Low-temperature spectra of one-band cytochrome c_1. (B) 61 μM in 50 mM phosphate buffer, pH 7.4; (C) 60 μM in 50 mM phosphate buffer, pH 7.4, containing 1% cholate and 0.1% β-mercaptoethanol; (D) 60 μM in 50 mM phosphate buffer, pH 7.4, containing 0.5% Tween 80. The sample was cooled in a gold-plated UV-transparent polyethylene cuvette in liquid nitrogen. The approximate cuvette temperature was −180 °C; the optical path was about 2 mm. Details of experiment were described under Experimental Procedures.

Discussion) no difference was observed between one-band c_1 and two-band c_1. The α-band, which showed only one band of 552.5 nm at room temperature, split into two absorption maxima at 552.5 and 550 nm with a distinct shoulder at 541.5 nm. The β-band, which showed three bands of absorption maxima at room temperature, displayed a spectrum showing maxima at 529, 523, 520.5, 518, 514, 510.5, and 506 nm.

However, the low-temperature splitting of both α and β maxima was dependent on the presence of detergent. When cytochrome c_1 was in the buffer medium containing 1% cholate and 0.1% β-mercaptoethanol, the α-band showed some change from that (Figure 3B) of c_1 in just buffer without detergent; i.e., the 552.5-nm peak almost diminished with a complete disappearance of the 541.5-nm band as presented in Figure 3C. When cholate and β-mercaptoethanol were removed from the cytochrome c_1 sample by dialysis, spectrum B (Figure 3) was recovered again. Figure 3D shows a spectrum obtained when cytochrome c_1 was in the buffer containing 0.5% Tween 80. The presence of 0.5% Tween 80 completely abolished the 552.5-nm peak from the α-band and the 518-nm peak from the β-band, and a new 538-nm peak in the α-band region and a 516-nm peak in the β-band region appeared. This detergent dependence on the low-temperature spectrum of cytochrome c_1 was observed in both one-band and two-band c_1 prepared by the present method. The low-temperature spectrum from

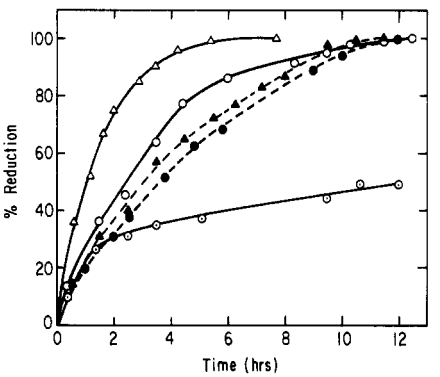

FIGURE 4: Photoreduction of cytochrome c_1. Cytochromes were photoreduced under anaerobic conditions as described under Experimental Procedures. The concentration of cytochrome c_1 is 9.8 μM, and that of cytochrome c is 10 μM. (△) One-band c_1 and (○) two-band c_1 in 50 mM phosphate buffer, pH 7.4; (▲) one-band c_1 and (●) two-band c_1 in 50 mM phosphate buffer, pH 7.4, containing 1% Emasol 1130; (⊙) cytochrome c in 50 mM phosphate buffer, pH 7.4.

two-band cytochrome c_1 prepared by the original method was not affected by detergent (King, 1983).

Photoreduction. Photoreductions of one-band c_1 and two-band c_1 were performed at pH 7.4 as shown in Figure 4. The

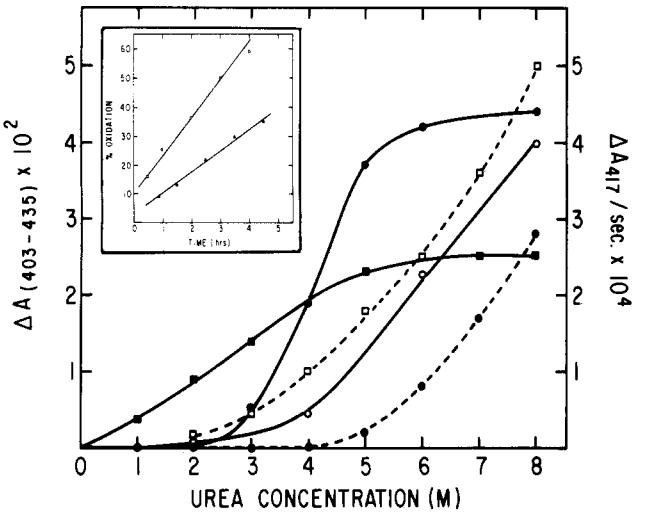

FIGURE 5: Effect of urea concentration on the Soret absorption and autoxidation of cytochromes c_1 and c. (■) 1.2 μM one-band ferricytochrome c_1, (●) 1.2 μM two-band ferricytochrome c_1, and (○) 1.3 μM ferricytochrome c for the Soret absorbance on the left ordinate; (□---□) 2.0 μM one-band ferrocytochrome c_1 and (●---●) 2.1 μM two-band ferrocytochrome c_1 on the right ordinate. All systems were in 0.1 M phosphate buffer, pH 7.4, 23 °C. Inset: Comparison of autoxidation rate of one-band c_1 and two-band c_1. (○) 6.3 μM one-band c_1; (△) 6.4 μM two-band c_1. All systems are in 50 mM phosphate buffer, pH 7.4, 23 °C.

rate of photoreduction in the absence of detergent (Emasol 1130) was much greater in one-band c_1 than in two-band c_1. We observed complete reduction of one-band c_1 at 6 h while two-band c_1 required 10 h. However, in the presence of Emasol 1130, the rate of photoreduction was decreased, showing complete reduction of both one-band and two-band c_1 at approximately 11 h, with no distinctive differences between them. It was obvious that the effect of Emasol 1130 on two-band c_1 was much smaller than that on one-band c_1. As a control, photoreduction of cytochrome c was conducted, and the results (cf. Figure 4) showed maximal reduction of only about 50% in 12 h. These results were in general agreement with those reported for two-band c_1 prepared by the original method (Yu et al., 1975).

Effect of Urea Concentration on Cytochrome c_1. As depicted in Figure 5, the denaturation of the oxidized cytochromes c_1 was monitored at the various urea concentrations shown. The rate of autoxidation of ferrocytochromes c_1 was also measured at the various urea concentrations. In the oxidized form of two-band c_1, an absorbance change of $\Delta A_{403-435}$ occurred at 3 M urea, and this change leveled off at 6 M urea. However, the oxidized form of one-band c_1 was more unstable than that of two-band c_1, showing a gradual change even at the lower urea concentration, but the extent of change was smaller than that of two-band c_1 at higher urea concentrations. In the case of ferrocytochromes c_1, the effect of urea occurred at 5 M urea in two-band c_1 and at 2 M urea in one-band c_1, where one-band c_1 was again more sensitive than two-band c_1.

The stability of one-band and two-band cytochrome c_1 was further examined by observing the rate of oxidation of the reduced forms of one-band and two-band c_1 at room temperature. The inset of Figure 5 shows the extent of oxidation of reduced one-band and two-band c_1 at the given time. Autoxidation of one-band c_1 was about 2 times faster than that of two-band c_1. These differences between one-band and two-band c_1 in urea treatment and autoxidation rate suggested that the conformations of these species might not be the same. The CD results substantiated this hypothesis. Circular dichroic

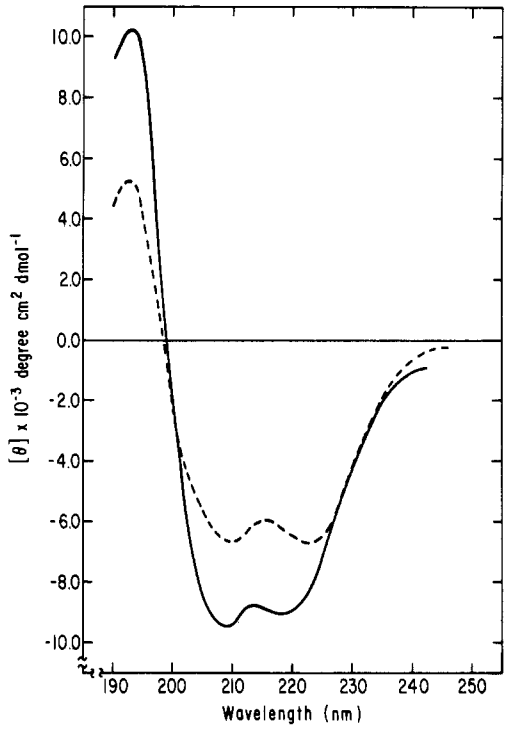

FIGURE 6: Circular dichroic spectra of the cytochrome c_1 in the UV region at pH 7.4 in 50 mM phosphate buffer containing 1% Emasol 1130: (---) one-band cytochrome c_1; (—) "two-band" cytochrome c_1.

spectra in the UV region (Figure 6) showed a significant difference between these two cytochromes c_1 [cf. King et al. (1983)], although those in the Soret region exhibited the same behavior.

Interaction with Cyanide. Investigation of a possible reaction of cyanide with cytochrome c_1 at pH 7.4 was conducted, and it was found that there was no reaction either with one-band or with two-band c_1. However, after cytochrome c_1 was denatured, for example, by sodium dodecyl sulfate, a reaction of c_1 with cyanide occurred. The denaturation was effected with as low as 0.03% sodium dodecyl sulfate. However, when cytochrome c_1 was denatured with 0.3% or higher sodium dodecyl sulfate, even dithionite could not reduce this cytochrome c_1. Figure 7 shows spectra of the 0.1% SDS-denatured one-band cytochrome c_1 in oxidized and dithionite-reduced forms, and its spectra after cyanide reaction are also depicted. Red shifts, ranging from 3 to 5 nm, in the α, β, and Soret regions were observed in the ferrocytochrome c_1–cyanide complex. These results are the same as those of the previous report of two-band c_1 prepared by the original method (Kaminsky et al., 1975). It should be noted that the present preparations of one-band and two-band cytochrome c_1 are more sensitive to SDS than two-band c_1 prepared by the original method.

Redox Potential of One-Band Cytochrome c_1. The midpoint potential (E_m at 7.4) for one-band c_1 was found to be +227 mV and was close to the value reported for two-band c_1 prepared by the original method (Chiang, 1976) and that of c_1 in submitochondrial particles (Dutton et al., 1970).

Enzymatic Oxidation and Reduction of Cytochrome c_1. Both the enzymatic oxidation and reduction of one-band cytochrome c_1 were determined with the heart muscle preparation as the electron carrier. Cytochrome c_1 in the reduced form was used as an electron donor and oxygen as an acceptor in oxidation, and in reduction, cytochrome c_1 in the oxidized form was used as an acceptor and succinate as a donor in the presence of a terminal inhibitor.

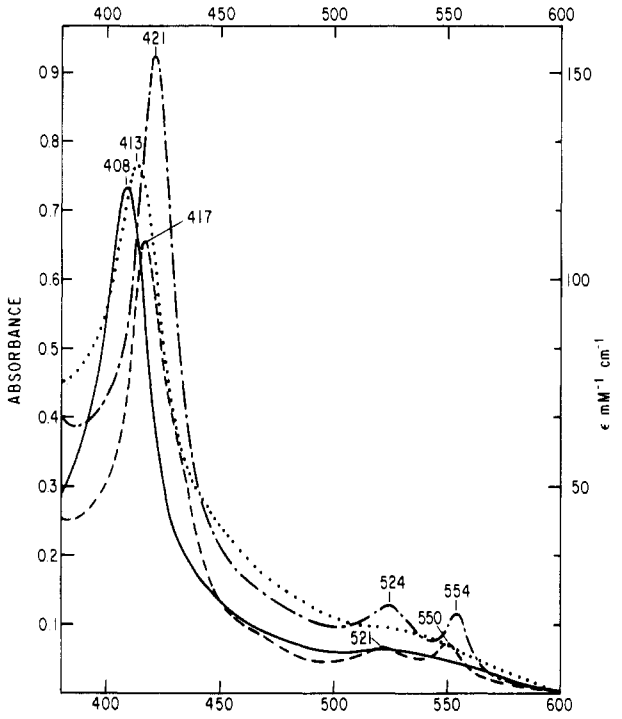

FIGURE 7: Spectra of cyanide-reacted cytochrome c_1 after denaturation. One-band cytochrome c_1 (7 μM) was denatured by 0.1% sodium dodecyl sulfate in 50 mM phosphate buffer, pH 7.4: (—) oxidized form; (- - -) reduced form; (···) oxidized form after cyanide reaction; (—·—) reduced form after cyanide reaction. Freshly prepared cyanide solution (neutralized) was added to make 0.3 M after SDS denaturation.

The oxidation of the reduced cytochrome c_1 by oxygen in the presence of the heart muscle preparation was dependent on the concentration of cytochrome c_1. Under these conditions, the K_m for one-band cytochrome c_1 was found to be 1.33 μM and the V_{max} to be 273 nmol of cytochrome c_1 per minute per milligram of the heart muscle preparation at 23 °C. The reduction of oxidized cytochrome c_1 by succinate catalyzed by the heart muscle preparation was also dependent on the concentration of c_1, showing the K_m for cytochrome c_1 to be 4.54 μM with a V_{max} of 200 nmol of cytochrome c_1 reduced per minute per milligram of the heart muscle preparation. These results indicated the cytochrome c_1 samples were enzymatically active.

Discussion

It should be emphasized that the yield of one-band c_1 increased by more washings on DEAE-cellulose with the sacrifice of that of two-band c_1. Nevertheless, insufficient washing may impair the purity of both two-band and one-band c_1 due to the incomplete removal of impurities. The protein concentration of concentrated c_1 fraction before the Ultrogel AcA 44 column seemed to be important for both the purity and yield of one-band and two-band cytochromes c_1. A protein concentration of 14–16 mg/mL usually gave good results and should not be more than 18 mg/mL.

The molecular weight of one-band cytochrome c_1 or a heme-containing subunit of two-band c_1 was estimated to be 29 000 by SDS–PAGE, and this is very close to M_r 27 924 obtained by the direct amino acid sequence determination (Wakabayashi et al., 1980, 1982b). The sedimentation equilibrium technique shows one-band cytochrome c_1 to be M_r 44 000 in 50 mM potassium phosphate buffer, pH 7.4, containing 1% cholate. König et al. (1980) have also reported M_r 45 000 by the ultracentrifuge technique and M_r 31 000 by SDS–PAGE. The higher value is possibly due to the formation of a cytochrome c_1–cholate complex and probably also a c_1–phosphate–detergent complex since phosphate binds with cytochrome c [see Taborsky & McCollum (1979) and footnote and references cited therein]. This hypothesis is consistent with the disparity between the observed and calculated isoelectric points. The recent report by Shimomura et al. (1985) showed M_r 25 600 calculated from the specific heme content and M_r 33 000 by the SDS–PAGE technique of Merle and Kadenbach (1980). Although the results of the different SDS–PAGE techniques allow the difference in the estimation of molecular weight, the value of M_r 25 600 from the specific heme content is rather low compared to the sequence data (Wakabayashi et al., 1980, 1982b). This could be one of the reasons why the purity of their cytochrome c_1 preparation was found to be 39 nmol/mg of protein even though the ratio of A_{417}/A_{277} was 2.31 which was also smaller than our result (see Table I).

Absorption spectra of one-band c_1 and two-band c_1 at either room temperature or liquid nitrogen temperature (Figure 3) do not show significant differences, implying the association of the hinge protein to cytochrome c_1 does not greatly change the electronic structure of cytochrome c_1 (Kim & King, 1981, 1983). However, one interesting observation from the spectra at liquid nitrogen temperature is that the α-band shows a split 552.5- and 550-nm peak (Figure 3B) without an additional 549-nm peak which is observed on the two-band c_1 prepared by the original method (Yu et al., 1972), and these bands are altered by detergents; with cytochrome c_1 associated with 1% cholate in 50 mM phosphate buffer, pH 7.4, the 552.5-nm peak was diminished, but the 550-nm peak was enhanced (Figure 3C), and this was demonstrated reversible by the removal of cholate. Moreover, 0.5% Tween 80 can completely abolish the peak at 552.5 nm (Figure 3D). We are inclined to consider this observation to be related to its polymerized state; probably completely polymerized cytochrome c_1 shows two distinct peaks of 550 and 552.5 nm, and the presence of 1% cholate can depolymerize cytochrome c_1, even though not completely, resulting in a decrease of the 552.5-nm peak. The 550-nm peak in the presence of Tween 80 may support our interpretation considering that cytochrome c_1 can be completely monomerized in Tween 80. This is further substantiated by the fact that two-band c_1 prepared by the original method (Yu et al., 1972; King, 1978) shows practically no effect of the detergent on α-band splitting. The disparity may be due to the more tightly bound polymerized form in these samples prepared by the original method [see references cited in King (1983)].

Our present cytochrome c_1 preparation exhibited similar photoreduction behavior as that shown in cytochrome c_1 prepared by the original method (Yu et al., 1975). The profile shown in Figure 4 may reflect some difference in the reactivity of one-band and two-band cytochrome c_1. Obviously, one-band c_1 is more sensitive to the light than two-band c_1 probably because the heme of one-band c_1 is not as fully covered by the protein moiety as that of two-band c_1. This is further demonstrated by the effect of the presence of Emasol 1130.

The reaction of cyanide with cytochrome c_1 is observed only when cytochrome c_1 is denatured (Kaminsky et al., 1975) while the reaction between the native form of cytochrome c and cyanide does take place (George & Tsou, 1952). Here again, the observation differed from the report by Schejter and Berke (1968).

The midpoint potential (+227 mV) for one-band c_1 is practically the same as that of two-band c_1 (+225 mV) (Chiang, 1976), and this suggests that the hinge protein does

not affect the redox potential (E_m) of cytochrome c_1. This observation is in agreement with the fact that no direct participation of the hinge protein is observed in the electron-transfer reaction between cytochromes c_1 and c (Kim et al., 1984) in rapid kinetic studies. Moreover, the enzymatic activity of present cytochrome c_1 preparations shows that either one-band or two-band cytochrome c_1 is active to serve as an electron donor as well as acceptor.

In conclusion, we have developed a simple and rapid method for the isolation of highly purified one-band and two-band cytochrome c_1 with substantially improved yield. The two-band cytochrome c_1 thus obtained is found to be a convenient starting material for the preparation of the hinge protein. Differences between two-band and one-band c_1 in certain properties may suggest a probable role of the hinge protein for stabilizing the conformation of cytochrome c_1 in the mitochondrial respiratory chain.

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Registry No. NH_2CONH_2, 57-13-6; CN^-, 57-12-5; cytochrome c_1, 9035-42-1.

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