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SPECTRAL EVIDENCE OF MULTIPLE CYTOCHROMES *b* PRESENT IN SUCCINATE:CYTOCHROME *c* REDUCTASE

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SUMMARY

1. Spectroscopic properties of succinate:cytochrome *c* reductase were examined at room temperature and near liquid nitrogen temperature. The reductase preparation did not catalyze NADH oxidation and was completely free of cytochrome oxidase. Consequently no terminal inhibitor, such as cyanide, was required to effect the reduction of the cychromes in the system.

2. Isolated cytochrome c_1, as well as the cytochrome c_1 existing in succinate: cytochrome *c* reductase, showed spectral splitting but not spectral shift at low temperatures, whereas cytochrome *b* showed blue shifts but no splitting.

3. At least three species of potentially distinguishable *b* cytochromes were observed in succinate:cytochrome *c* reductase. These species showed α bands at (I) 565, (II) 561.5 and (III) 558 nm at room temperature and (I) 562, (II) 559 and (III) 555 nm at about −160°C, respectively. Succinate reduced principally the cytochrome *b* of type II. The assignment of two *b* cytochromes with absorption maxima of 558 and 565 nm is mainly because the absorbance ratio of 565 nm:558 nm was not constant in the preparations examined.

INTRODUCTION

The evidence of the existence of multiple cytochromes *b* in mitochondria and submitochondrial particles has been recently presented[1–9]. Several approaches, such as selective inhibitor[3], kinetic measurements[4,6,7] and especially potentiometric titration[1,2,4] have been used to distinguish the different species.

In these mitochondrial preparations have been observed three α bands centered approximately at 558, 561 and 565 nm at room temperature. It is currently agreed that the one with α band of 561 nm is the classical cytochrome *b* as Keilin originally proposed and thus named cytochrome b_K, sometimes known as high potential cytochrome *b* (E_m approx. 125 mV[1,2]). However, it is not agreed whether the α maxima of 558 and 565 nm are derived from one or more species. Sato *et al.*[8,*] are of the opinion that only one cytochrome is involved but possesses double α maxima at 558 and 565 nm. This second cytochrome *b* is related to energy transduction and has been

* Refs 8 and 9 were not available to us when we originally submitted the manuscript (December 7th, 1971).

named cytochrome b_T or low potential cytochrome *b*. Its midpoint oxidation potential is dependent upon its energy state; E_m has been found to be approximately −30 and +250 mV in the presence and absence of ATP in coupled preparations, respectively. Other workers, represented by Slater and co-workers[7] and Wikström[9,*], have suggested at least two cytochrome *b* species for the α bands of 558 and 565 nm. More recently Davis and Hatefi[5] have shown that the cytochrome *b* in Complex II is somewhat spectrophotometrically different from that of Complex III.

With respect to cytochrome *b*, we have systematically studied the spectrophotometric behavior of a segment of the respiratory chain, succinate:cytochrome *c* reductase[10]. This segment is completely free of cytochrome oxidase and does not catalyze the oxidation of NADH nor contain acid-extractable FMN but is active in the oxidation of succinate with a remarkable antimycin A sensitivity[11]. The employment of this preparation for the study of multiplicity of *b* cytochromes possesses obvious advantages over the submitochondrial particles or mitochondria due to the elimination of the possible complication that a partition of cytochrome *b* may be argued only to be involved in the NADH chain (*i.e.* different from the cytochrome *b* in the succinate chain). Moreover, no terminal inhibitor (such as cyanide) is required to effect the reduction of the cytochromes in the system.

This communication describes the evidence of the existence of multiple cytochromes *b* in the succinate:cytochrome *c* reductase segment by using a simple method of difference spectrophotometry. Low temperature spectroscopic measurements were employed to substantiate the results. The cytochrome *b* species found in succinate:cytochrome *c* reductase are somehow different from those described in Complexes II and III[5].

METHODS

Succinate:cytochrome *c* reductase, cytochrome c_1 and Complex II were prepared according to the methods of Takemori and King[10], Yu *et al.*[12] and Hatefi and co-workers[13,14], respectively. Cytochrome c_1 was estimated from the difference spectra of the sample reduced by ascorbate *minus* that oxidized by ferricyanide; a millimolar extinction coefficient of 17.5 for $A^{552.5}_{red} - A^{540}_{red}$ was used[12]. Cytochrome *b* was estimated from the difference spectra of the dithionite-reduced *minus* the ascorbate-reduced forms; a millimolar extinction coefficient of 28.5 for $A^{562}_{red} - A^{577}_{red}$ was used[15].

The spectroscopic measurements were carried out on a Cary Spectrophotometer, Model 14, at approximately 23 °C. The low temperature spectrophotometry was conducted in a Cary Spectrophotometer, Model 14, equipped with Scattered Transmission Accessory, RCA 06217 Photomultiplier and Sylvania DXL quartz halogen lamp. The sample was placed in a cuvette of 2–3-mm optical path (adjustable by spacers placed between the double observation windows) which 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 placed in the cuvette and the other in ice–water. The set-up checked with crystalline cytochrome *c* and gave somewhat higher intensification factor but otherwise essentially the same results were obtained as those reported by Estabrook[16].

* See footnote, p. 300.

RESULTS

Succinate:cytochrome *c* reductase, which was usually made at 5–10 mg/ml, showed visible spectra as depicted in Fig. 1. The α and β bands of cytochrome c_1 appeared in the ascorbate-reduced form at 552.5 and 524 nm, respectively and those of cytochrome *b* in the dithionite-reduced form appeared at 562 and 531 nm. The difference spectra of the ascorbate-reduced *minus* the oxidized forms and the dithionite-reduced *minus* the ascorbate-reduced forms (see Fig. 2) gave the same results with a better resolution than the absolute spectra.

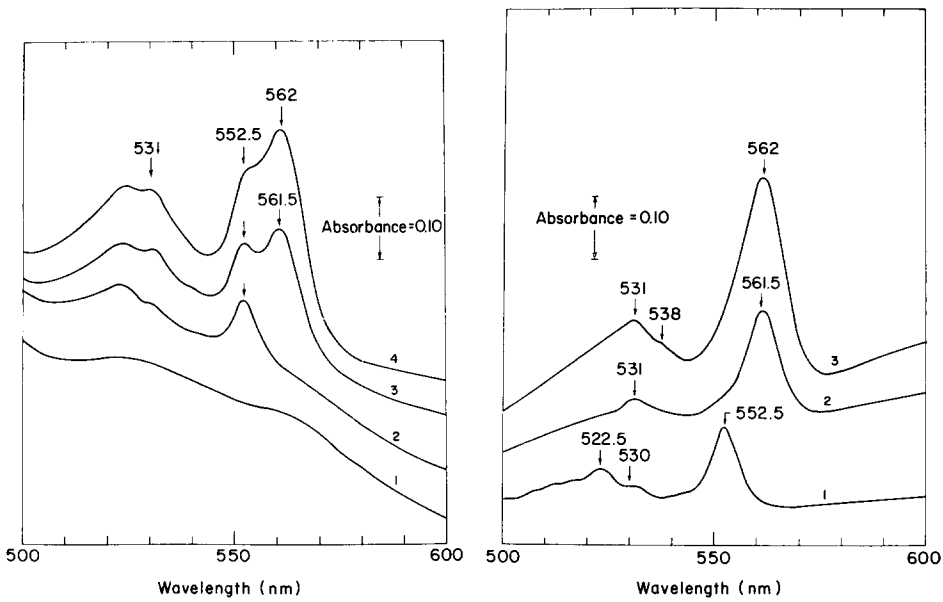

Fig. 1. Visible spectra of succinate:cytochrome *c* reductase. The spectra were measured at about 23 °C in a 1-cm cuvette. Reductase was dissolved in 0.1 M phosphate buffer containing 0.5% cholate and protein concentration was 5 mg/ml. Curve 1, oxidized; Curve 2, ascorbate-reduced; Curve 3, succinate-reduced; and Curve 4, dithionite-reduced. The reference cell contained phosphate buffer.

Fig. 2. Difference spectra of succinate:cytochrome *c* reductase. The conditions were the same as those of Fig. 1. Curve 1, the ascorbate-reduced *minus* the oxidized forms; Curve 2, the succinate-reduced *minus* the ascorbate-reduced forms; and Curve 3, the dithionite-reduced *minus* the ascorbate-reduced forms.

When the spectral measurement was conducted at —160 °C the bands became sharper and the absorbance was intensified as expected (Figs 3 and 4). In the case of cytochrome c_1 (*i.e.* the ascorbate-reduced *minus* the oxidized, Curve 1), a very colorful spectrum was observed. The α band, which was relatively symmetrical at room temperature, split into at least three absorption bands with maxima at 552.5, 550 and 549 nm. The β band of cytochrome c_1, which showed some fine structure even at room temperature, split into a minimum of 9 bands. Those discernible included maxima at 528, 523, 521, 514, 510 and 506 nm and shoulders at 541, 524, 516 and 452 nm. The intensification of the α and β bands at low temperatures was about 6–7-

fold. In contrast to cytochromes *c* and *b*, no blue shift of these bands at low temperatures was found in cytochrome c_1. Identical results were obtained in the purified preparation of cytochrome c_1[12], as shown in Fig. 5.

Cytochrome *b*, however, showed completely different spectral behavior at —160 °C compared to the spectrum at room temperature; all the absorption bands exhibited blue shifts of 2 or 3 nm. The α peak shifted from 562 to 560 nm and no splitting was detected. One of the isolated cytochrome *b* preparations (unpublished results of this laboratory) showed its α band at 557 nm at —160°C (560 nm at 23 °C). This difference may indicate some denaturation or modification of the cytochrome having occurred during the isolation.

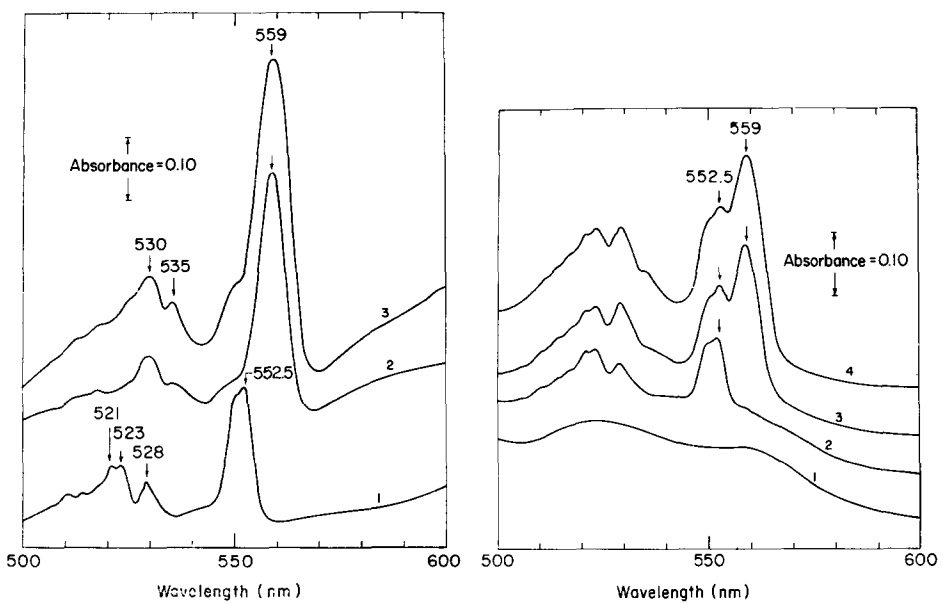

Fig. 3. Low temperature difference spectra of succinate:cytochrome *c* reductase. The spectra were measured at —160 °C in a cuvette with a light path of about 2 mm at 10 mg protein per ml. Curve 1, the ascorbate-reduced *minus* the oxidized forms; Curve 2, the succinate-reduced *minus* the ascorbate-reduced forms; and Curve 3, the dithionite-reduced *minus* the ascorbate-reduced forms.

Fig. 4. Low temperature spectra of succinate:cytochrome *c* reductase. The conditions were the same as those given in Fig. 3 except protein concentration was 7 mg/ml. Curve 1, the oxidized form; Curve 2, the ascorbate-reduced; Curve 3, the succinate-reduced; and Curve 4, the dithionite-reduced form. The reference cuvette contained buffer only.

If more than one species of cytochrome *b* are present in succinate cytochrome *c* reductase and each of them has different redox potential, then reduced with different reductant, one might be able to detect the different degrees of reduction for each species. As depicted in Fig. 2, succinate reduced only 60–75 % of the dithionite-reducible cytochrome *b* in the reductase. The reduction was completed within a period of minutes. The α band of the succinate-reduced cytochrome *b* *minus* the oxidized was found at 561.5 nm, which is about 0.5 nm shorter than that of the dithionite-reduced. Since succinate-reduced cytochrome *b* did not have the same α band as that of dithio-

nite-reduced cytochrome *b*, it is possible that dithionite and succinate do not reduce the same species of cytochrome *b* present in the reductase or alternatively they reduce the same species but to a different extent. The difference spectra of dithionite-reduced *minus* the succinate-reduced reductase showed two distinguishable α bands at 565 and 558 nm at room temperature (Fig. 6, Curve 1) and 3 bands at 562, 555 and 549 nm at near liquid nitrogen temperatures (see Fig. 6, Curve 2). A rather symmetrical α band was found in the difference spectra of the succinate-reduced *minus* the ascorbate-reduced forms with a maximum at 561.5 nm at room temperature and 559 nm at low temperatures. These facts clearly showed that succinate-reduced cytochrome *b* consisted of mainly one species of cytochrome *b* of α band at 561.5 nm at room temperature and 559 nm at about −160 °C. A portion of other species of succinate-reducible cytochrome *b* with α bands of 565 and 558 nm was also observed.

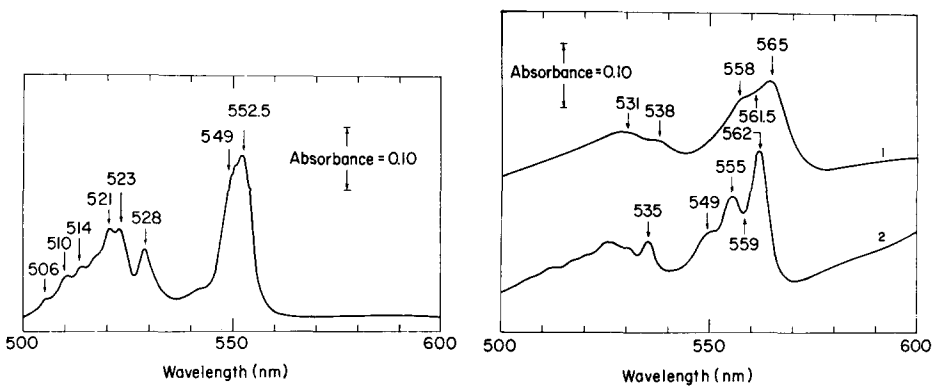

Fig. 5. Low temperature spectra of purified cytochrome c_1. A 23 μM cytochrome c_1 solution which contained 25 nmoles/mg protein in 0.1 M phosphate buffer was used. The conditions were the same as those in Fig. 3.

Fig. 6. Difference spectra of the dithionite-reduced *minus* the succinate-reduced forms of succinate: cytochrome *c* reductase at room and low temperatures. Curve 1, room temperature, the same conditions as those in Fig. 3; Curve 2, at −160 °C, the same conditions as those in Fig. 3.

The evidence of such portions of cytochrome *b* reduced by succinate was also demonstrated by the titration of succinate. Taking advantage of the slow reduction of cytochrome *b* by succinate, a family of difference spectra was observed by measuring the reduction of cytochrome *b* at different times after addition of succinate *versus* the succinate fully reduced sample. As shown in Fig. 7A, succinate first reduced the cytochrome *b* with an α band of 561.5 nm and then reduced cytochrome *b* with bands of 565 nm and 558 nm, judging by the α band moving from 561.5 to 565 nm with a shoulder at 558 nm when the reduction approached completion.

A similar result was also obtained by succinate titration, by measuring the spectra at a given time with various concentrations of succinate instead of using a fixed concentration of succinate and measuring the spectra at different times. Since the extent of reduction of the 558 and 565 bands of cytochrome *b* by succinate was rather small, it was difficult to discern the appearance of such cytochrome *b* if the difference spectra were measured from the succinate-reduced *minus* the ascorbate-reduced forms. Therefore, we used in this particular case the succinate fully reduced

sample as a reference. Experiments on partial reduction of cytochrome *b* with dithionite also revealed that cytochrome *b* with α band of 561.5 nm was reduced prior to other species of cytochromes *b* (Fig. 7B). In other words, the cytochrome *b* reduced by succinate had a higher redox potential than other species of cytochromes *b*.

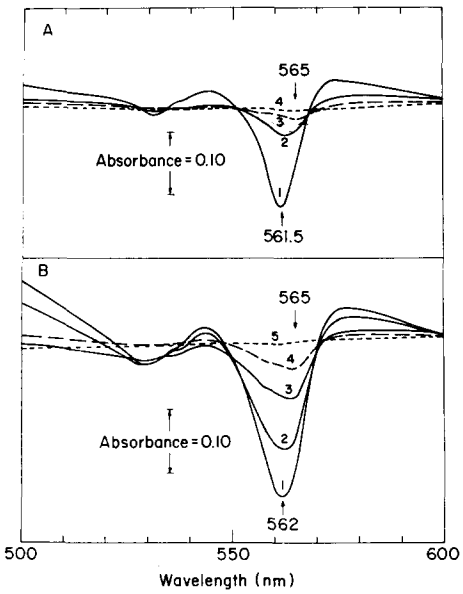

Fig. 7. Titration of succinate:cytochrome *c* reductase with succinate (A) and dithionite (B) in the presence of ascorbate. In A, Curve 1, difference spectra of the ascorbate-reduced *minus* the ascorbate- *plus* the succinate-reduced forms; the amount of the succinate present was about 100 times in excess and was added 20 min before addition of ascorbate. Curve 2, the ascorbate and succinate partially reduced *minus* the ascorbate and succinate fully reduced form. In the sample cuvette, succinate was added immediately after the addition of ascorbate, whereas in the reference cuvette succinate was added 20 min prior to the addition of ascorbate. Curves 3 and 4, same conditions as in Curve 2, except the spectra were measured at 3 and 10 min after addition of reductants. In B, Curve 1, the ascorbate-reduced *minus* the ascorbate and dithionite fully reduced forms. Curves 2, 3, 4 and 5, same conditions as in Curve 1, except gradually increased amounts of dithionite added to ascorbate-reduced sample.

Addition of antimycin A caused a red shift of about 2 nm but the total amount of the succinate-reducible cytochrome *b* remained unchanged. Similar results were also observed when the antimycin A was added to the dithionite-reduced preparation. It has been reported that in pigeon heart mitochondria[8] the antibiotic caused spectral shift only for the succinate-reducible *b*-cytochrome but not the *b*-cytochrome with the double α band at 558 and 565 nm at room temperature. If that were also true in the case of succinate:cytochrome *c* reductase, we should have seen no difference in the difference spectra obtained from the succinate-reduced *minus* the dithionite-reduced preparation regardless of whether antimycin A was present or not. Our results showed a slight difference in the difference spectra obtained in the afore-mentioned treatment, in spectral shape as well as the absorbance ratio of 565 nm:558 nm. The difference spectra obtained in the presence of antimycin A showed a lower absorption ratio of 565 nm:558 nm and with a better peak resolution than the difference spectra obtained in

the absence of antimycin A. Therefore, that antimycin A has no effect on *b* cytochrome with α band at 565 and 558 nm must be considered with caution, at least in the case of succinate:cytochrome *c* reductase reported here. Indeed, a species[7] of cytochrome *b* called b_i^{2+}·antimycin is differentiated from cytochromes b_T and b_K.

DISCUSSION

The results show clearly that isolated cytochrome c_1 possesses similar spectral properties to the cytochrome c_1 present in the intact or functionally active succinate: cytochrome *c* reductase. No shift in the absorption bands has been observed in the low temperature spectra. Since the purified cytochrome c_1 has been conclusively shown to be only one hemoprotein[12], it is quite convincing that there is only one kind of cytochrome c_1 present in the succinate:cytochrome *c* reductase. These properties also strongly suggest the isolated cytochrome c_1 is not modified. More importantly, the identity of the spectra indicates the lack of the direct interactions between cytochrome c_1 and other components. Otherwise the spectrum of cytochrome c_1 in the reductase should be expected to be different from that of the isolated. But most probably the existing interactions do not affect the spectrum of cytochrome c_1.

On the other hand, judging by the spectral behavior described, cytochrome *b* is not a single species. At least three (*cf.* also ref. 9), perhaps four (*cf.* also ref. 7), species of spectrally distinguishable cytochromes *b* are present in the reductase. The spectral properties described above are in agreement with those described in the pigeon heart mitochondria[8]. The *b* cytochrome with α absorption band at 565 and 558 nm appears concurrently in the course of titration. These two bands could well, as suggested by Sato *et al.*[8], belong to a single species of *b* cytochrome. However, there is evidence which is more in favor of Wikstrom's observation[9]; *i.e.* each absorption band may belong to a single *b* cytochrome. The ratio of the two absorption bands was found to vary from preparation to preparation. If they are derived from a single species a constant absorption ratio should be expected.

Neither the results described here nor those reported in the literature can, however, differentiate whether these species are due to one hemoprotein with various ligands or to the existence of several hemoproteins of different nature. Preliminary results of our recent experiments showed that the reducibility of succinate-reducible cytochrome *b* was very much dependent upon the lipid content of the reductase preparations. As expected, the ATP showed no enhancement on the reducibility of *b* cytochrome by succinate on the reductase (as in uncoupled mitochondria[9] preparations). These observations might suggest that the variety of spectral species of *b* cytochrome in electron transfer and energy conservation may simply be derived from a single hemoprotein in different environments rather than multiplicity of hemoproteins of different nature.

We were unable to detect a cytochrome *b* with an α band at 553 nm at low temperature[5]. This cytochrome *b*, which has been reported to be present in the Complex III, may be mistaken from cytochrome c_1; in succinate:cytochrome *c* reductase the 552.5 nm band was found to be reducible by ascorbate and can be completely subtracted out spectrophotometrically by the isolated cytochrome c_1 or ascorbate-reduced reductase. Complex II, prepared according to the method of Baginsky and Hatefi[13] or Davis and Hatefi[14], shows two distinguishable α bands, one at 557 nm and

the other at 549 nm in the dithionite-reduced *minus* oxidized spectra at low temperature. In our judgment, both bands were due to cytochromes *b* rather than one of them, 549 nm, being due to cytochrome c_1. At the present time, we are unable to offer an explanation why the 557-nm band was only reduced by succinate to such a small extent. If cytochrome *b* with an α band of 557 nm is also involved in succinate:cytochrome *c* reductase, we should be able to find it in such a preparation; and if it belongs to the band at 561.5 nm (the cytochrome *b* of Complex II at room temperature showed α band at 560 nm), it should express the succinate reducibility higher than the reported value of 20%. It may not be neglected, however, that Complex II prepared either by us or by Hatefi's laboratory contains large amounts of cytochrome c_1, up to 2 nmoles per mg of protein.

Since cytochrome *b* with an α band at 565 nm is only slightly reduced by succinate, it may not be directly involved in electron transfer. It seems very likely that it is the same as cytochrome b_T, which is believed to be primarily involved in energy conservation. The nonreducibility of cytochrome b_T or the species of 565 nm by succinate has been claimed by Wilson, Chance, and co-workers (for example, refs. 1, 2, 8, 17 and 18) due to its low redox potential. Therefore, they argue that the nonreducibility cannot be used to exclude cytochrome b_T from the main pathway. However, the system, such as we and other workers have used, contains usually excess succinate in millimolar quantities, whereas the cytochrome b_T present is only in micromolar concentration. If E_m for succinate and cytochrome b_T in uncoupled preparations is 20 and −30 mV, respectively, one would expect a considerable reduction of b_T because of the concentration effect in overcoming the unfavorable thermodynamics. Actually, it is little reduced. Moreover, further addition of succinate does not increase the reduction. At any rate, the succinate reducible cytochrome *b* is most probably the same as that described as cytochrome b_K which is involved in electron transfer.

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