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STEPWISE REDUCTION OF CYTOCHROMES *b*-562, *b*-566 AND *b*-558 IN RAT LIVER MITOCHONDRIA*

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SUMMARY

1. Addition of KCN to aerobic, rotenone-inhibited rat liver mitochondria without addition of substrate caused reduction of cytochromes *b*-562 (having an α-band at 562 nm at room temperature), $c + c_1$ and $a + a_3$. The effect of KCN on cytochrome *b*-562 was reversed by pentachlorophenol, though the effect of KCN on cytochromes $c+c_1$ and $a+a_3$ was not reversed by this uncoupler.

2. Addition of ATP to aerobic, rat liver mitochondria inhibited with 500 μM KCN under conditions where cytochromes *b*-562, $c+c_1$ and $a+a_3$ were reduced, caused reduction of cytochrome *b*-566. The absorbance spectrum of cytochrome *b*-566 had an α-band at 565.5 nm, a β-band at 538 nm and a γ-band at 431 nm, but no shoulder around 558 nm at room temperature.

3. Addition of succinate to rotenone-KCN-inhibited and ATP-treated rat liver mitochondria under conditions where cytochromes *b*-566, *b*-562, $c+c_1$ and $a+a_3$ were already fully reduced, caused reduction of cytochrome *b*-558 (having an α-band at 558 nm, a β-band at 527 nm and a γ-band at 426 nm at room temperature) after exhaustion of molecular oxygen in the reaction medium, without any contribution from a long-wavelength species (cytochrome *b*-566).

4. It was concluded that the 558-nm band is not a short-wavelength shoulder of cytochrome *b*-566, but is due to a different species from cytochrome *b*-566.

INTRODUCTION

The multiplicity of *b*-type cytochromes in mitochondria has been shown by results on the effect of antimycin A [1–8], oxidants [9–16], energization [2, 17–22] and potentiometric titration [23–28]. Chance and Schoener [17] and Slater et al. [2] found independently that ATP causes the reduction of a type of cytochrome *b* which is not cytochrome *b*-562. There are several reports [18, 19, 25, 29–33] that the absorption spectrum of the cytochrome *b* which is reduced by ATP has a peak at 566 nm and a

* The terms cytochromes *b*-562, *b*-566 and *b*-558 used are based on the absorbance maxima of the α-bands of these components at room temperature, according to the recommendation of the I.U.B. Enzyme Commission.

** Deceased.

shoulder around 558 nm at room temperature. Sato et al. [29, 30] supposed that both the long-wavelength maximum at 566 nm and the short-wavelength shoulder at 558 nm are due to the same species, cytochrome b_T: that is, cytochrome b_T has a double-headed α-peak. On the other hand, Slater [32], Wikström [18] and Yu et al. [33] suggested independently that these two maxima are due to two different species, *b*-566 and *b*-558, because the A_{566}/A_{558} ratio is variable. However, as the variation in this ratio could result from complicating redox changes in the *b*-562 component it is still uncertain whether these two α-peaks are due to two different species of *b*-type cytochrome or to a single species with a double-headed α-peak (cf. review by Wikström [34]).

To examine this question, the present work subjects cytochromes *b*-562, *b*-566 and *b*-558 to stepwise reduction.

MATERIALS AND METHODS

Rat liver mitochondria were isolated by the method of Hogeboom [35], as described by Myers and Slater [36]. Protein was determined by the biuret method as described by Cleland and Slater [37]. All reactions were carried out in medium consisting of 30 mM Tris · HCl buffer, 70 mM sucrose, 200 mM mannitol and 20 mM KCl, with other components as indicated in the legends to the figures, in a final volume of 3 ml at pH 7.4. Absorbance changes of cytochrome components were examined in a Hitachi, Model 356, two-wavelength spectrophotometer. Two procedures were used to obtain difference spectra. In Procedure A, difference spectra were obtained in a Union, Model SM-401, High Sensitivity Split-beam Spectrophotometer (with a slit width of 1.2 nm) equipped with a Union, Model SM-4012, baseline corrector equipped with two memory systems. Spectral data, digitized at 0.1-nm intervals, are stored in the memory core of the baseline corrector and can be read out into an X-Y recorder as individual curves or as differences between any pair of spectral curves. Unless otherwise stated, the difference spectra presented here are corrected for the difference spectrum obtained by adding rotenone to mitochondria in both cuvettes. In Procedure B, difference spectra were obtained by scanning measuring wavelengths, taking 575 nm as the reference wavelength using a Hitachi, Model 356, two-wavelength spectrophotometer (with a slit width of 1.0 nm) equipped with a Hitachi, Model QPD_{201}, X-Y recorder. The wavelength scale of the Hitachi instrument (Model 356) and the Union instrument (Model SM-401) were calibrated using the line spectrum from a D_2 lamp (656.1 nm). All measurements were carried out at room temperature (approximately 25 °C).

RESULTS AND DISCUSSION

Reduction of cytochrome b-562 induced by KCN in aerobic, rotenone-inhibited rat liver mitochondria

The cytochrome *b* which had been reduced by endogenous substrates without added substrate was partially oxidized by addition of rotenone. Subsequent addition of 1 mM KCN caused complete reduction of cytochrome $a+a_3$ (data not shown) and cytochrome $c+c_1$, with a biphasic increase in the absorbance at 562 nm minus 575 nm, as shown in Fig. 1A. The increase in the absorbance at 562 nm minus 575 nm was re-

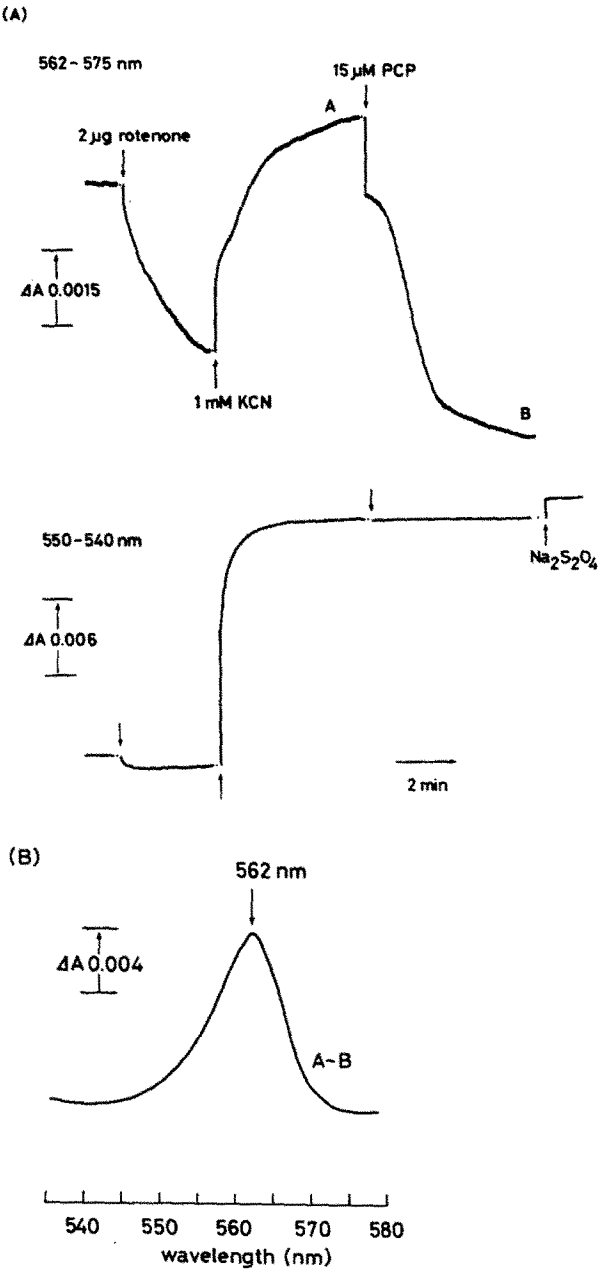

Fig. 1. (A) Effects of KCN and uncoupler on the redox state of cytochromes *b*-562 and $c+c_1$ in aerobic, rotenone-inhibited rat liver mitochondria. The rat liver mitochondrial suspension contained 3.3 mg protein/ml. Experimental conditions are described in the text. (B) Absorption spectrum of cytochrome *b*-562. The rat liver mitochondrial suspension in both the sample and reference cuvettes (7.1 mg protein/ml) was supplemented with 4 μg of rotenone and 1 mM KCN and then 15 μM pentachlorophenol (PCP) was added to the reference cuvette only. The difference spectrum was recorded by Procedure A 5 min after addition of pentachlorophenol.

versed biphasically by 15 μM pentachlorophenol to a lower level of reduction than that induced by addition of rotenone without added substrate. However, cytochromes $a+a_3$ and $c+c_1$ which had been reduced by KCN were not oxidized by the uncoupler. The difference spectrum in the absence and presence of the uncoupler (A minus B in Fig. 1A) mainly indicates oxidation of cytochrome *b*-562 (having an α-band at 562 nm at room temperature), as shown in Fig. 1B. The reduction of cytochrome *b*-562 induced by KCN was insensitive to oligomycin (data not shown). It seems likely, therefore, that the species of *b*-type cytochrome reduced by addition of KCN was *b*-562, and that reduction of *b*-562 was affected by the energy state of the mitochondria. This is in a good accordance with the results reported by Wikström [18].

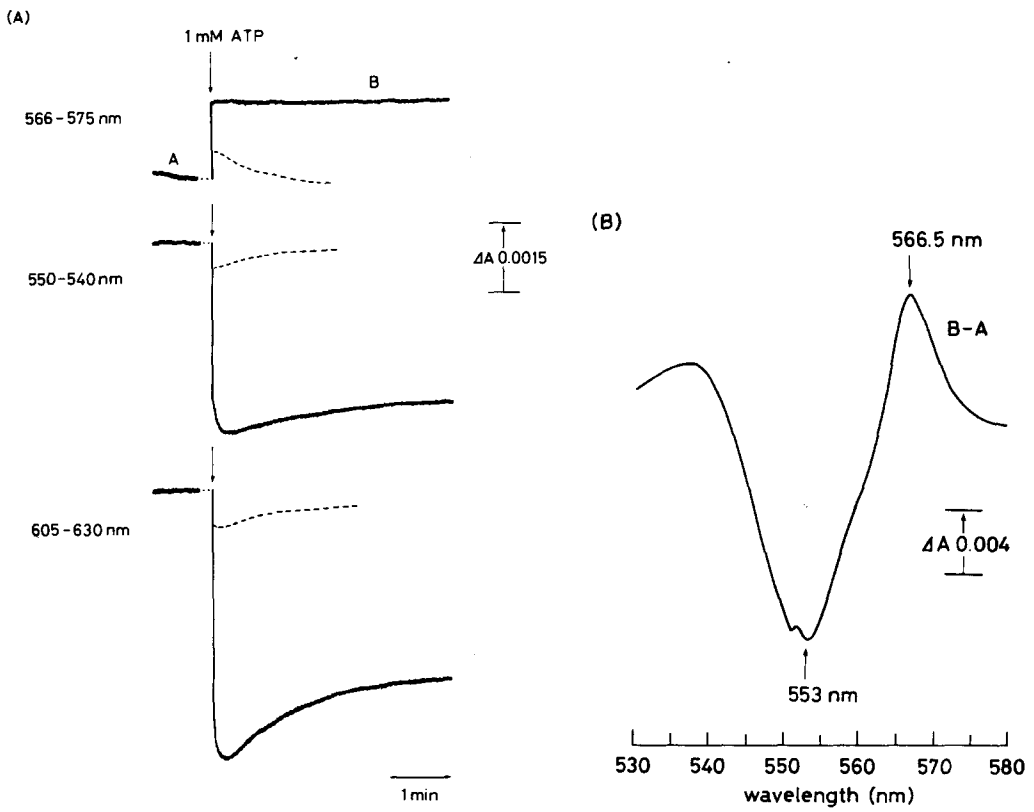

Fig. 2. (A) Effects of ATP and oligomycin on the redox state of cytochromes *b*-566, $c+c_1$ and $a+a_3$ in aerobic, rotenone-KCN-inhibited rat liver mitochondria. The rat liver mitochondrial suspension (2.9 mg protein/ml) was supplemented with 2 μg of rotenone, 1 mM KCN and 1 mM ATP as indicated in Figs 1A and 2A. Dotted line, with 3.2 μg of oligomycin. (B) Difference spectrum. The rat liver mitochondrial suspension in both the sample and reference cuvettes (9.0 mg protein/ml) was supplemented with 4 μg of rotenone and 1 mM KCN and then 1 mM ATP was added to the sample cuvette only. The difference spectrum was recorded by Procedure A immediately after addition of ATP.

Reduction of cytochrome b-566 induced by ATP in aerobic, rotenone-KCN-inhibited rat liver mitochondria

The effect of ATP was tested on aerobic, rotenone-KCN-inhibited rat liver mitochondria under conditions (A in Fig. 1A) where cytochromes *b*-562, $c+c_1$ and $a+a_3$ were fully reduced by endogenous substrates without added substrate. Fig. 2A shows that on addition of ATP the absorbance at 566-nm minus 575 nm increased, while cytochromes $a+a_3$ and $c+c_1$ were oxidized. These effects of ATP were prevented by inhibitors of respiratory chain phosphorylation, such as oligomycin (Fig. 2A) and uncouplers (data not shown). The difference spectrum in the presence and absence of ATP (B minus A in Fig. 2A) indicates reduction of cytochrome *b*-566 and

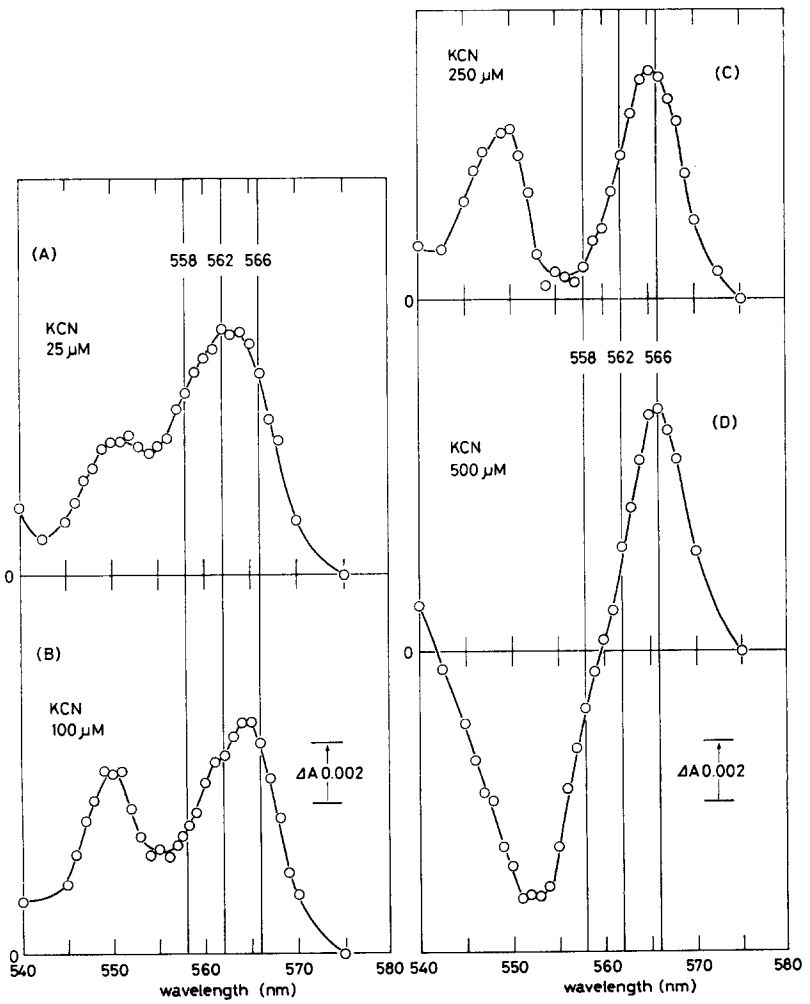

Fig. 3. Effect of ATP in the presence of various concentrations of KCN on the redox state of cytochromes *b*-562, *b*-566 and $c+c_1$ in aerobic, rotenone-inhibited mitochondria. The conditions were as for Fig. 2A except that 9.0 mg protein/ml of rat liver mitochondria and KCN at the indicated concentrations were used. Difference spectra were obtained by Procedure B, as B minus A of Fig. 2A.

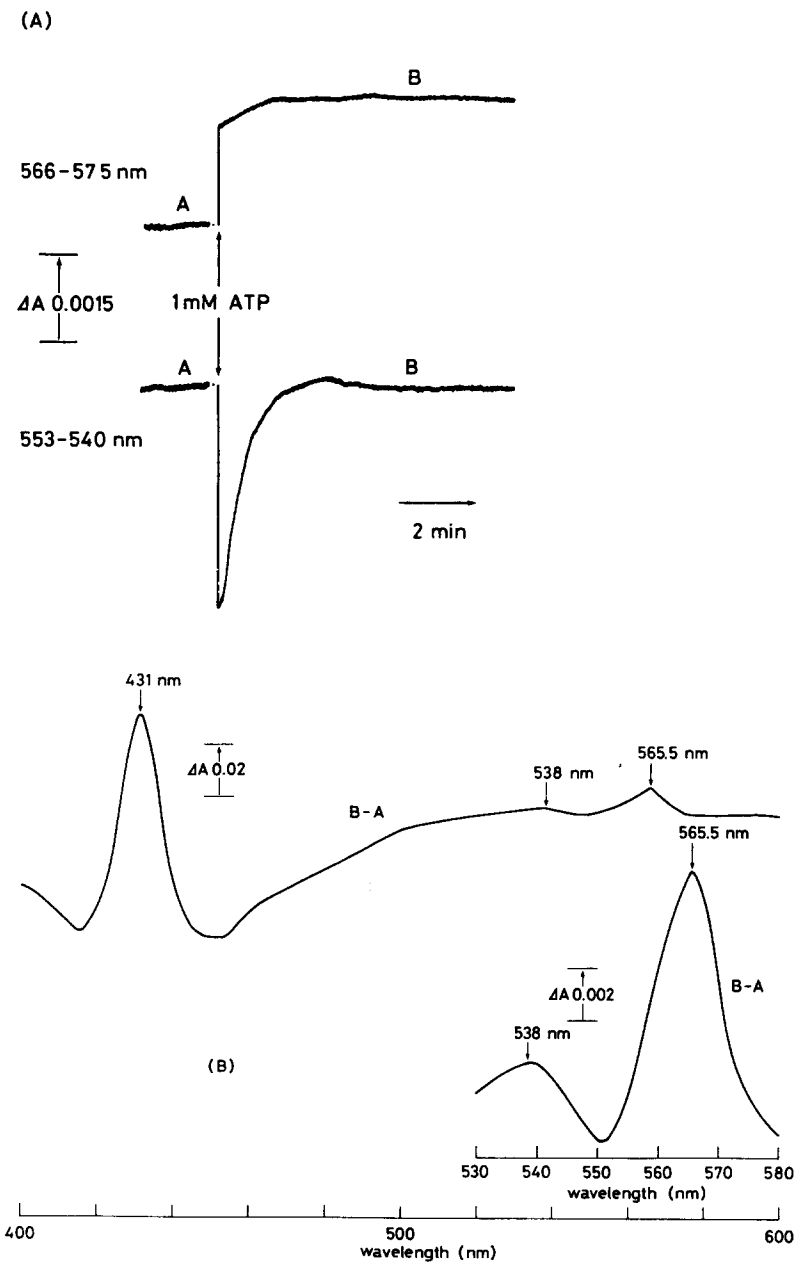

Fig. 4. (A) Effect of ATP on the redox state of cytochromes *b*-566 and c_1 ($+c$) in aerobic mitochondria inhibited with 500 μM KCN and rotenone. The conditions were as for Fig. 2A except that 2.3 mg protein/ml of rat liver mitochondria and 500 μM KCN were used. (B) Absorption spectrum of cytochrome *b*-566. The rat liver mitochondrial suspension in both the sample and reference cuvettes (8.9 mg protein/ml) was supplemented with 4 μg of rotenone and 500 μM KCN and then 1 mM ATP was added to the sample cuvette only. The difference spectrum was taken by Procedure A, 5 min after addition of ATP.

oxidation of cytochrome c_1 ($+c$), as shown in Fig. 2B. This spectrum gave no evidence that cytochrome *b*-566 has a shoulder around 558 nm, but this cannot be decided definitely from this result alone, because the difference spectrum in which cytochrome c_1 ($+c$) became oxidized relative to the reference while the cytochrome *b*-566 became reduced resulted in erasure of any spectral bands in between. Thus, to find experimental conditions to minimize the influence of other species of cytochromes on the α-band of cytochrome *b*-566, the effect of ATP was tested on aerobic, rotenone-inhibited mitochondria in the presence of various concentrations of KCN. Fig. 3 shows that addition of ATP mainly caused reduction of cytochromes *b*-562 and *c* in the presence of 25 μM KCN, reduction of cytochromes *b*-562, *b*-566 and *c* in the presence of 100 μM KCN, reduction of cytochromes *b*-566 and *c* in the presence of 250 μM KCN and reduction of cytochrome *b*-566 and oxidation of cytochrome c_1 ($+c$) in the presence of 500 μM KCN. Addition of ATP caused oxidation of cytochrome $a+a_3$ also in the presence of KCN at concentrations of 25 to 1000 μM (data not shown). Fig. 3C suggests that cytochrome *b*-566 has no shoulder around 558 nm. To confirm this, we tried to find experimental condition in which only one species of cytochrome *b*-566 appeared. Fig. 4A shows that the effect of ATP on cytochrome c_1 ($+c$) in aerobic, 500 μM KCN-inhibited mitochondria was reversed with time, while its effect on cytochrome *b*-566 was not. The difference spectrum in the presence and absence of ATP (steady state, B minus A in Fig. 4A) indicates clearly that cytochrome *b*-566 (having an α-band at 565.5 nm, a β-band at 538 nm and a γ-band at 431 nm) has no shoulder around 558 nm, as shown in Fig. 4B. The ratio of the absorbance at 431 nm to that at 565.5 nm was approximately 8.0.

Reduction of cytochrome b-558 induced by the aerobic and anaerobic transition on adding succinate to rotenone-KCN-inhibited and ATP-treated rat liver mitochondria

The present findings suggest that the short-wavelength band (558 nm), which Sato et al. [29, 30] described as shoulder of cytochrome *b*-566 may appear on addition of substrate to aerobic, rotenone-KCN-inhibited and ATP-treated rat liver mitochondria. Next, the effect of addition of substrate was tested on aerobic, rotenone-KCN-inhibited and ATP-treated rat liver mitochondria under conditions (B in Fig. 3A) in which cytochromes *b*-562 and *b*-566 were fully reduced by endogenous substrates without added substrate. Fig. 5A shows that succinate caused a slow increase in the absorbance at 558 nm minus 575 nm after exhaustion of molecular oxygen in the reaction medium. The rate of the transition from state A to state B in Fig. 5A was dependent on the concentration of KCN added before addition of succinate and was accelerated with decreasing concentration of this inhibitor. If the molecular oxygen was put in the reaction medium in the state B in Fig. 5A, the absorbance at 558 nm minus that at 575 nm was reversed to the level of state A in Fig. 5A. The difference spectrum in the presence and absence of 10 mM succinate (B minus A in Fig. 5A) indicates reduction of cytochrome *b*-558 having an α-band at 558 nm, a β-band at 527 nm and a γ-band at 426 nm, without any contribution from a long-wavelength species (*b*-566), as shown in Fig. 5B. The ratio of the absorbance at 426 nm to that at 558 nm was approximately 6.5. It seems likely that the spectral properties of cytochrome *b*-558 are different with those of *b*-type cytochromes of the outer membrane or intermembrane space [38, 39] but resemble those reported previously [40, 41]. Cytochrome *b*-558 was reduced much more slowly than pigments of the respiratory chain,

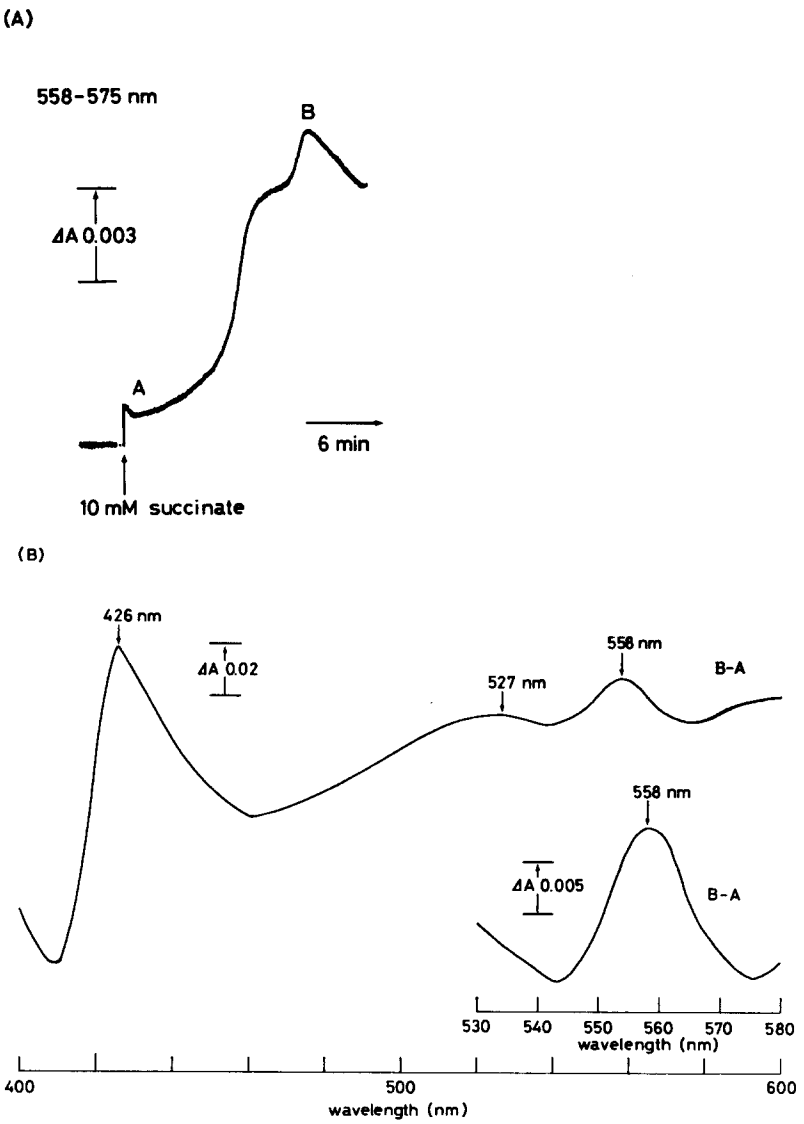

Fig. 5. (A) Effect of succinate on the redox state of cytochrome *b*-558 in rotenone-KCN-inhibited and ATP-treated rat liver mitochondria. The rat liver mitochondrial suspension (6.2 mg protein/ml) was supplemented with 4 μg of rotenone, 1 mM KCN and 2 mM ATP as indicated in Figs 1A and 2A and then 10 mM succinate was added as indicated. To minimize contamination with oxygen, the surface of the reaction mixture was covered with liquid paraffin. (B) Absorption spectrum of cytochrome *b*-558. The rat liver mitochondrial suspension in both the sample and reference cuvettes (9.5 mg protein/ml) was supplemented with 4 μg of rotenone, 1 mM KCN and 2 mM ATP. Then 10 mM succinate was added to the sample cuvette. After incubation for 15 min, succinate was added to the reference cuvette also and then the spectrum was measured. The surface of the reaction mixture in the sample cuvette was covered with liquid paraffin. The difference spectrum (B minus A in Fig. 5A) was obtained by Procedure A, 15 min after addition of succinate to the sample cuvette and corrected by the difference spectrum obtained by adding rotenone, KCN and ATP to mitochondria in both cuvettes using a Union, Model SM-4012, baseline corrector.

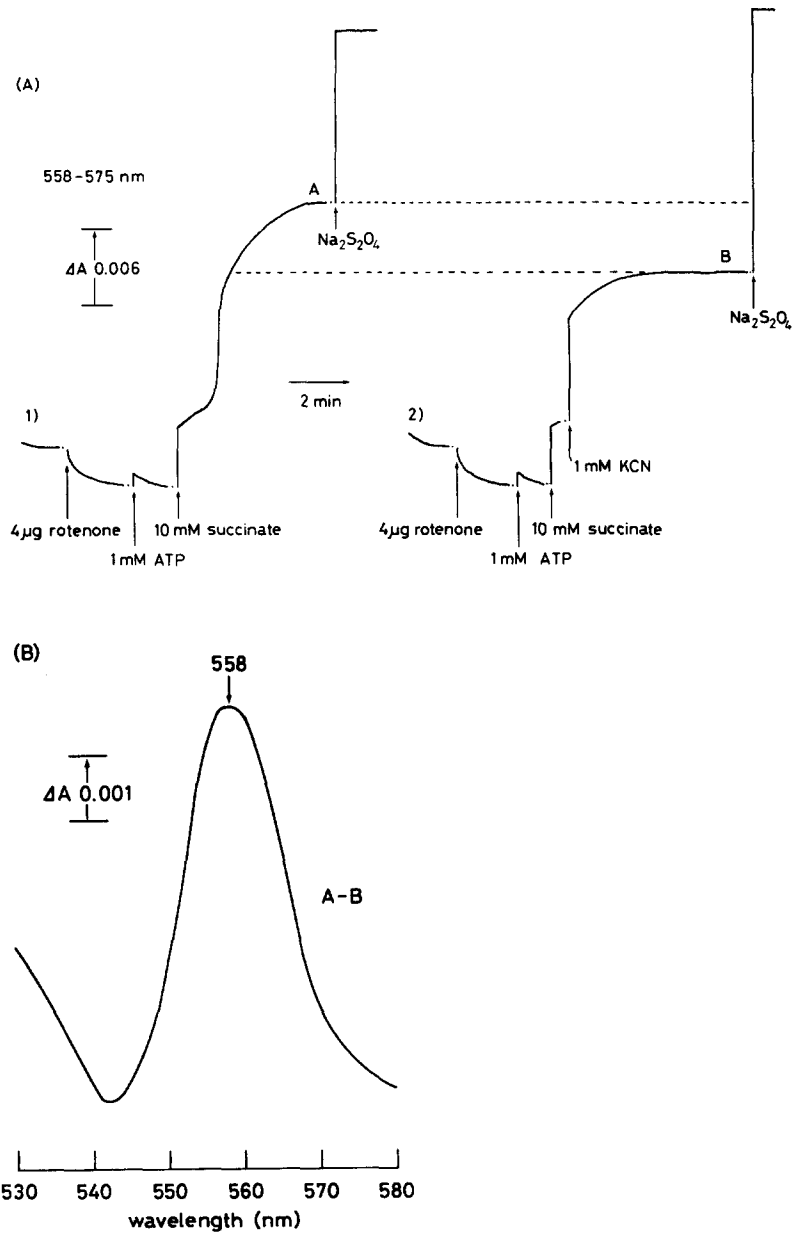

Fig. 6. (A) Effect of KCN on the redox state of cytochrome *b*-558 in succinate-reduced, rotenone-inhibited and ATP-treated mitochondria. The rat liver mitochondrial suspension (6.0 mg protein/ml) was supplemented with 4 μg of rotenone, 2 mM ATP and 10 mM succinate for traces (1) and (2) and with 1 mM KCN for trace (2) only, as indicated. (B) Difference spectrum. The rat liver mitochondrial suspension in both the sample and reference cuvettes (6.0 mg protein/ml) was supplemented with 4 μg of rotenone, 2 mM ATP and 10 mM succinate and then 1 mM KCN was added to the reference cuvette under aerobic conditions. The difference spectrum (A minus B in Fig. 6A) between the sample and reference cuvettes was obtained by Procedure A, 6 min after addition of succinate.

so it seems questionable whether it is actually a respiratory-chain-linked pigment. However, as shown in Fig. 6B, the difference spectrum between anaerobic, succinate-reduced and ATP-treated mitochondria and aerobic, KCN-inhibited, succinate-reduced and ATP-treated mitochondria (A minus B in Fig. 6A) indicates reduction of cytochrome *b*-558 having an α-band at 558 nm and a β-band at 527 nm (data not shown). Therefore, these results indicate that cytochrome *b*-558 was reduced quickly, like pigments of the respiratory chain, by the aerobic and anaerobic transition caused by succinate, if KCN was not added. Thus, the present findings suggest that cytochrome *b*-558 may be readily autoxidizable while cytochromes *b*-566 and *b*-562 show little if any autoxidizability. This may explain why we could reduce cytochromes *b*-566 and *b*-558 separately. Figs 7 and 8 show that addition of 10 μM pentachlorophenol to the conditions of States A and B of Fig. 6A before addition of dithionite caused oxidation of cytochromes *b*-566 (+*b*-558) partially and only cytochrome *b*-566 partially, respectively. We also found that the redox state of cytochrome *b*-558 in energized rat liver mitochondria was affected by the membrane potential [42]. At present, however, we cannot exclude the possibility that cytochrome *b*-558 is not linked to the respiratory chain and this requires further study. Studies are also required on the relashionship between the cytochrome *b*-558 and cytochrome *b*-555 (555 nm at 77 °K) observed by Chance and Schoener [17] in energized pigeon heart mitochondria.

The present findings show that the 558 nm band is due to a different species from cytochrome *b*-566 and is not a short-wavelength shoulder of the latter. Therefore, it seems highly probable that the high-potential form of cytochrome b_T is not a single molecular entity, but consists of several cytochrome *b* components (*b*-566,

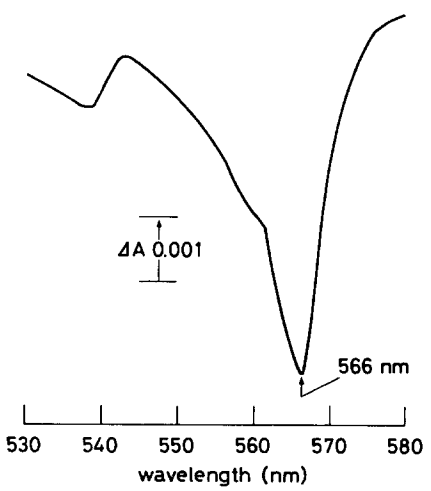

Fig. 7. Effect of uncoupler on the redox states of *b*-type cytochromes in energized mitochondria under anaerobic conditions. The rat liver mitochondrial suspension in both the sample and reference cuvettes (6.6 mg protein/ml) was supplemented with 4 μg of rotenone, 2 mM ATP and 10 mM succinate and then 10 μM pentachlorophenol was added to the sample cuvette under anaerobic conditions. The difference spectrum in the presence and absence of pentachlorophenol was obtained by Procedure A and corrected by the difference spectrum under anaerobic conditions obtained by adding rotenone, ATP and succinate to mitochondria in both cuvettes using a Union, Model SM-4012, baseline corrector.

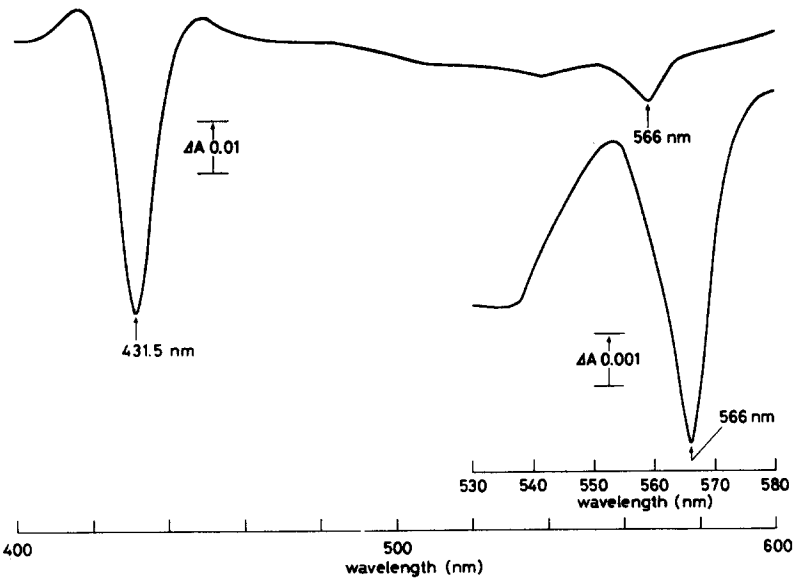

Fig. 8. Effect of uncoupler on the redox states of *b*-type cytochrome in energized mitochondria under aerobic conditions. The rat liver mitochondrial suspension in both the sample and reference cuvettes (6.6 mg protein/ml) was supplemented with 4 μg of rotenone, 2 mM ATP, 10 mM succinate and 1 mM KCN and then 10 μM pentachlorophenol was added to the sample cuvette under aerobic conditions. The difference spectrum in the presence and absence of pentachlorophenol was obtained by Procedure A and corrected by the difference spectrum under aerobic conditions obtained by adding rotenone, ATP, succinate and KCN to mitochondria in both cuvettes using a Union, Model SM-4012, baseline corrector.

b-558 and also *b*-562). The so-called cytochrome b_T hypothesis, proposed by Chance et al. [24], implies that the high-potential form of cytochrome b_T is a single molecular entity but if, as our results indicate, this is not so, the b_T hypothesis must be reconsidered.

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