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REDUCTION AND OXIDATION OF LONGER-WAVELENGTH CYTOCHROME *b* (b_{566}) IN RAT LIVER MITOCHONDRIA UNDER THE INFLUENCE OF EXTERNAL OXIDANTS

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SUMMARY

The reduced form of longer-wavelength cytochrome *b* (b_{566}) was identified in rat liver mitochondria in State 5 on addition of ferricyanide. Addition of menadione caused oxidation of b_{566} and reduction of cytochrome c_1 ($+ c$). In the presence of menadione, ferricyanide induced reduction of b_{566} and oxidation of cytochrome $c_1 + c$, and exhaustion of the added ferricyanide resulted in oxidation of b_{566} and reduction of cytochrome $c_1 + c$.

Antimycin A enhanced the extent of the ferricyanide-dependent redox change of b_{566} from 20 to 68 % of the total absorbance at 566 nm.

Uncouplers hardly affected the change of b_{566} in the presence of antimycin A, but lowered the reduced level in the absence of antimycin A.

INTRODUCTION

In 1952, Chance[1] found that oxygen induced the reduction of cytochrome *b* in antimycin A-treated yeast cells. In the presence of antimycin A a similar phenomenon was observed by Pumphrey[2] in electron transfer particles, by Rieske[3] in the cytochrome b–c_1 complex, and by Wilson *et al.*[4] in succinate–cytochrome *c* reductase and in a cholate-treated preparation of chicken heart mitochondria. In the light of recent findings on *b*-type cytochromes made in several laboratories[5-8], Wilson *et al.*[4] reported that the cytochrome *b* reduced under these experimental conditions was longer-wavelength cytochrome *b* (b_{566}) and that the reduction of b_{566} required activation of electron transport through cytochrome c_1. More recently, Erecińska *et al.*[9] measured the rate of reduction of b_{566} and that of oxidation of cytochrome c_1 by oxygen in anaerobic, uncoupled pigeon heart mitochondria. They postulated that the aerobic reduction of cytochrome b_{566} is directly related to energy conservation at Site II.

In studies on the effects of various oxidants on the redox state of cytochrome components in rat liver mitochondria oxidizing succinate, we obtained spectral evidence indicating that ferricyanide caused reduction of cytochrome b_{566} and oxidation of cytochrome $c_1 + c$ and that menadione caused oxidation of cytochrome b_{566} and reduction of cytochrome c_1 ($+ c$) under conditions where the effects of

energy coupling sites at Site I and Site III were eliminated by rotenone and KCN, respectively.

MATERIALS AND METHODS

Rat liver mitochondria were isolated by the method of Hogeboom[10], as described by Myers and Slater[11]. Protein was determined by the biuret method as described by Cleland and Slater[12]. All reactions were carried out in one of the following media: Medium A, 25 mM Tris–HCl buffer, 50 mM sucrose, 5 mM $MgCl_2$, 2 mM EDTA and 15 mM KCl; Medium B, 30 mM Tris–HCl buffer, 70 mM sucrose, 200 mM mannitol and 20 mM KCl. The other components used are indicated in the legends to the figures. The final volume of the mixture was 3 ml and the pH was 7.4. Measurements of the absorbance changes of cytochrome components were made with a Hitachi, Model 356, two-wavelength spectrophotometer using the following wavelength pairs: cytochrome *b* (classical cytochrome *b*, b_{560}), 560 nm *minus* 575 nm; cytochrome b_{566}, 566 nm *minus* 575 nm; cytochrome $c_1 + c$, 550 nm *minus* 540 or 575 nm. Difference spectra were obtained by one of following two procedures. Procedure A was the two-wavelength/double beam method described previously[13]. In Procedure B difference spectra were obtained by scanning wavelengths, taking 575 nm as a reference wavelength.

RESULTS AND DISCUSSION

A highly reduced state of the respiratory chain components in mitochondria was induced by addition of succinate with rotenone *via* State 2 → 3 → 4 → 5 (ref. 19). On addition of ferricyanide to State 5 mitochondria cytochrome $c_1 + c$ was almost 100% oxidized in the presence of KCN, whereas a considerable proportion of the cytochrome *b* components remained in the reduced state (37% at 560 nm and 62% at 566 nm). Fig. 1 shows that the difference spectrum between mitochondria oxidized with ferricyanide and mitochondria in State 2 has a peak at 565 nm (Curve B) and the absorption due to b_{566} vanishes on adding uncouplers, such as flufenamic acid[14] (Curve C).

Subsequently, the effect of menadione on the redox state of cytochrome components was tested using various wavelength pairs, since menadione is known as a mediator of electron flow from various dehydrogenases by interaction with respiratory chain components on the substrate side of the antimycin site (*cf.* refs 15, 16). In the presence of rotenone, antimycin A and KCN, menadione added to anaerobic mitochondria oxidizing succinate induced three phases in the redox state of b_{566} after the rapid oxidation process, as shown in B, C and D in Fig. 2A. In phases B and C, cytochrome b_{566} was oxidized, whereas cytochrome $c_1 + c$, measured at 550–575 nm, was highly reduced. In phase D full reduction was observed for all components, such as b_{566}, b_{560} (not shown), cytochrome $c_1 + c$ and pigment-558 (refs 17, 8). The results obtained by the two-wavelength method were confirmed by the difference spectrum shown in Fig. 2B. The difference spectrum (before and after addition of menadione in phase C) indicated the oxidation of b_{566} and reduction of cytochrome $c_1 + c$, as shown in Curve C–A, though little change was observable in cytochrome b_{560}. Curve D–C indicates the reduction of

b_{566}, b_{560} and pigment-558 during the transition from phase C to phase D. The oxidation of b_{566} may be caused by the transfer of the reducing equivalents from b_{566} to menadione and the simultaneous reduction of cytochrome $c_1 + c$ is caused

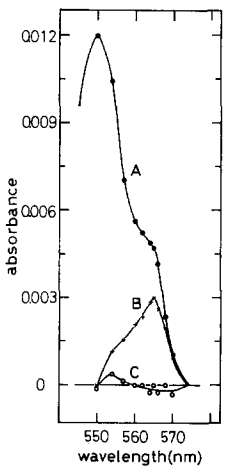

Fig. 1. Effect of ferricyanide on the cytochrome *b* spectrum in rat liver mitochondria. Ferricyanide (4 mM) was added to State 5 mitochondria in the presence of 3 mM KCN. State 5 was induced by 10 mM succinate with 4 μg rotenone in Medium A containing 500 μM ADP and 10 mM inorganic phosphate. $2.5 \cdot 10^{-4}$ M flufenamic acid was added after treatment with ferricyanide. Difference spectra were obtained by Procedure A, taking State 2 mitochondria as reference material. Rat liver mitochondria, 2.46 mg/ml. Curve A, State 5 mitochondria; Curve B, with ferricyanide; Curve C, with ferricyanide and flufenamic acid.

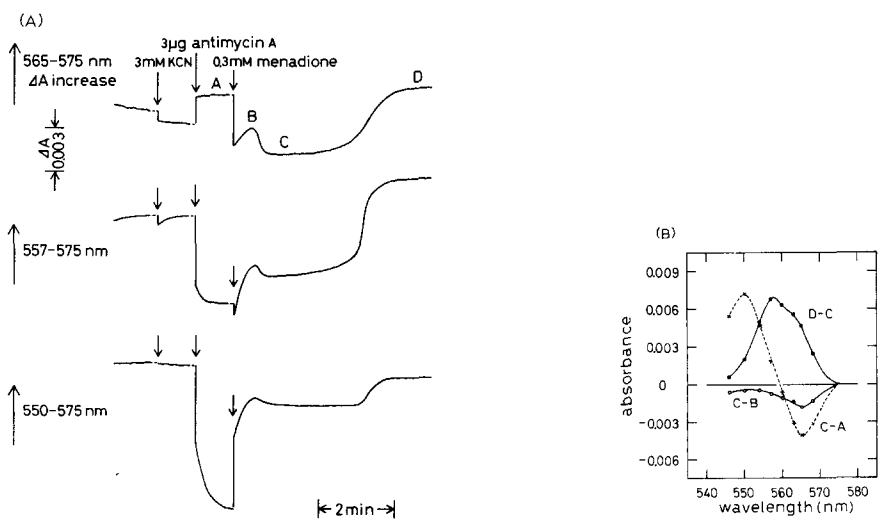

Fig. 2. (A) Effect of menadione on the redox state of cytochrome components. Rat liver mitochondria (7.0 mg/ml) were suspended in Medium B and State 5 was induced by 10 mM succinate with 4 μg rotenone. Menadione was added to State 5 mitochondria after addition of KCN and antimycin A. (B) Absorption spectra of cytochrome components. The wavelength scanning was performed point by point with a separate incubation for each wavelength couple taking 575 nm as reference wavelength. Curve C–B, b_{566} was oxidized after addition of menadione in phase B; Curve C–A, b_{566} was oxidized in phase C ($+\Delta A_{550\text{ nm}}/-\Delta A_{565\text{ nm}} = 1.7$; point of zero absorbance change, 559 nm); Curve D–C, cytochrome components were reduced in phase D.

by the acceptance of reducing power from succinate and also from b_{566} *via* menadione through an antimycin-insensitive path.

It is well known that ferricyanide is reduced preferentially by cytochrome *c* (ref. 18). When ferricyanide was added to a reaction system containing menadione, an electron flow occurred even in the presence of antimycin A. Fig. 3A shows that ferricyanide added in the reduced state scarcely affected b_{566} in the absence of antimycin A. However, oxidation of b_{566} occurred when ferricyanide (yellow) was converted to ferrocyanide (colorless). The difference in absorbance immediately after addition of ferricyanide and after its conversion to ferrocyanide (ferri = o) indicated oxidation of b_{566} and reduction of cytochrome $c_1 + c$, as shown in Curve B–A of Fig. 3B. Classical cytochrome *b* did not show an anomalous change in redox state under the same conditions, but changed like cytochrome $c_1 + c$, though to a lesser extent (not shown). This was also confirmed by the fact that the point of zero absorbance change was at 561 nm in Curve B–A. Antimycin A caused reduction of b_{566} and a decrease in absorbance at 552 nm, presumably due to oxidation of cytochrome c_1 and it induced a shift of the point of zero absorbance change from 561 nm to 559 nm (Curve C–B). Moreover, reduction of b_{566} on addition of ferricyanide was greater in the presence of antimycin A. Namely, the extent of the redox change of b_{566}, as a percentage of the total difference between the absorbance on reduction with $Na_2S_2O_4$ and on oxidation with rotenone in the

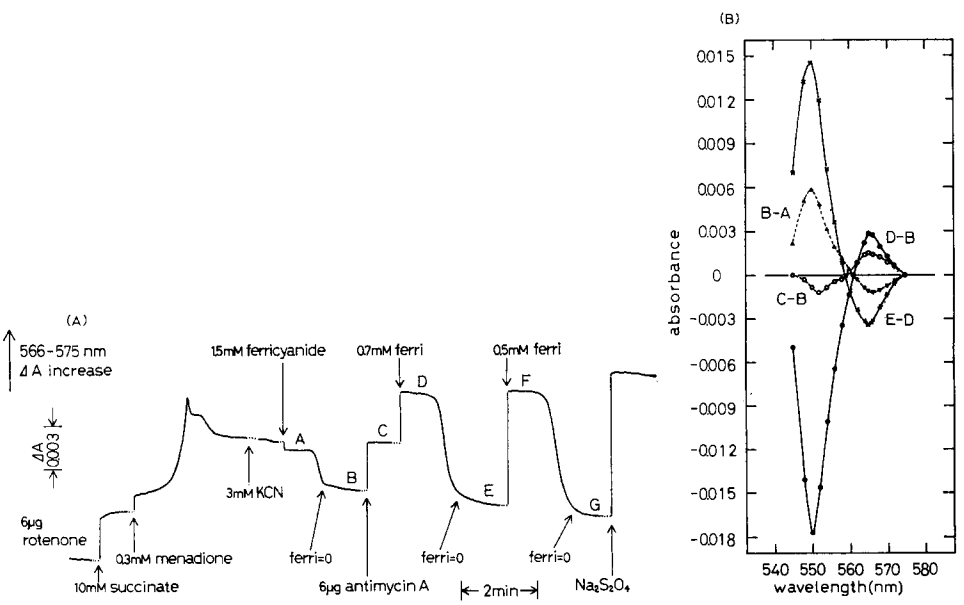

Fig. 3. (A) Effect of ferricyanide on the redox state of cytochrome b_{566} (measured at 566 nm *minus* 575 nm) in the presence of menadione. Rat liver mitochondria (6.8 mg/ml) were suspended in Medium B. (B) Absorption spectra of cytochrome components. Difference spectra were obtained by Procedure B, with 575 nm as the reference wavelength. The conditions were as for A except that 3.4 mg/ml of rat liver mitochondria were used. Curve B–A, b_{566} was oxidized on exhaustion of added ferricyanide ($+\Delta A_{550\ nm}/-\Delta A_{565\ nm} = 5.0$); Curve C–B, b_{566} was reduced by antimycin A (point of zero absorbance change, 559 nm); Curve D–B, b_{566} was reduced after ferricyanide ($-\Delta A_{550\ nm}/+\Delta A_{565\ nm} = 6.2$; point of zero absorbance change, 561 nm); Curve E–D, b_{566} was oxidized when ferricyanide was consumed ($+\Delta A_{550\ nm}/-\Delta A_{565\ nm} = 4.3$; point of zero absorbance change, 559 nm).

absence of substrate, was 20 % in the absence of antimycin A and 68 % in its presence. The redox change induced by ferricyanide was repeatable, as indicated in D, E, F and G in Fig. 3A and the reduction and oxidation of b_{566} were usually accompanied by oxidation and reduction, respectively, of cytochrome $c_1 + c$ (Curve D–B and Curve E–D in Fig. 3B). The redox change of b_{566} is probably not controlled by the ferricyanide/ferrocyanide ratio, since the amount of ferricyanide added did not influence the level of reduced b_{566} but only the duration of its reduced state, and since addition of ferrocyanide to b_{566} in the reduced state did not cause any change of the degree of reduction. Thus the transition of b_{566} from the reduced to the oxidized state is caused by a stoppage of electron flow resulting from exhaustion of the electron acceptor. In other words, the reduction of oxidation of b_{566} depends strictly on whether there is an electron flow through cytochrome $c_1 + c$ to ferricyanide.

There was a distinct difference between the absorbance change of b_{566} in the presence of pentachlorophenol (+ PCP, solid line in Fig. 4) and in its absence (– PCP, dotted line in Fig. 4) when antimycin A was absent. Namely, in the presence of the uncoupler the absorbance at 566 nm became much lower than in the system without uncoupler. This may be due to the inhibition by uncoupler of the energy-dependent reduction of b_{566}, as reported by several workers[20,21]. Then ferricyanide induced a decrease in absorbance at 566 nm and this was followed by an increase in absorbance on exhaustion of ferricyanide (A, B in Fig. 4). This change was different in direction from that in the uncoupler-free system, as shown by the dotted curve in Fig. 4 and was similar to those of b_{560} and cytochrome $c_1 + c$. Considering the contribution of the classical cytochrome b to the absorbance at 566 nm (refs 9, 21), it seems that this change is mainly due to the absorbance change of b_{560} at 566 nm, although spectral evidence for this is not yet available. As shown in D, E, F and G in Fig. 4, however, pentachlorophenol did not influence the ferricyanide-induced reduction of b_{566} in the presence of antimycin A. It was also noted that the oxidant-dependent reduction of b_{566} was hardly affected by previous addition of ATP or oligomycin to the reaction system containing menadione and antimycin A.

Results presented here suggest that the absorbance changes at 566 nm involve two types of redox change of cytochrome b_{566}, which may be described as the oxidant-induced reduction and the energy-dependent reduction, respectively. How-

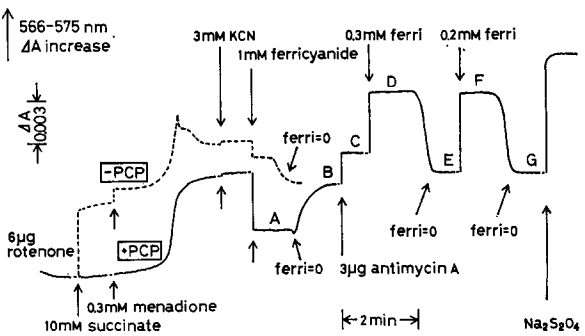

Fig. 4. Effect of uncoupler on the redox state of cytochrome b_{566}. Rat liver mitochondria (6.8 mg/ml) were suspended in Medium B. + PCP, 10 μM of pentachlorophenol.

ever, it remains uncertain whether they are based on different or essentially the same mechanisms. The oxidant-induced type was characterized by a larger redox change at 566 nm compared with the energy-dependent reduction. Moreover, the former change was usually accompanied by a redox change in the opposite direction of the counter component such as cytochrome $c_1 + c$, but little is known about behavior of the counter component(s), *e.g.* in ATP-dependent reduction of b_{566} (ref. 21). The presence of both menadione and antimycin A was required for demonstration of the typical type of oxidant-induced reduction in the present experiments. Under these conditions the redox change of b_{566} and cytochrome $c_1 + c$ could be repeated by addition of a limited amount of ferricyanide, since menadione induced an electron flow from substrate to ferricyanide in the presence of antimycin A. The energy-dependent change of b_{566} could be differentiated from the oxidant-dependent change by use of uncoupler in some cases, such as in Fig. 4, since uncoupler abolished the energy-dependent type and not the ferricyanide-induced reduction. However, it was rather difficult to distinguish the two types in systems without menadione and antimycin A, as seen in Curves B and C in Fig. 1. In these spectra, the identification of b_{566} by ferricyanide can probably be explained as the oxidant-induced type. The disappearance the b_{566} peak in the presence of uncoupler may be due to a release of the energy coupling at Site II, resulting in a slight reduction of cytochrome c_1 and oxidation of b_{566}, though the possibility of an uncoupler effect on the energy-dependent reduction can not be ruled out.

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