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EVIDENCE FOR THE OCCURRENCE IN SUBMITOCHONDRIAL PARTICLES OF A DUAL RESPIRATORY CHAIN CONTAINING DIFFERENT FORMS OF CYTOCHROME *b*

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SUMMARY

1. Addition of ascorbate + *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) to beef-heart submitochondrial particles (Mg–ATP particles) in the presence of KCN causes extensive reduction of cytochromes $c_1 + c$ and $a + a_3$, and a partial reduction of cytochrome *b*. Addition of ATP results in an increased cytochrome *b* reduction, with a slight decrease in the level of reduced cytochrome $a + a_3$. Subsequent addition of NAD^+ causes a further substantial reduction of cytochrome *b* and a partial oxidation of cytochrome $a + a_3$. The total amount of cytochrome *b* reduced at this stage is close to that obtained by adding NADH or succinate to the particles in the presence of KCN.

2. Cytochrome *b* reduced upon the addition of TMPD and NAD^+ has an absorption maximum at 562 nm, and that reduced upon the addition of ATP at 565 nm, with a shoulder at 558 nm. All three phases of cytochrome *b* reduction are inhibited by antimycin, and the ATP- and NAD^+-induced phases are also inhibited by FCCP and oligomycin. The NAD^+-induced cytochrome b_{562} reduction is ATP-dependent, and is abolished by rotenone and by pyruvate + lactate dehydrogenase, indicating that it proceeds by way of NADH generated through reverse electron transport *via* cytrochrome b_{565} (+ b_{558}). The effect of NAD^+ in inducing cytochrome b_{562} reduction is duplicated by fumarate in a non-additive fashion.

3. The three phases of cytochrome *b* reduction are accompanied by roughly proportional extents of reduction of ubiquinone and flavoprotein. The amounts of cytochrome *b*, ubiquinone and flavoprotein, reduced upon the addition of ascorbate + TMPD, ATP and NAD^+, are close to the total enzymically (NADH and succinate) reducible contents of these components in the particles.

4. The results are interpreted to indicate that submitochondrial particles contain a dual respiratory chain, one including cytochrome b_{565} (+ b_{558}) and a functional Coupling Site II, and another, including cytochrome b_{562} and no functional Coupling Site II. The possible significance of these results for the functional organization of mitochondria is discussed.

Abbreviations: TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

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INTRODUCTION

In recent years evidence has accumulated[1-21] for the occurrence in the mitochondrial respiratory chain of multiple forms or species of cytochrome *b*. These can be distinguished spectrophotometrically by the position of their α-bands. One of them has an α-band with a maximum at 562 nm, and is probably identical with the classical cytochrome *b* first described by Keilin[22]. Another type of cytochrome *b*, which may consist of two components[15,19], has α-bands with maxima at 565 and 558 nm. It has been proposed that the latter[4,5,12-14] or both[8-11,15,19] types of cytochrome *b* may participate in energy conservation in the cytochrome *b*→cytochrome c_1 span (Coupling Site II) of the respiratory chain, and that the two types of cytochrome *b* are members of the same electron-transport system, in which they interact directly in a sequential[12,21] or cyclic[8,20] fashion.

Over the past few years, the function of cytochrome *b* has been studied in this laboratory[23-28] particularly with respect to its relationship to ubiquinone. Recently we have briefly reported evidence[29] indicating that in submitochondrial particles the two types of cytochrome *b* are located in two separate electron-transport systems, only one of which, that containing cytochrome b_{565} (+ b_{558}), includes Coupling Site II. Both electron-transport systems were shown to contain flavoprotein and ubiquinone, and to be connected to cytochromes c_1, *c* and $a+a_3$. The purpose of the present paper is to describe in detail the evidence supporting this conclusion, and to discuss some of its implications for the functional organization of the mitochondrial respiratory chain.

MATERIALS AND METHODS

Submitochondrial particles from "heavy" beef-heart mitochondria were prepared by sonication in the presence of Mg^{2+} and ATP ("Mg–ATP particles") as described by Löw and Vallin[30]. After separation by differential centrifugation the particles were washed once with 0.25 M sucrose containing 10 mM $MgSO_4$ and the final pellet was suspended in the same medium to yield a protein concentration of 25–30 mg/ml.

Reactions were measured at 30 °C in an incubation medium consisting of 170 mM sucrose, 4 mM $MgSO_4$ and 50 mM Tris–acetate, pH 7.5. Further additions are specified in the table and figure legends.

Measurements of cytochromes, flavoprotein and NADH were carried out in an Aminco-Chance dual-wavelength spectrophotometer. Cytochrome $a+a_3$ was measured at 605–630 nm, cytochrome c_1+c at 553–540 nm, cytochrome *b* at 562–575 nm, flavoprotein at 475–510 nm, and NADH at 340–375 nm, using extinction coefficients[31-33] of 12.0, 19.1, 20.0, 10.6 and 6.2 $mM^{-1}\cdot cm^{-1}$, respectively. Ubiquinone and ubiquinol were measured after extraction from the particles by methanol–light petroleum as described by Kröger and Klingenberg[34]. In the absence of added substrate the particles were found to contain no ubiquinol. Protein was determined by the biuret method[35].

Tables I and II summarize some basic parameters of the particles that are of relevance in the present context. The contents of various respiratory-chain components, listed in Table I, are in fair agreement with those earlier reported[36] for a similar

TABLE I

RESPIRATORY-CHAIN COMPONENTS OF Mg–ATP PARTICLES

Enzymically reducible amounts of flavoprotein were determined in the presence of antimycin (1 μg/mg protein) by the sequential additions of 5 mM succinate and 1 mM NADH. The figure given below for NADH refers to the increase in flavoprotein reduction obtained after previous reduction by succinate. In the case of the other components, the enzymically reducible amounts were determined from the reduction obtained after the addition of NADH and/or succinate in the presence of 1.67 mM KCN. Particle-protein concentration was 1 mg/ml in a final volume of 3 ml. Chemically reducible amounts of flavoprotein and cytochromes refer to the total amounts reduced after addition of $Na_2S_2O_4$ to the particles in the absence of substrate. In the case of ubiquinone, reduction was done with KBH_4 after extraction of the quinone as described in ref. 34.

Component		*nmoles/mg protein*	
		Enzymically reducible	*Chemically reducible*
Flavoprotein	Succinate 0.29 NADH 0.10	0.39	0.86
Ubiquinone		5.0	5.5
Cytochrome *b*		0.52	0.71
Cytochrome $c_1 + c$		0.53	0.58
Cytochrome $a + a_3$		1.07	1.07

TABLE II

RESPIRATORY RATES AND RESPIRATORY-CONTROL RATIOS OF Mg–ATP PARTICLES

Respiratory rates were derived from the times required for anaerobiosis to occur, assuming linear rates of oxygen consumption. Reduction of cytochromes $a + a_3$ (605–630 nm) was used as the parameter measured. The reaction mixture consisted of 170 mM sucrose, 4 mM $MgSO_4$, 50 mM Tris–acetate, pH 7.5, 1 mg particle protein/ml, and 1 mM NADH, 5 mM succinate or 5 mM ascorbate + 0.3 mM TMPD. When indicated, 3 μg oligomycin and 1.6 μM FCCP were added. Final volume, 3 ml. Temp., 30 °C.

Substrate	*Respiratory rate (natoms O/min per mg protein)*			*Respiratory control ratio (C/B)*
	(A) None	*(B) Oligomycin*	*(C) Oligomycin + FCCP*	
NADH	209	180	710	3.9
Succinate	236	231	394	1.7
Ascorbate + TMPD	354	296	394	1.3

preparation (ETP_H). The particles exhibited respiratory control with NADH, succinate and ascorbate + *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) as substrates, and this was only little influenced by oligomycin (Table II). The respiratory-control ratios were similar to those reported[37] for EDTA particles in the presence of oligomycin.

All chemicals used were commercial products of highest available purity.

RESULTS

As shown in Fig. 1, addition of ascorbate + TMPD to Mg–ATP particles in the presence of KCN caused extensive reduction of cytochromes $a + a_3$ and $c_1 + c$,

and a partial reduction of cytochrome *b* (measured at 562–575 nm). The extent of this cytochrome *b* reduction increased with increasing concentration of TMPD and was prevented by antimycin (not shown). Addition of ATP resulted in an increased reduction of cytochrome *b* and a concomitant, slight oxidation of cytochrome $a + a_3$. Subsequent addition of NAD^+ caused a further, rapid oxidation of cytochrome $a + a_3$, and a significant further reduction of cytochrome *b*; the latter was relatively slow. The extent of reduction of cytochrome *b* in the presence of ascorbate, TMPD, ATP and NAD^+ was not increased significantly by the further addition of NADH and succinate, and was approximately equal to that obtained with either of the latter substrates when added alone or in combination with ascorbate and TMPD (Fig. 2); addition of ATP caused little increase in cytochrome *b* reduction (< 10 % measured at 562–575 nm) in the presence of succinate or NADH, in accordance with previous reports[8,9,18,38].

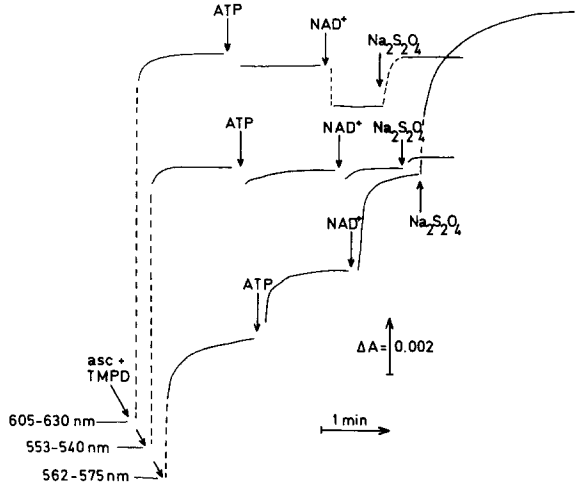

Fig. 1. Comparison of the redox states of cytochromes *b* (562–575 nm), $c_1 + c$ (553–540 nm) and $a + a_3$ (605–630 nm) after the addition of various compounds to Mg–ATP particles. The reaction mixture consisted of 170 mM sucrose, 4 mM $MgSO_4$, 50 mM Tris–acetate, pH 7.5, 1.7 mM KCN, and 1 mg Mg–ATP particles/ml. When indicated, 5 mM ascorbate (asc), 0.3 mM TMPD, 3.3 mM ATP, 16.7 μM NAD^+ and a few grains of solid $Na_2S_2O_4$ were added. Final volume, 3 ml. Temp., 30 °C.

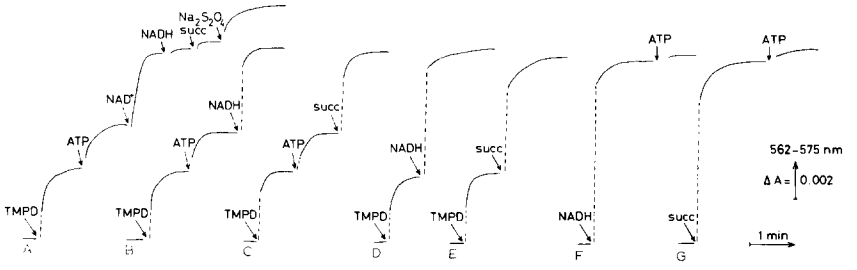

Fig. 2. Extents of cytochrome *b* reduction after various additions to Mg–ATP particles. In Traces A–E, 5 mM ascorbate was included in the reaction mixture. When indicated, 1 mM NADH and 5 mM succinate (succ) were added. Other conditions were the same as in Fig. 1.

The NAD+-induced reduction of cytochrome *b* in the presence of ascorbate and TMPD was dependent upon ATP as shown in Fig. 3. NAD+, in the absence of ATP, had no effect on the redox state of cytochrome *b*. However, subsequent addition of ATP resulted in the reduction of an amount of cytochrome *b* that was larger than

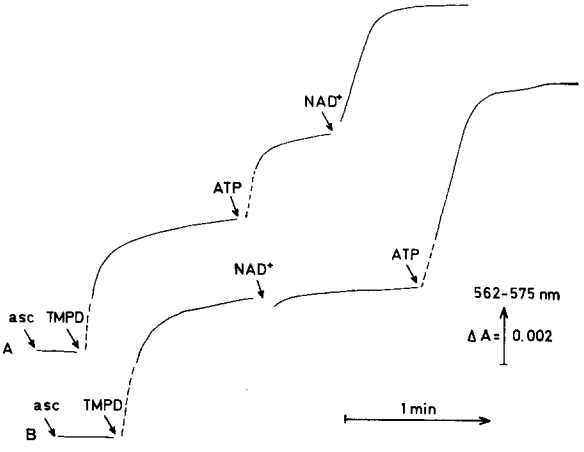

Fig. 3. ATP dependency of the NAD+-induced cytochrome *b* reduction. Conditions were the same as in Fig. 1, except that 1.2 mM ATP and 10 μM NAD+ were used.

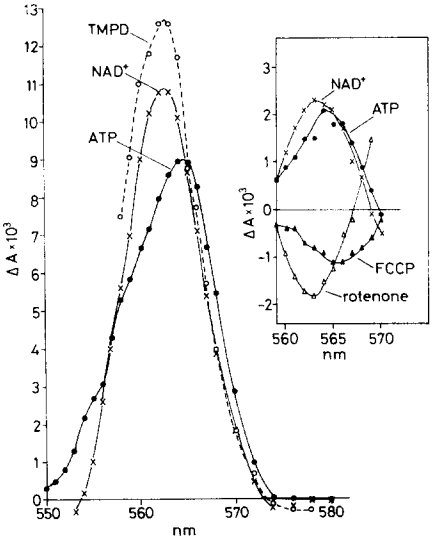

Fig. 4. Spectral analysis of cytochrome *b* reduced after various additions. The reaction mixture was the same as in Fig. 1, except that, in the main figure (not in the inset) the particle-protein concentration was 4 mg/ml. The spectra in each figure were obtained from the same sample after serial addition of the following reagents: main figure: 5 mM ascorbate, 0.3 mM TMPD, 3.3 mM ATP, 20 μM NAD+; inset: 5 mM ascorbate, 0.3 mM TMPD, 3.3 mM ATP, 20 μM NAD+, 3.2 μM rotenone, 1.6 μM FCCP. Curves marked TMPD, ATP, NAD+ (main figure), and ATP, NAD+, rotenone, FCCP (inset) are the difference spectra found after each addition, with the system prior to that addition as the reference. After each addition the sample was incubated for sufficient time to reach new steady state. The spectra were obtained by changing the measuring wavelength as indicated, with the reference wavelength fixed at 575 nm.

that obtained with ATP alone and approximately equal to the sum of the amounts of cytochrome *b* reduced upon the sequential addition of ATP and NAD^+. As will be shown later (*cf.* Fig. 7), the NAD^+-induced cytochrome *b* reduction, but not that induced by ATP, was prevented and reversed by rotenone, whereas both types of cytochrome *b* reduction were prevented and reversed by carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP). These findings suggested that the two types of cytochrome *b* reduction involved different components of cytochrome *b*.

In Fig. 4 it is shown that, indeed, the positions of the α-bands for the two *b*-type cytochromes were different. Cytochrome *b* reduced in the presence of ATP had an absorbance maximum near 565 nm, with a shoulder at 558 nm (*cf.* ref. 8), whereas that reduced in the presence of NAD^+ had a maximum near 562 nm; these will be referred to in the following as cytochromes b_{565} and b_{562}, respectively. Cytochrome *b* reduced by TMPD was of the cytochrome b_{562} type. As expected, reversal of the NAD^+-induced cytochrome *b* reduction by rotenone resulted in the disappearance of cytochrome b_{562}, and subsequent reversal of the ATP-induced cytochrome *b* reduction of FCCP resulted in the disappearance of cytochrome b_{565} (Fig. 4, inset).

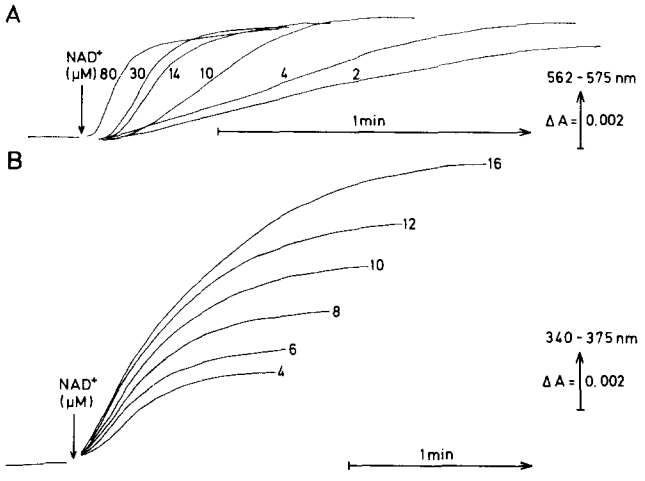

Fig. 5. Effect of increasing NAD^+ concentration on the reduction of cytochrome *b* and NAD^+. The experiment was performed as in Fig. 3, Trace A, except that the concentration of added NAD^+ was varied as indicated.

Reduction of cytochrome b_{562} required only catalytic amounts of NAD^+ (Fig. 5). Concentrations of NAD^+ from 2 to 80 μM resulted in the same steady-state levels of cytochrome b_{562} reduction, even though these concentrations were limiting as shown by the concentration dependency of the rates both cytochrome *b* and NAD^+ reduction. Low concentrations of NAD^+ were used throughout the present experiments since it was found that commercial preparations of NAD^+ contained a component capable of reducing cytochromes $c_1 + c$ and $a + a_3$ in KCN-blocked particles, when NAD^+ was added at a concentration of 1 mM. This component also reduced cytochrome *b* in a transient fashion, and the extent of reduction was enhanced by antimycin.

The NAD^+-induced reduction of b_{562} was prevented when an NAD^+-generating system [pyruvate + lactate dehydrogenase (L-lactate: NAD^+ oxidoreductase,

EC 1.1.1.27)] was included in the incubation mixture (Fig. 6). Furthermore, when the NAD^+-generating system was added after completion of the NAD^+-induced b_{562} reduction, reoxidation of the cytochrome occurred. These data indicated that b_{562} reduction took place by way of NADH which is generated during reverse electron transport from cytochromes $c_1 + c$ *via* b_{565}, and thus, that cytochromes b_{562} and b_{565} may be located in two separate electron-transport chains (*cf.* Fig. 11).

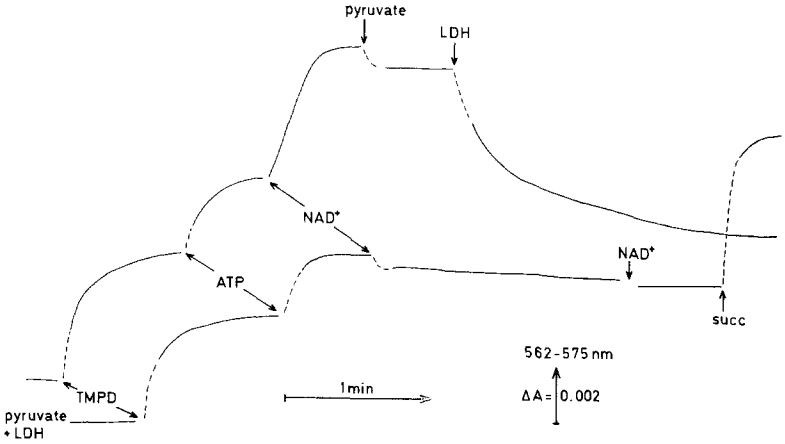

Fig. 6. Effect of pyruvate and lactate dehydrogenase on the NAD^+-induced reduction of cytochrome *b*. Conditions were the same as in Fig. 3. Ascorbate, 5 mM, was present in the incubation mixture. When indicated, 6.7 mM pyruvate and 18 units crystalline pig-heart lactate dehydrogenase (LDH) (Boehringer) were added; succ = succinate.

As would be expected under these conditions, the NAD^+-dependent reduction of b_{562} was prevented by rotenone (Fig. 7A), which blocks electron transfer from b_{565} to NAD^+, probably between ubiquinone and NADH dehydrogenase [NADH: (acceptor) oxidoreductase, EC 1.6.99.3]. Antimycin, which inhibits electron-transfer between cytochromes c_1 and *b*, caused a reoxidation of the latter (Fig. 7B) presumably by way of ubiquinone (*cf.* Fig. 9). Accordingly, subsequent addition of NAD^+ resulted in some reduction of b_{562}. The uncoupler, FCCP, and the energy-transfer inhibitor, oligomycin, prevented, as expected, the NAD^+-dependent b_{562} reduction, and also caused a reoxidation of b_{565} (Figs 7C and 7D); the latter was relatively rapid in the case of FCCP and slow in the case of oligomycin, and was in both cases accompanied by an increased reduction of cytochromes $a + a_3$ (not shown).

When added after NAD^+, rotenone caused a slow but complete reversal of the NAD^+-induced reduction of b_{562} (Fig. 7E; *cf.* also Fig. 4, inset). Addition of antimycin after NAD^+ resulted in a relatively rapid reoxidation of a part of cytochrome *b*, followed by a lag period and a subsequent, slow further oxidation (Fig. 7F). FCCP and oligomycin, when added after NAD^+, caused a reoxidation of cytochrome *b* only after a well-marked lag period (Figs 7G and 7H). The length of this lag period increased with the duration of the previous presence of NAD^+ (Fig. 8), and presumably reflects the time required for the NADH accumulated to become reoxidized through the KCN-inhibited respiratory chain. A residual rate of NADH oxidation was observed even in the presence of KCN and rotenone, corresponding to approx. 0.2 % of that of the uninhibited NADH oxidase. It may also be seen in Figs 7 and 8 that the addition

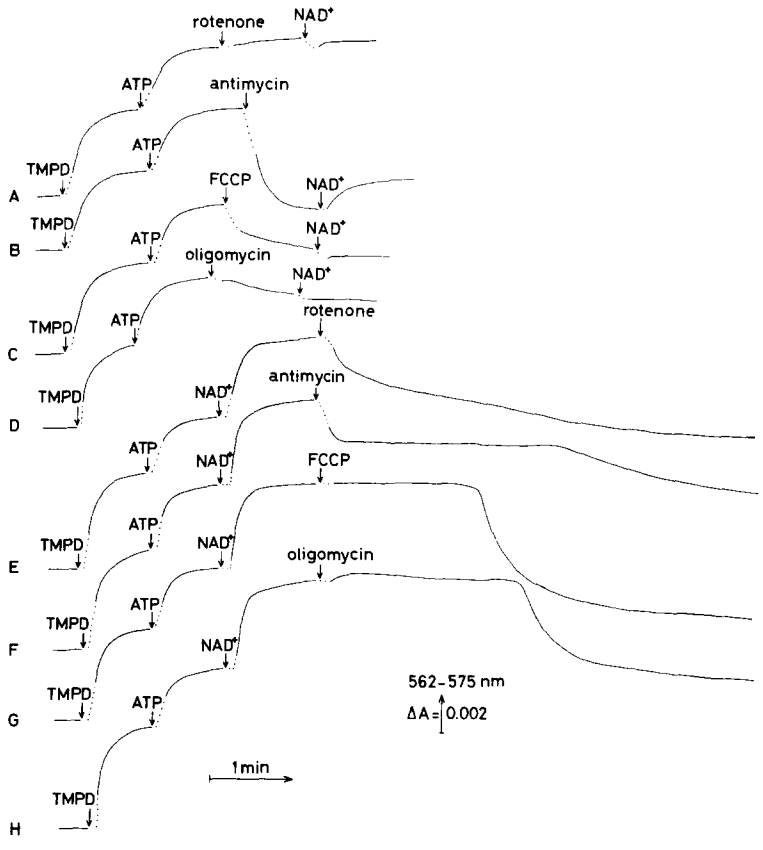

Fig. 7. Effects of rotenone, antimycin, FCCP and oligomycin on the ATP- and NAD^+-induced reduction of cytochrome *b*. Conditions were the same as in Fig. 3. Ascorbate, 5 mM, was present in the incubation mixture. When indicated, 3.2 μM rotenone, 0.8 μg antimycin, 1.2 μM FCCP, and 3 μg oligomycin were added.

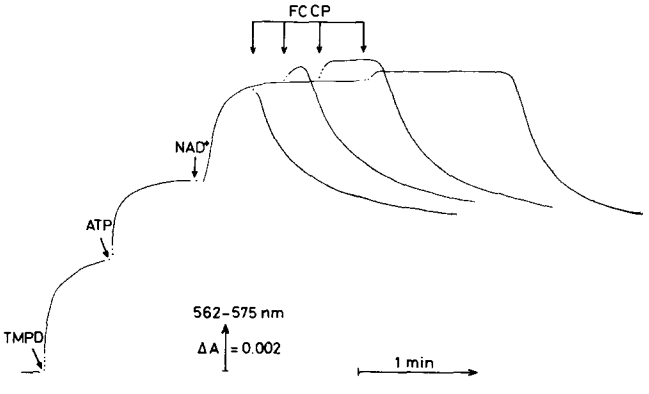

Fig. 8. Effect of FCCP added after different time periods on the ATP- and NAD^+-induced cytochrome *b* reduction. Conditions were the same as in Fig. 6. At the time indicated 1.2 μM FCCP was added to each sample.

of oligomycin and FCCP following the addition of NAD^+ occasionally resulted in a transient increase in cytochrome *b* reduction. The possible significance of this phenomenon will be considered later (*cf.* Discussion).

Fig. 9 shows that addition of NAD^+ to ATP-energized particles in the presence of KCN, ascorbate and TMPD resulted in a reduction not only of cytochrome *b* but also of ubiquinone and flavoprotein. The data are expressed as the percent of total component in the reduced state. In the case of ubiquinone, the percentage of reduction was determined after extraction of the quinone as described in Materials and Methods. In the case of cytochrome *b* and flavoprotein, the amount reduced after addition of sodium dithionite was taken as 100 %.

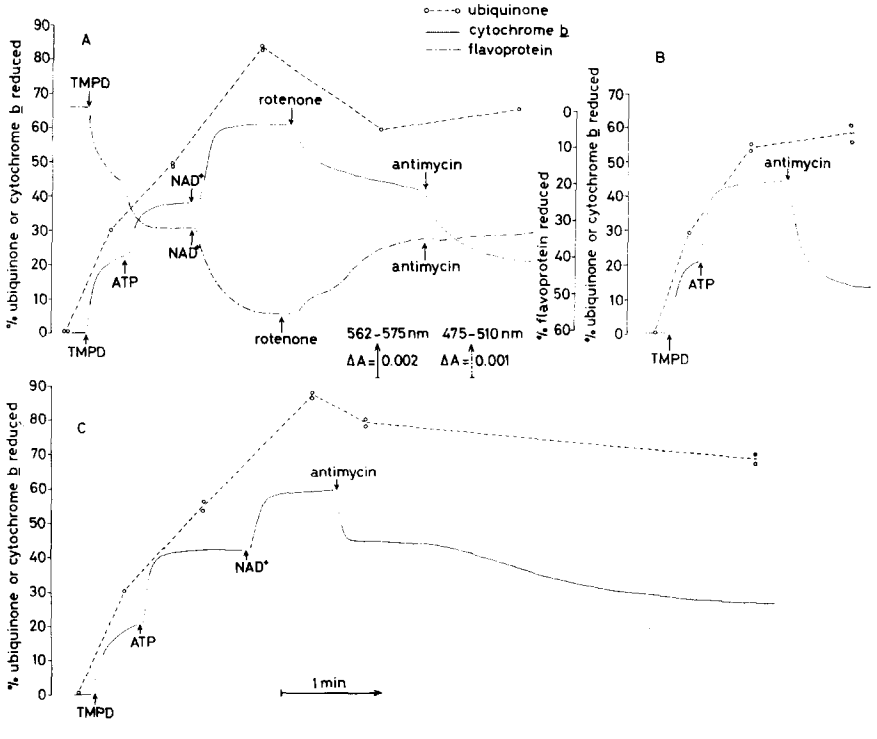

Fig. 9. Effects of various compounds on the reduction of cytochrome *b*, ubiquinone and flavoprotein in Mg–ATP particles. Conditions were the same as in Fig. 3. Ascorbate, 5 mM, was present in the incubation mixture. When indicated, 3.2 μM rotenone and 0.8 μg antimycin were added. Samples for ubiquinone determination were removed at the times indicated.

Addition of TMPD in the presence of ascorbate and KCN resulted, as already shown (*cf.* Figs 1–3), in a partial reduction of b_{562}, accompanied by a likewise partial reduction of ubiquinone and flavoprotein (Fig. 9A). Subsequent addition of ATP caused a reduction of b_{565} (*cf.* Figs 1–3), as well as a further reduction of ubiquinone and flavoprotein. When NAD^+ was now added, b_{562} was further reduced (*cf.* Figs 1–3), and this again was accompanied by a further reduction of both ubiquinone and flavoprotein. At this stage, about 60 % of the total cytochrome *b*, 80 % of the ubiquinone and 50 % of the flavoprotein were in the reduced state. These precentages were close

to the enzymically (succinate + NADH) reducible portions of these components (*cf.* Table I).

The portions of cytochrome *b*, ubiquinone and flavoprotein, reduced upon the addition of NAD^+, represent those which are available to NADH formed by reverse electron transport, and which are not available under the prevailing conditions to reducing equivalents coming directly from cytochrome c_1. These results suggested that b_{562} is associated with an electron-transport system separate from that containing b_{565}, and that both systems contain ubiquinone and flavoprotein.

As expected, rotenone, when added after the addition of NAD^+, reversed the effects of the latter (Fig. 9A). The reoxidation of flavin was clearly biphasic. Rotenone did not induce reoxidation of cytochrome b_{565}, or of those portions of cytochrome b_{562}, ubiquinone and flavoprotein which had been reduced prior to the addition of NAD^+.

Addition of antimycin, either after rotenone (Fig. 9A) or in the absence of NAD^+ (Fig. 9B) caused a reoxidation of cytochrome b_{565}, accompanied by a further reduction of ubiquinone, and no change in the redox state of flavoprotein. These results show that in the absence of reducing equivalents from NADH, antimycin brings about a re-equilibration between b_{565} and ubiquinone. A similar, antimycin-induced alteration of the equilibrium between cytochrome *b* and ubiquinone has recently been described by Boveris *et al.*[40]. When antimycin was added after the addition of ATP and NAD^+, in the absence of rotenone (Fig. 9C), the relatively rapid reoxidation of b_{565} was followed, as already shown (*cf.* Fig. 7F), by a slow reoxidation of b_{562}, and was accompanied by a likewise slow reoxidation of ubiquinone.

In view of the biphasic reoxidation of flavoprotein upon the addition of rotenone following reduction in the presence of NAD^+ (*cf.* Fig. 9A) it was of interest to test the effect of fumarate in replacing NAD^+. Indeed, as shown in Fig. 10, addition of fumarate to the particles in the presence of KCN, ascorbate, TMPD and ATP resulted in a reduction of cytochrome *b*, ubiquinone and flavoprotein. However, the effects of fumarate occurred much more slowly than those of NAD^+, and subsequent addition

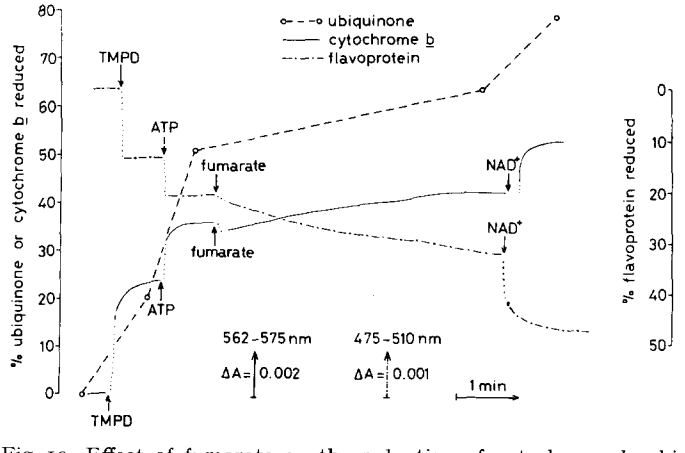

Fig. 10. Effect of fumarate on the reduction of cytochrome *b*, ubiquinone and flavoprotein in Mg–ATP particles. Conditions were the same as in Fig. 1. Ascorbate, 5 mM, was present in the incubation mixture. When indicated, 0.4 mM fumarate was added. Samples for ubiquinone determination were removed at the times indicated.

of NAD^+ resulted in a further substantial reduction of all three components. The final steady states were equal to those reached with NAD^+ alone (not shown).

DISCUSSION

Our interpretation of the results reported in this paper is summarized schematically in Fig. 11.

The results strongly suggest that Mg–ATP particles from beef-heart mitochondria contain two separate systems for electron transport between substrates (NADH and succinate) and cytochrome c_1. The two systems involve different forms or species of cytochrome b. One involves cytochrome b_{565} $(+ b_{558})$, presumably identical with the cytochrome b_T of Chance *et al.*[12], which has a relatively low midpoint potential[13], and the reduction of which by ascorbate + TMPD requires ATP.

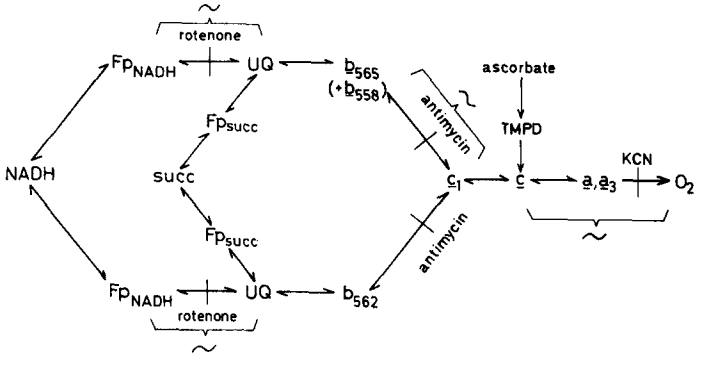

Fig. 11. Hypothetical scheme of electron-transport pathways in Mg–ATP particles. Location of energy-coupling sites is indicated by ~. For further explanation, see text. Fp = flavoprotein; UQ = ubiquinone; succ = succinate.

This reduction is inhibited by FCCP, oligomycin and antimycin, and is probably due to an energy-linked reversal of electron transport from cytochrome c_1 to cytochrome b_{565} $(+ b_{558})$ over Coupling Site II. Whether cytochromes b_{565} and b_{558} represent one[38] or two[15,19] molecular entities, and whether their reduction by cytochrome c_1 proceeds by way of a "high-potential" form of the b cytochrome(s) as postulated by Wilson and Dutton[14] (*cf.*, however, ref. 41), or an alkalinization of the membrane by ATP as recently suggested by Azzi and Santato[18], cannot be decided from the present data. This system will be referred to below as the cytochrome b_{565} pathway.

The second system involves cytochrome b_{562} presumably identical with Keilin's[22] cytochrome b or the cytochrome b_K of Change *et al.*[12], and will be referred to below as the cytochrome b_{562} pathway. This b cytochrome is reduced partially by ascorbate + TMPD, and its degree of reduction is not increased by the addition of ATP, but is increased by the subsequent addition of NAD^+. The ascorbate + TMPD-induced cytochrome b_{562} reduction seems to proceed *via* cytochrome c_1, as indicated by its sensitivity to antimycin. Its extent can be increased by increasing the TMPD concentration. This reduction thus appears to take place by simple equilibration between cytochromes c_1 and b_{562} (the latter having a relatively high midpoint potential as

compared to cytochrome b_{565} ($+ b_{558}$); *cf.* refs 13 and 42), without the involvement of ATP-linked energy coupling. The NAD^+-induced b_{562} reduction is abolished by pyruvate + lactate dehydrogenase, as well as by agents that would be expected to inhibit NADH formation through ATP-dependent reversal of electron transport from cytochrome c_1 to NAD^+, such as FCCP, oligomycin, antimycin and rotenone. This reduction thus appears to proceed by way of NADH formed through reverse electron transport *via* the b_{565} pathway. Of the inhibitors mentioned, rotenone abolishes the NAD^+-induced b_{562} reduction in two ways, by blocking both NAD^+ reduction by b_{565} ($+ b_{558}$), and b_{562} reduction by NADH, as will be discussed below.

Our data concerning ubiquinone and flavoprotein are consistent with the proposed scheme, by showing that both the ATP-induced reduction of b_{565} ($+ b_{558}$) and the TMPD- and NAD^+-induced reductions of b_{562} were accompanied by the reduction of ubiquinone and flavoprotein. The flavoproteins on both pathways seem to include both NADH dehydrogenase and succinate dehydrogenase (succinate: (acceptor) oxidoreductase, EC 1.3.99.1), as indicated by the findings that fumarate duplicated the effect NAD^+ in inducing b_{562} reduction, and that the two effects were not additive. The involvement of two flavoproteins is also indicated by the biphasic flavin oxidation following the addition of rotenone to the NAD^+-supplemented system (*cf.* Fig. 9A). The first phase presumably involves the reoxidation of succinate dehydrogenase located on the b_{562} pathway, the reduction of which by NADH is blocked by rotenone, whereas NADH dehydrogenase is reoxidized only when the NADH accumulated had disappeared *via* KCN and rotenone leaks.

The amounts of flavoprotein and ubiquinone associated with the two pathways appear to be roughly proportional to the amounts of cytochrome *b*, and account together for nearly all enzymically reducible flavoprotein, ubiquinone and cytochrome *b* present in the particles. It is quite possible, in fact probable, that the measurements regarding flavoprotein include nonheme iron as well, which may contribute to the absorbance changes observed at the wavelengths here employed (*cf.* ref. 43). An attempt to evaluate this contribution and, in general, to elucidate the role of nonheme iron in the two pathways is in progress by means of EPR spectroscopy.

The reduction of NAD^+ by cytochrome c_1 *via* the b_{565} pathway required energy supply at both Coupling Sites I and II. When ATP was replaced by alkali, this resulted in a reduction of b_{565} ($+ b_{558}$), in accordanc with Azzi and Santato[18], but not a reduction of NAD^+; evidently, reversal at Coupling Site I does not occur under these conditions. The occurrence of a coupling site between NAD^+ and ubiquinone on the b_{562} pathway is not clearly shown by the present data, but indications for this may be deduced from the effect of FCCP in reversing b_{562} reduction in the presence of NAD^+. The transient increase in b_{562} reduction observed under these conditions (*cf.* Fig. 8) may be interpreted as the result of an increased electron flux from NADH to b_{562} (as long as NADH is present) due to uncoupling of an energy-conservation site between NADH dehydrogenase and ubiquinone on the b_{562} pathway. Thus, the b_{562} pathway seems to have a functional Coupling Site I but not Coupling Site II.

Whether the two pathways link to a common $c_1 \rightarrow c \rightarrow a,a_3$ chain (as indicated in Fig. 11) or two separate chains, cannot be decided at the present stage. Earlier observations[37] that, in energized particles in the presence of partially inhibitory concentrations of KCN, the reductions of $c_1 + c$ and $a + a_3$ by NADH and succinate are biphasic, may be taken as an indication for separate chains. Chance *et al.*[44] have

provided additional kinetic evidence for two cytochrome chains in submitochondrial particles. Davis and Hatefi[17] have reported that upon fractionation of submitochondrial particles into so-called complexes, a short-wavelength cytochrome *b* (with an α-peak at 560–561 nm) is found in Complex II, together with succinate dehydrogenase, whereas b_{565} and b_{558} are recovered in Complex III, together with cytochrome c_1. More recently, however, Davis *et al.*[45] concluded that both b_K and b_T are present in Complex III; the short-wavelength *b* in Complex II may be identical with the succinate-reducible cytochrome *b* found in ubiquinone-deficient submitochondrial particles, earlier described in this laboratory[23]. Our finding that antimycin inhibits the reduction of both b_{562} and b_{565} $(+ b_{558})$ by reduced cytochrome c_1 is consistent with the notion[46] that antimycin acts on a component not identical with cytochrome *b* but associated with Complex III and present in a molar amount equivalent to cytochrome c_1. Indications of a "U-turn" in the substrate → *b* sequent of the respiratory chain, similar to that described here, has been reported by Hinkle *et al.*[47], but this has not been interpreted in terms of two separate electron-transport systems. Slater[20] has recently considered the existence of two electron-transport chains, involving different types of cytochrome *b*, but these were envisaged to interact with each other in a cyclic fashion, with a direct electron transfer between the two *b* cytochromes.

A question of considerable interest is whether the two electron-transport pathways here described are normal constituents of intact mitochondria, or whether they are only found in submitochondrial particles. Such an artifact might arise from fragmentation of mitochondria resulting, for example, in two populations of submitochondrial particles. Both of these would have a full respiratory chain, but one would contain b_{565} $(+ b_{558})$, with a regular Coupling Site II, whereas the other would contain b_{562}, with no functional Coupling Site II. Linkage of reverse electron transport through the first type of chain to forward electron transport through the second would require an external electron mediator such as NAD^+ or fumarate. The occurrence of such an artifact, however, appears unlikely for the following reasons: (1) Intact mitochondria contain the same types of cytochrome *b* as do submitochondrial particles[15], and even though the midpoint potentials of these appear to be somewhat shifted upon fragmentation[13], in both cases b_{565} $(+ b_{558})$ is involved in Site II energy coupling whereas b_{562} is not[12]. (2) Both in mitochondria[15] and submitochondrial particles[15] (*cf.* also Fig. 2), substrates can fully reduce the different types of *b* cytochromes. If, as postulated by Chance *et al.*[12] on the basis of studies with intact mitochondria, the energy-transducing and non-energy-transducing *b* cytochromes would be located sequentially on one chain, the oxidation of both (by O_2) should be slow in the energized state and accelerated upon uncoupling. The same should hold if both cytochromes b_{562} and b_{565} $(+ b_{558})$ would be involved in energy transduction, as envisaged by Slater *et al.*[8]. As revealed by the published data[12], only b_{565} $(+ b_{558})$ showed a slow rate of oxidation in the energized state which was greatly enhanced upon uncoupling, whereas the oxidation of b_{562} was rapid in both states. (3) The particles used in the present study exhibited respiratory-control efficiencies comparable to the parent mitochondria. Earlier attempts[37] to separate populations of submitochondrial particles with different respiratory-control efficiencies by means of density gradient centrifugation gave negative results. It should finally be pointed out that the fact that the b_{562} pathway lacks Coupling Site II, is not necessarily in conflict with its possible occurrence in intact mitochondria, since, in most mito-

chondrial preparations, NADH and succinate are not oxidized at full phosphorylating efficiency, *i.e.*, with a P/O ratio of 3.0 and 2.0, respectively.

It would appear from the above points that the present findings cannot be explained in terms of two populations of particles and, consequently, that the two electron-transport systems are present in the same membrane. Alternatively, one would have to assume that the mitochondrial preparation from which the particles are derived contains two functionally separate types of inner membrane, or even two types of mitochondria, differing in their cytochrome *b* components.

In conclusion, the present results indicate the existence in the mitochondrial inner membrane of two electron-transport chains, with different forms or species of cytochrome *b*, and where only one contains Coupling Site II, as well as the occurrence of two pools of ubiquinone and flavoprotein. These results seem to open important new aspects of mitochondrial structure and function which deserve further exploration.

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