The Respiratory Chain of Plant Mitochondria

XII. SOME ASPECTS OF THE ENERGY-LINKED REVERSE ELECTRON TRANSPORT FROM THE CYTOCHROMES $c$ TO THE CYTOCHROMES $b$ IN MUNG BEAN MITOCHONDRIA

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ABSTRACT

The cytochromes $c$ of mung bean (Phaseolus aureus) mitochondria become reduced when sulfide, a cytochrome oxidase inhibitor free from uncoupling side effects, is added to the aerobic mitochondrial suspension in the absence of added substrate. The cytochromes $b$ remain largely oxidized. Subsequent addition of ATP results in partial oxidation of the cytochromes $c$ and partial reduction of the cytochromes $b$ due to ATP-driven reverse electron transport through the second site of energy conservation, or coupling site, of the respiratory chain. Cytochrome $a$ is also oxidized under these conditions, but there is no concomitant reduction of the flavoprotein components, of ubiquinone, or of endogenous pyridine nucleotide. The reaction is abolished by oligomycin. The reducing equivalents transported from the cytochromes $c$ and $a$ in ATP-driven reverse electron transport are about 2-fold greater than those which appear in the cytochromes $b$. It is suggested that the equivalents not accounted for are present in a coupling site enzyme at the second site of energy conservation which interacts with the respiratory chain carriers by means of the dithiol-disulfide couple; this couple would not show absorbance changes with redox state over the wavelength range examined. With succinate present, reverse electron transport can be demonstrated at both coupling sites in both the aerobic steady state and in anaerobiosis. ATP-driven reverse electron transport in anaerobiosis maintains cytochrome $a$ $30\%$ oxidized while endogenous pyridine nucleotide is $50\%$ reduced.

When mung bean mitochondria, oxidizing succinate in the presence of sulfide through the alternate, cyanide- and sulfide-insensitive terminal oxidase, become anaerobic, cytochrome $b_{557}$, which has remained largely oxidized, becomes slowly reduced. The slow reduction is observed in coupled, energized mitochondria and in uncoupled mitochondria; the time course parallels the reduction of cytochrome $a$, under the same conditions. It appears that sulfide-ligated, oxidized cytochrome $a_2$ may be in close enough proximity to cytochrome $b_{557}$ in the membrane to inhibit the reduction of the latter.

Electron transport from substrate to oxygen through the coupled mitochondrial respiratory chain conserves the available free energy in a way which can then be utilized for ATP formation from ADP and $P_i$; the reaction is reversible, in that input of free energy in the form of ATP can reverse the flow of electrons through the respiratory chain carriers (7, 11, 26). Reversed electron transport can also utilize directly the high energy intermediates generated during coupled substrate oxidation as was shown some years ago in mammalian mitochondria (5, 8, 12--15, 17, 28) for the energy-linked reduction of endogenous pyridine nucleotide by succinate. This reaction has recently been studied in some detail in plant mitochondria (39, 41), using mitochondria from etiolated mung bean (P. aureus) seedlings, and two paths for the reduction were found. One involves the first energy conservation site of the respiratory chain and is entirely analogous to the pathway in mammalian mitochondria. The second pathway operates independently of the respiratory chain and of $a$ in the mitochondria of endogenous NAD from m.dite derived in turn from the fumarate formed by succinate oxidation. The second pathway is inoperative in the absence of added $P_i$, so that it does not interfere with studies of the interaction of ATP with the plant respiratory chain, which are of necessity carried out in the absence of $P_i$ to maintain a high phosphate potential (17).

The reduction of endogenous NAD by succinate in mung bean mitochondria treated with sulfide and mCLAM$^2$ to inhibit the two terminal oxidases (31) and utilizing ATP as the free energy source demonstrates the activity of the first energy conservation site between the respiratory chain's NADH dehydrogenase and the ubiquinone/flavoprotein/cytochrome $b$ region of the chain. Succinate is required for this reaction; no reduction of NAD occurs with ATP alone, in contrast to the situation observed in pigeon heart mitochondria (11, 17) where endogenous cytochrome $c$ can provide the reducing equivalents when it is in turn reduced in the presence of added sulfide. The oxidation with added ATP of the cytochromes $c$ reduced in anaerobiosis by endogenous substrate has also been demonstrated in mung bean mitochondria (20); this oxidation was accompanied by a concomitant reduction of the cytochromes $b$.

The second energy conservation site in plant mitochondria has been located between the $b$ cytochromes and the $c$ cytochromes by application of the crossover theorem (16, 19) to the observed changes in redox state of these carriers during the state 4 to state 3 transition (4) and during the state 4 to uncoupled state transition (43). This location is also supported by measurement of the midpoint potentials of these cytochromes (20). The observed oxidation of the cytochromes $c$ and reduction of cytochromes $b$ on addition of ATP to anaerobic mitochondria implies that the redox state of the carriers 1

$^1$ The phosphate potential is given by the free energy term RT

$\ln \{(ATP)/(ADP)\cdot(P_i)\};$ as used in this paper, the term high phospho-

$^2$ Abbreviations: E$_{mT'}$ Oxidation/reduction midpoint potential |

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at the second coupling site can equilibrate with the phosphate potential with relatively little interference from the first coupling site. This reaction could thus provide an additional probe for studying the second coupling site and, hopefully, clarify the role of the three cytochromes \( b \). This paper reports a study of the reverse electron transport reaction between the \( c \) and \( b \) cytochromes with ATP as the source of energy.

**MATERIALS AND METHODS**

Mitochondria were prepared from the hypocotyls of 5- or 6-day-old etiolated seedlings of mung bean (Phaseolus aureus), using substantially the method described by Bonner (3) and Ikuma and Bonner (24), with the modifications of Storey and Bahr (42). The mitochondria were assayed for respiratory control in a medium containing 0.3 M mannitol, 10 mM TES, 5 mM \( P \), and brought to pH 7.2 with KOH. This medium is designated TP; the same medium without phosphate is designated T. All experiments in this paper were carried out in medium T, unless otherwise specifically noted. Oxygen consumption by the mitochondrial suspension with succinate or malate as substrate was measured in a closed cuvette with a Clark electrode (Yellow Springs Instrument Co.) as described by Estabrook (23). Mitochondrial protein content was determined by a modified Lowry method (29).

Adenine nucleotides and NADH were obtained from Boehringer Mannheim Corp.; succinic acid from Aldrich Co.; sodium sulfide, potassium cyanide, and mannitol from J. T. Baker Chemical Co. These were used without further purification. The uncoupler 1799 was generously supplied by Dr. Peter Heytlery of E. I. du Pont de Nemours Co., and a sample of mCLAM was generously provided by Dr. Gregory Schonbaum of the University of Alberta.

Absorbance changes corresponding to the reduction or oxidation of the respiratory chain carriers were recorded using a dual wavelength spectrophotometer (6) with a compensation circuit to reduce noise from light source fluctuations (18). The following wavelength pairs were used for the various carriers: 468 to 490 nm for flavoprotein; 282 to 295 nm for ubiquinone; 549 to 540 nm for \( c_{m7} \); 556 to 540 nm for \( b_{o7} \); 560 to 540 nm for \( b_{o7} \); and 565 or 566 to 540 nm for \( b_{o7} \). The subscripts give the reduced minus oxidized difference absorbance maxima for the cytochromes in spectra obtained at 77 K (32).

Fluorescence changes corresponding to the oxidation and reduction of low potential, fluorescent flavoprotein \( F_p \) (36, 40) were recorded by an Eppendorf fluorimeter equipped with an Heraeus (Hanau) mercury arc No. ST-75 as described in earlier papers (21, 40, 42). For this work, the primary filter for the excitation light was an interference filter with maximal transmittance at 463 nm with 10-nm band width; the transmittance of the secondary filter for the emitted light lay between 490 and 3000 nm.

Difference spectra of the mitochondrial suspensions were obtained at liquid nitrogen temperature with the split beam spectrophotometer described by Chance (6), using the technique developed by Estabrook (22) as subsequently modified by Bonner (1).

**RESULTS**

The cytochromes \( c \) and cytochrome \( a \) in aerobic mung bean mitochondria become partially reduced upon treatment with cyanide (37) or sulfide, the reducing equivalents coming from endogenous substrate. There is very little reduction of the cytochromes \( b \) or of flavoprotein under these conditions. Coupled mitochondria with cytochromes \( c \) partially reduced and with cytochromes \( b \) essentially all oxidized are a convenient system for examining reverse electron transport, since it is not necessary to work under anaerobic conditions as was done in the experiments previously reported (20). Oxidation of cytochrome \( c_{m7} \) by reverse electron transport utilizing free energy from ATP is shown in Figure 1A. Addition of sulfide to mung bean mitochondria suspended in medium free of \( P \), and treated with mCLAM to block the alternate, cyanide-insensitive oxidase (31) results in 50% reduction of the cytochrome \( c \). Addition of ATP causes some 60% oxidation of the cytochrome, and this effect is reversed by addition of the uncoupler 1799. Reduction is complete on the addition of NADH.

**FIG. 1.** Changes in redox state of cytochrome \( c_{m7} \) in mung bean mitochondria as monitored at 549 to 540 nm with the dual wavelength spectrophotometer. A: Reduction of \( c_{m7} \) by endogenous substrate of addition of sulfide, followed by oxidation upon addition of ATP due to energy-linked reversed electron transport. Addition of the uncoupler 1799 abolishes the energy-linked reaction and the cytochrome becomes reduced. Addition of NADH and succinate gives full reduction of \( c_{m7} \), and serves to quantitate the total amount in the mitochondrial suspension. B: The same reaction conditions are employed as in A, but cyanide is substituted for sulfide. C: The same reaction conditions as in A, but oligomycin is added prior to ATP.
and succinate. The extent of reduction attained in the presence of ATP and 1799 but absence of substrate is 76%, compared to the 60% observed in energy-depleted mitochondria (37). The use of cyanide (Fig. 1B) gives results similar to those obtained with sulfide (Fig. 1A), but the extent of cytochrome oxidation on addition of ATP is less, and the reduction on addition of uncoupler is more rapid and shows a slight overshoot. These differences are consistent with the known uncoupling effects of cyanide (25). For this reason, sulfide, known to have no uncoupling effects on pigeon heart mitochondria (17) and apparently also free of such effects on mung bean mitochondria, was used as the inhibitor for cytochrome oxidase throughout this study.

The oxidation of cytochrome c<sub>323</sub> by added ATP is inhibited by oligomycin, as shown in Figure 1C. In fact, isolation of the respiratory chain from intramitochondrial ATP by oligomycin results in a further reduction of the cytochrome in the absence of sulfide; addition of ATP and 1799 have little further effect.

The reduction of the cytochromes b accompanying the oxidation of the cytochromes c appears in the four experimental records of Figure 2, which were all obtained with the same mitochondrial preparation. Reduction of cytochrome c<sub>323</sub> on addition of sulfide and its oxidation on addition of ATP are shown in Figure 2A, with the use of the appropriate wavelength pair 549 to 540 nm. Cytochrome c<sub>323</sub> behaves identically under these experimental conditions. The same experiment is repeated in Figure 2B with the wavelength pair 556 to 540 nm which records the redox changes of b<sub>323</sub> but which also records in part the redox changes of c<sub>323</sub>; the slight increase in reduction on addition of ATP corresponds to b<sub>323</sub> reduction which leads to an increase in absorbance at 556 nm and to c<sub>323</sub> oxidation which leads to a decrease. The total absorbance change at this wavelength pair attributable to b<sub>323</sub> is about two times that attributable to c<sub>323</sub> (20); the resulting absorbance change observed is a small net increase in absorbance at 556 nm and virtually no change on addition of uncoupler. There is little interference from cytochrome c<sub>323</sub> with the absorbance changes recorded at 560 to 540 nm, the wavelength pair suitable for monitoring the redox state of cytochrome b<sub>323</sub>. A definite reduction of this cytochrome is evident on ATP addition (Fig. 2C), which is reversed by uncoupler. The wavelength pair 566 to 540 records changes in the redox state of cytochrome b<sub>323</sub> with some interference from c<sub>323</sub>. The experiment using this wavelength pair is shown in Figure 2D. Addition of ATP does give some reduction of b<sub>323</sub>, but the slow phase seen with b<sub>323</sub> (Fig. 2C) is missing. Uncoupler causes rapid reoxidation, and subsequent succinate addition causes little further reduction of this cytochrome (Fig. 2D).

Addition of succinate to the mung bean mitochondria after uncoupler results in essentially complete reduction of the cytochromes c and b<sub>323</sub> (Fig. 2, A and B). But the reduction of b<sub>323</sub> by succinate is somewhat slower and incomplete (Fig. 2C) when interference from c<sub>323</sub> with the absorbance changes at 560 to 540 nm is taken into account. There is very little reduction of b<sub>323</sub> by succinate under these conditions (Fig. 2D). These results are in good agreement with those obtained using energy-depleted mung bean mitochondria with cyanide as inhibitor of cytochrome oxidase and exogenous NADH as the source of reducing equivalents (37).

A difference spectrum obtained at room temperature showing the effect of adding sulfide to mung bean mitochondria suspended in medium T is shown in Figure 3A. The two cytochromes c and cytochrome a become reduced, yielding difference absorbance maxima at 550 and 600 nm. The presence of sulfide shifts the latter maximum 1 to 2 nm to shorter wavelengths; as pointed out by Chance et al. (9), all plant mitochondria examined so far have the reduced-minus-oxidized difference absorbance maximum of cytochrome a at 601 to 603 nm rather than at 605 nm as observed with mammalian mitochondria. There is a difference absorbance maximum at 482 nm which is partially attributable to the cytochromes, but must also contain some contribution from some other component, possibly an iron-sulfur protein. The minimum is not at the proper position for flavoprotein (10). Addition of ATP to the sulfide-containing suspension reduces the amplitude of the maximum attributable to cytochromes c and a, corresponding to oxidation, and a new absorbance band at 562 nm appears, corre-

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![Figure 2](image-url)  
*Fig. 2. Comparison of redox changes in cytochromes c<sub>323</sub>, b<sub>323</sub>, b<sub>566</sub>, and b<sub>553</sub> on initiation of reversed electron transport by addition of ATP to sulfide-treated mung bean mitochondria. The same mitochondrial suspension was used in all four experiments. Note that the records C and D were obtained at twice the recording sensitivity as those of A and B.*
sponding to reduction of the cytochromes b (Fig. 3A). There is some change in the shape of the absorbance minimum at 481 to 482 nm, but little change in amplitude. The same difference spectrum obtained at −196 C is shown in Figure 3B. All three cytochromes b are partially reduced, with bmax barely detectable, as expected from the experimental records of Figure 2 obtained with the dual wavelength spectrophotometer.

One peculiarity of the reversed electron transport from the cytochromes c to the cytochromes b is that rather less bmax and bint are reduced than would be expected from the oxidation of cint and cmax on the basis of absorbance changes. This is evident from the records of Figure 2. The absorbance change observed on adding uncoupler to the ATP-treated mitochondria—which corresponds to the full difference of coupled minus uncoupled—is 4.3 × 10⁻³ at 549 to 540 nm (Fig. 2A), while an estimate of the sum of the absorbance changes attributable to the b cytochromes in the other three records is about 2.8 × 10⁻³. It is even more evident from Figure 3A, where some 0.018 absorbance units of the cytochromes c at 550 nm and of cytochrome a at 600 nm disappear on addition of ATP, to be replaced by 0.0052 unit of the cytochromes b at 562 nm. It is difficult to quantitate these changes because the extinction coefficients of the various cytochromes at their difference absorbance maxima are not known with certainty (27). If one assumes that all the extinction coefficients are approximately the same, as was done in a previous kinetic study with quite reasonable results (32), one finds that about half the equivalents lost by the cytochromes c and cytochrome a on addition of ATP appear in the cytochromes b.

An attempt to search for the remainder of these equivalents in ubiquinone or flavoprotein is shown in the experiments of Figure 4. Because of the high intrinsic absorbance of ATP in the ultraviolet region of the spectrum where ubiquinone is measured, the order of addition of sulfide and ATP was reversed in the experiments of Figure 4 compared to those of Figure 2, the ATP being added first in the former. Addition of sulfide to a suspension of mitochondria in medium T containing ATP (Fig. 4A) gives a transient absorbance change with the wavelength pair 282 to 295 nm; subsequent addition of uncoupler has no effect. Addition of succinate causes an absorbance decrease at 282 nm, corresponding to complete ubiquinone reduction, as determined from separate control experiments. The response of cytochrome cint in the same mitochondrial preparation to the same series of additions is also shown in Figure 4A, in the record directly below that of the ubiquinone responses. In the presence of ATP, addition of sulfide produces relatively little reduction of the cytochrome: the reduction occurs on addition of uncoupler, showing that energy-linked reversed electron transport is maintaining the cytochrome oxidized. This order of addition does not change the response of the mitochondria to the combination of sulfide + ATP; only the initial phase of reduction seen in Figure 2A on addition of sulfide is missing.

The flavoprotein responses to ATP in the presence of sulfide are shown in Figure 4B, together with the cytochrome cint response obtained from the same mitochondrial preparation under the same experimental conditions. It is evident from the higher degree of cint oxidation maintained in the presence of ATP + sulfide than that maintained in the presence of these reagents plus uncoupler that reversed electron transport from the cytochromes c to the cytochromes b is occurring. It is also evident from the absorbance and fluorescence changes diagnostic of the redox state of the flavoproteins that electron transport into or out of these components cannot be detected with any certainty. There is virtually no change in flavoprotein fluorescence. The increase in absorbance at 468 nm on addition of sulfide to the mitochondrial suspension is due to the 482-nm component observed in Figure 3A. The same change is also observed on addition of cyanide. From previous work (36, 37), the absorbance decrease at 468 nm on addition of succinate can be ascribed primarily to Fp₆₅. This set of experiments shows that electron transport from the cytochromes c to the cytochromes b does not involve the flavoproteins or ubiquinone. Those equivalents from the cytochromes c which are not found in the cytochromes b must be in some component with an absorbance spectrum that does not change detectably on change of redox state.

If the order of succinate addition is changed such that this substrate is added directly to sulfide-treated mitochondria, the effect on the cytochromes b is that shown in Figure 5. Cytochrome bmax is rapidly reduced (Fig. 5A). The subsequent addition of ATP produces a very small increase in reduction which is uncoupler sensitive. By comparison with Figure 5B, this change can be attributed in large part to cytochrome bmax, which shows the same time course for the change, but a much

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**Figure 3.** A: Difference spectra of mung bean mitochondria obtained at room temperature. The dashed line is the spectrum obtained on adding sulfide to the aerobic mitochondrial suspension; the solid line is the spectrum obtained on subsequent addition of ATP. The aerobic mitochondrial suspension contained 12 mg protein/ml plus 1 mM mCLAM; this was the reference sample. Sulfide was added to the measuring cuvette at 0.5 mM and the difference spectrum obtained. ATP was then subsequently added to the measuring cuvette, and the second spectrum was obtained. B: Difference spectrum at liquid nitrogen temperature corresponding to the solid line spectrum in A. The aerobic mitochondrial suspension contained 20 mg protein/ml and 1 mM mCLAM; this served as reference. The measuring cuvette contained this suspension plus 0.5 mM sulfide and 1 mM ATP. The optical path length was 1.0 cm in A and 0.2 cm in B.
Fig. 4. A: Effect of reverse electron transport from the cytochromes \(c\) on the redox state of ubiquinone. The upper record shows the effect on ubiquinone of adding: first sulfide, then the uncoupler 1799 to the mitochondrial suspension containing ATP added 2 min prior to the start of the experiment. The downward deflection observed on addition of succinate corresponds to ubiquinone reduction; the extent of the deflection is equal to the total change from oxidized to reduced for ubiquinone as determined separately. Note that these experiments were carried out in Medium T which contains no \(P_i\), so that interference from pyridine nucleotide reduction is avoided. The parallel experiment for cytochrome \(c_{547}\) with the same mitochondrial suspension is shown in the lower record. B. Effect of reverse electron transport from the cytochromes \(c\) on the redox state of flavoprotein. The upper record shows the effect on flavoprotein of adding sulfide, followed by ATP, 1799, and succinate in that order. The reaction conditions are identical to that in A. The parallel experiment for cytochrome \(c_{547}\) with the same mitochondrial suspension is shown in the lower record.

larger absorbance change at 560 to 540 nm than at 556 to 540 nm. Conversely, the rapid reduction seen at 560 to 540 nm (Fig. 5B) on the addition of succinate is associated with the same time course but a smaller absorbance change than that seen at 556 to 540 nm; the rapid reaction can be attributed in large part to cytochrome \(b_{560}\). There is a very small, slower absorbance change observed at 565 to 540 nm on addition of succinate to sulfide-treated mitochondria (Fig. 5C). At this wavelength pair, both \(b_{560}\) and \(b_{547}\) are recorded, and this absorbance change and time course, by comparison with those shown in Figure 5B, can be attributed to \(b_{547}\); there is no reduction of \(b_{560}\) under these conditions. This is the result to be expected on the basis of the different midpoint potentials of the cytochromes \(b\) (20). Comparison of the relatively large and qualitatively similar absorbance change in Figures 5B and 5C on addition of ATP shows that both \(b_{547}\) and \(b_{560}\) are reduced by reversed electron transport with succinate present. This reduction is sensitive to uncoupler, as shown by the reoxidation observed on addition of 1799.

The records of Figure 5 were obtained with a mitochondrial suspension of high enough protein concentration that anaerobiosis was achieved in a relatively short time on addition of NADH, even with both mCLAM and sulfide present to inhibit both terminal oxidases. At the far right side of each of these three records, after an extended steady state, a slow increase in absorbance relative to 540 nm is observed. Previous work (37) with energy-depleted mung bean mitochondria has demonstrated that this change is due primarily to the slow reduction of cytochrome \(b_{547}\) in anaerobiosis in the presence of either cyanide or sulfide. This slow reduction occurs in the presence of high substrate concentrations and thus represents a different phenomenon from the slow reduction of cytochrome \(b_{547}\) observed in oxygen pulse experiments which are carried out with succinate as substrate at high concentrations of malonate (32). This behavior is somewhat puzzling, since there is no evidence for a specific inhibition by cyanide or sulfide of the reduction of this cytochrome; its midpoint potential \(E_{\text{m,547}} = +42\) mv is close enough to that of cytochrome \(b_{547}\) with \(E_{\text{m,547}} = +75\) mv (20) that no qualitative difference in behavior would be expected. Since the previous experiments had been carried out with energy-depleted mung bean mitochondria, it was necessary to examine the reduction rate of \(b_{547}\) in coupled mitochondria in the presence of sulfide to ascertain the effect of energization. The records from this set of experiments are shown in Figure 6. Addition of sulfide to the mitochondrial suspension results in partial reduction of cytochrome \(a\) and cytochrome \(c_{547}\) (Fig. 6, A and D), with little effect on cytochromes \(b_{560}\) and \(b_{547}\) (Fig. 6, B and C). Addition of ATP causes oxidation of cytochromes \(a\) and \(c_{547}\) with concomitant reduction of \(b_{547}\) and \(b_{560}\), demonstrating that the mitochondria are indeed coupled and capable of reversed electron transport at the second site of energy conservation. Addition of succinate causes reduction of all components (with a slight overshoot) to their redox levels characteristic of the coupled energized aerobic steady state with inhibited cytochrome oxidase. Oxygen is consumed via the alternate, sulfide-insensitive oxidase pathway, and the suspension becomes anaerobic. Cytochrome \(a\) then becomes slowly reduced, as shown in Figure.
Fig. 5. Reverse electron transport to cytochrome $b_{553}$ (A), cytochrome $b_{557}$ (B), and $b_{562}$ (C) driven by ATP in the presence of added succinate.

6A; the process requires about 4 min, in general agreement with previous results obtained with cyanide as inhibitor (35). Comparison of the records of Figures 6B and 6A shows that reduction in anaerobiosis of cytochrome $b_{553}$ is also slow and follows nearly the same time course as the reduction of cytochrome $a$. The same is true of $b_{557}$ (Fig. 6C), but the smaller absorbance change indicates only partial reduction when interference at 566 to 540 nm from $b_{553}$ is taken into account. Cytochrome $c_{2}$ attains nearly its full degree of reduction in the aerobic steady state (Fig. 6D), and little further reduction occurs on anaerobiosis. These results with coupled mung bean mitochondria, held at high phosphate potential by added ATP in the absence of P$_{i}$ and supplied plentifully with substrate, demonstrate that the slow rate of reduction of cytochrome $b_{553}$ in the presence of sulfide or cyanide is not due to an energy-linked process: it is also observed in energized, coupled mitochondria.

The set of experiments parallel to those of Figure 6, with uncoupler added prior to succinate, are presented in Figure 7. The same mitochondrial preparation was used in both sets of experiments. Comparison of the records of Figures 7A and 7B again shows that $b_{553}$ is reduced in anaerobiosis with nearly the same time course as cytochrome $a$. With uncoupler present, less $b_{553}$ is reduced in the aerobic steady state with succinate (Fig. 7B) than in the energized coupled state (Fig. 6B), the difference being attributable to reversed electron transport driven by ATP in the latter case. With uncoupler present, there is at best only a partial reduction of cytochrome $b_{553}$, as shown by the smaller upward deflection of the trace seen in Figure 7C compared to Figure 6C. Cytochrome $c_{2}$ is completely reduced by succinate in the uncoupled aerobic steady state (Fig. 7D); no further reduction is observed in anaerobiosis.

Comparison of Figures 6A and 6D with Figures 7A and 7D allows one to assess the extent to which ATP-driven reverse electron transport operates from the cytochromes $c$ and cytochrome $a$ with succinate present. The operation of this reversed electron transport in both the aerobic steady state and in anaerobiosis is particularly well illustrated by comparison of the records of Figures 6A and 7A. The magnitude of the slow absorbance change corresponding to cytochrome $a$ reduction in anaerobiosis is the same in both cases, as expected, but the over-all change observed with the energized mitochondria is less. Cytochrome $a$ remains partially oxidized in the presence of ATP in anaerobiosis as well as in the aerobic steady state; the extent of oxidation is about 30% as calculated from these records. The same is true of cytochrome $c_{2}$ (compare Figs. 6D and 7D), but the extent of oxidation in the energized state is less than that of cytochrome $a$, as would be expected from the more positive midpoint potential of $c_{2}$ (20). It has been shown (41) that, under the conditions of the experiment of Figure 6, endogenous pyridine nucleotide is about 15% reduced in the aerobic steady state and becomes 50% reduced in anaerobiosis. Endogenous pyridine nucleotide, therefore, acts as the ultimate acceptor for reducing equivalents in reversed electron transport driven by ATP, but cytochromes $b_{553}$ and $b_{557}$ also maintain a more reduced steady state (Fig. 5). With the first and second sites of energy con-
have cytochromes the same is true, however, than does the mCLAM, which was reduced in anaerobiosis, in the aerobic condition. (Further, the state of 50% reduction is obtained with ATP and succinate when mCLAM is also present in the sulfide-blocked system (41). This is further evidence that only the first energy conservation is operative in plant mitochondria treated with cyanide or sulfide which oxidize substrate through the alternate oxidase.

**DISCUSSION**

The demonstration of ATP-driven reverse electron transport between the cytochromes c and the cytochromes b of mung bean mitochondria, added to the evidence from the crossover experiments (4) and potential measurements (20) cited earlier, confirms the location of the second energy conservation site between these respiratory chain carriers. The pattern of the reverse electron transport helps to resolve some of the fine structure of this site. The high potential carriers involved are cytochromes c_{\text{ox}}, c_{\text{red}}, and a, which all respond in like manner to energization with ATP and de-energization with uncoupler. Cytochrome a is also the low potential carrier of the third coupling site. This is in accord with their measured midpoint potentials: E_{\text{m},c} = +235 mV for the two c cytochromes and +190 mV for cytochrome a. Cytochrome c_{\text{ox}} is the c cytochrome extractable with salt, while c_{\text{red}} remains bound to the mitochondrial membrane (2, 27). Cyto-

**Fig. 6.** Reverse electron transport from cytochromes a (A) and c_{\text{ox}} (D) to cytochromes b_{56} (B) and b_{55} (C) driven by ATP in the presence of sulfide but absence of mCLAM, followed by succinate addition to give the aerobic steady state of succinate oxidation which is, in turn, followed by transition to anaerobiosis, with the mitochondria in the coupled, energized state. Oxygen consumption is through the alternate, sulfide-insensitive oxidase. The order of addition is sulfide, ATP, and succinate as indicated in the record of D.

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chrome $c_{22}$, corresponds, therefore, to mammalian cytochrome $c$, while $c_{22}$ corresponds to mammalian $c_{1}$. Wohlrab (44) has demonstrated that cytochrome $c$ is required for the cytochrome $a$ pool to equilibrate rapidly with itself and with cytochrome $c_{1}$; by analogy, $c_{22}$ is assigned this role in plant mitochondria. This, in turn, places cytochrome $c_{22}$ as the high potential carrier acting directly at the second energy conservation site. Cytochrome $c_{22}$ maintains this cytochrome and cytochrome $a$, the low potential carrier of the third site, essentially in equilibrium.

The low potential part of the second energy conservation or coupling site comprises the cytochromes $b$. The pattern of ATP-driven reverse electron transport indicates that all three cytochromes can accept electrons from the high potential carriers, but that $b_{22}$, as expected from its low midpoint potential ($E_{1/2} = -77$ mV) (20), is reduced to a very limited extent. The results of the experiments reported here, added to the kinetic measurements of the oxidation of the $b$ cytochromes (32), are compatible with the placement of both $b_{22}$ and $b_{11}$, as the low potential carriers acting directly at the second energy conservation site. It is suggested tentatively that reducing equivalents from NADH-linked substrates pass through the first coupling site and reach the second site through cytochrome $b_{22}$ to $b_{11}$, while reducing equivalents from succinate or exogenous NADH reach cytochrome $b_{22}$ through ubiquinone and Fp ($37$). Both cytochromes $b_{22}$ and $b_{11}$ can be oxidized via the coupling site by the cytochromes $c$; the extent with which they actually interact with each other is still problematical.

When reverse electron transport is induced by ATP in mung bean mitochondria treated with sulfide but without added substrate (Figs. 1–3), more reducing equivalents are taken from the cytochromes $c_{22}$, $c_{21}$, and $a$, all acting as a single pool, than appear in the cytochromes $b$ by a factor of 2 or so. As pointed out above, these equivalents do not appear in either flavoprotein or ubiquinone. If energy conservation is to occur between the cytochromes $b$ and cytochromes $c$, there must be some sort of coupling site enzyme which can transform the free energy of the oxidation/reduction reactions mediating electron transport into the free energy of an acid anhydride eventually convertible to ATP. This problem has been analyzed in detail (33, 38), and the chemical hypothesis derived from this analysis predicts that each coupling site enzyme should partake in the electron transport process by means of the dithiol-disulfide redox couple. In order to function, the coupling site enzyme must exist as two species, one of low energy and one of high energy. The hypothesis predicts that the low energy species has the lower midpoint potential and undergoes redox reactions with low potential carriers of the coupling site; the high energy species has the higher midpoint potential and interacts with the high potential carriers. It is the oxidized disulfide form of the high energy species which conserves the free energy and makes it available for energy-linked reactions and ATP synthesis; it also provides a mechanism which, at the second site, can react with reduced cytochrome $c$ to become reduced to the dithiol, thereby oxidizing two molecules of the cytochrome. The reduced forms of the two species are freely interconvertible, thereby mediating reverse electron flow. In summary, the hypothesis predicts that energization of the coupling site enzyme, as has been done with ATP in these experiments, will produce the oxidized energized form which is the direct oxidant for cytochrome $c$. The energized state is characterized, therefore, by oxidation of the cytochromes $c$ and reduction of both the cytochromes $b$ and the coupling site enzyme. The latter would not show any absorbance change over the wavelength range currently available for mitochondrial studies. The amount of coupling site enzyme at the second site can be estimated from the cytochrome content (27) and the apparently lost equivalents to be about 0.1 to 0.2 nmole/mg protein. While these experiments scarcely prove the existence of such a coupling site enzyme in mung bean mitochondria, they do suggest evidence for it.

The slow reduction of cytochromes $b_{22}$ and $b_{11}$ which is observed in the presence of cyanide or sulfide appears to correlate with the redox state of cytochrome $a_{2}$ in both the coupled energized (Fig. 6) and uncoupled states (Fig. 7). In the coupled energized state, the situation is somewhat complicated by ATP interaction at both the first and second coupling sites, which maintains pyridine nucleotide partially reduced and cytochrome $a$ partially oxidized; part of the reduction of the cytochromes $b$ is due to this reverse electron transport. In the uncoupled state, only about 30% of cytochrome $b_{22}$ is reduced in the aerobic steady state, and the rest becomes reduced in the slow, somewhat biphasic reaction seen in the record of Figure 7B. The apparent ability of cytochrome $b_{22}$ to "sense" the redox state of cyanide- or sulfide-ligated cytochrome $a_{2}$ suggests that these two carriers are in close proximity in the membrane and that oxidized cytochrome $a_{2}$ with sulfide or cyanide bound to the inhibitory site of that carrier, can somehow inhibit the reduction of $b_{22}$. The idea of a close interaction between cytochrome $a_{2}$ and the cytochromes $b$ of the respiratory chain is an explicit part of the chemical hypothesis for energy conservation in the mitochondrial membrane (33, 38). The hypothesis points out that a fourth site of energy conservation may operate between cytochrome $a_{2}$ and oxygen; experimental support for this idea has been put forward by Muraoko and Slater (30). This site is postulated to be thermodynamically coupled to the second and third coupling sites by utilizing the free energy of the reaction to bind the water molecules generated at the coupling sites during the energy-conserving reaction accompanying electron transport. (Ref. 38 gives full details of this mechanism.) Such a formulation requires close physical proximity of cytochrome $a_{2}$ to the second coupling site, as well as to the third coupling site of which it is a part. To the extent that the foregoing suggestion of inhibition of $b_{22}$ reduction by sulfide-ligated, oxidized $a_{2}$ has any validity, this observation may be interpreted as evidence for such proximity.

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