The Respiratory Chain of Plant Mitochondria

IX. OXIDATION-REDUCTION POTENTIALS OF THE CYTOCHROMES OF MUNG BEAN MITOCHONDRIA

P. Leslie Dutton and Bayard T. Storey
Johnson Research Foundation, University of Pennsylvania, Philadelphia, Pennsylvania 19104

ABSTRACT

The oxidation-reduction potentials of the cytochromes of the respiratory chain of mung bean (*Phaseolus aureus*) mitochondria have been measured under strictly anaerobic conditions with a combined spectrophotometric/potentiometric method. The midpoint potentials at pH 7.2 are as follows: cytochrome a: +190 millivolts; a_3: +380 millivolts; b_{553}: +75 millivolts; b_{557}: +42 millivolts; b_{562}: −77 millivolts; c_{547} and c_{549}: +235 millivolts. (The subscripts refer to the difference absorbance maxima observed for these cytochromes in reduced-minus-oxidized difference spectra recorded at 77 K.) The same values of midpoint potentials at pH 7.2 are obtained with mitochondria depleted of energy by aerobic incubation with ADP and uncoupler in the presence of inorganic phosphate, or with coupled mitochondria energized with ATP in the absence of inorganic phosphate. Coupling site II is placed between b_{553}/b_{557} and c_{549}/c_{547} in these mitochondria, and coupling site III is placed between a and a_3.

Central to the resolution of both electron transport and energy conservation in mitochondria is the problem of determining the midpoint redox potentials of the electron transport carriers. These midpoint potentials provide the basis for calculation of the free energy available in the energy conservation process. In conjunction with kinetic studies, they provide a means for fitting together the interactions between the electron transport carriers of the respiratory chain and for determining the path of electron transport from substrate to oxygen. The need for reliable midpoint potentials in order to sort out the complex interactions of cytochromes b in plant mitochondria has been pointed out in previous papers of this series (19, 28). A combined spectrophotometric-potentiometric technique developed by Dutton (17) for measuring the midpoint potentials of membrane-bound carriers by using redox mediators under strictly anaerobic conditions has been used effectively by Wilson and Dutton (33, 34) to determine the midpoint potentials of the cytochromes in rat liver mitochondria. More recently, the potentials of cytochromes in pigeon heart mitochondria and sonicated submitochondrial particles from beef heart (15, 18) have been measured with this method. Of particular interest is the fact that cytochrome b in animal mitochondria, once thought to be but a single component, has proliferated into two, and possibly three, components with different midpoint potentials. One of these in turn, designated b_T (15), can exist in an energized form with a midpoint potential at pH 7, of +245 mv and in a de-energized

form with a midpoint potential of −55 mv in rat liver mitochondria (34) and about 0 mv in pigeon heart mitochondria.

Multiplicity of b cytochromes has long been recognized in plant mitochondria, which have three such cytochromes resolvable in reduced-minus-oxidized difference spectra obtained at 77 K (2, 3) and by means of kinetic experiments (25). Now that animal mitochondria appear to have joined the ranks of plant mitochondria by expansion of their cytochrome b components, it becomes of even greater interest to determine the midpoint potentials of the plant mitochondrial cytochromes in order to see which ones are common to the two types and which ones differ between the two types. In this paper, we report the values for midpoint potentials of the cytochromes of mung bean (*Phaseolus aureus*) mitochondria measured at pH 7.2 and 24 C.

MATERIALS AND METHODS

Mitochondria were prepared from the excised hypocotyls of 6-day-old etiolated mung bean (*P. aureus*) seedlings, following the methods of Bonner (4) and Ikuma and Bonner (21) with the modifications described by Storey and Bahr (30). All chemicals used were of the purest grade available commerically. The uncoupler 1799[1] was kindly supplied by Dr. P. Heytler of E. I. DuPont de Nemours Company. The respiratory activity and respiratory control quotient of each mitochondrial preparation was determined polarigraphically to ensure that the mitochondria were isolated intact (20). For this determination, mitochondria were suspended at 0.6 to 1.2 mg of protein/ml in a medium containing 0.3 M mannitol, 10 mM TES and 4 mM phosphate adjusted to pH 7.2 with KOH. This is designated medium TP; the same medium with phosphate omitted is designated medium T. The potential measurements were performed in either medium TP or medium T as stated in the text. The protein content of each preparation was determined by a modified Lowry method (24).

The potential measurements were carried out with a platinum measuring and calomel reference electrode. Since the cytochromes are bound to the mitochondrial membrane, they cannot react with the platinum electrode, and redox mediators are required. These also serve to poise the system through a range of potentials. The redox mediators used to act between the membrane-bound cytochromes and the platinum electrode

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[1] Abbreviations: 1799: bis(hexafluoroacetonyl) acetone; TES: tris-(hydroxymethyl) methylamino ethylsulfonic acid; TMPD: N,N,-N′,N′-tetra-methyl-*p*-phenylenediamine: DAD: diaminodurene; PMS: phenazine methosulfate; PES: phenazine ethosulfate; DQ: duroquinone; E_h: measured potential referred to the normal hydrogen electrode; E_m: midpoint potential of a compound capable of undergoing oxidation-reduction, defined as that potential where the concentration of oxidized and reduced forms are equal; $E_{m7.2}$: midpoint potential at pH 7.2; MBM: mung bean mitochondria.

were the following, with midpoint potentials at pH 7 and number of electrons, n, involved in the redox reaction as listed: potassium ferricyanide, +430 mv, n = 1 (Baker Chemical Co., Philadelphia, Pa.); N,N,N',N'-tetra-methyl-p-phenylenediamine (TMPD), +260 mv, n = 1 (Eastman Organic Chemicals, Rochester, N.Y.); diaminodurene (DAD), +240 mv, n = 2 (generous gift from Dr. C. P. Lee); phenazine methosulfate (PMS), +80 mv, n = 2 (Sigma Chemiacl Co.); phenazine ethosulfate (PES), +55 mv, n = 2 (K & K Laboratories, Plainview, N.Y.); duroquinone (DQ), +34 mv, n = 2 (Eastman Organic Chemicals); pyocyanine, −34 mv, n =2 (K & K Laboratories); 2-hydroxy-1,4-naphthoquinone, −145 mv, n = 2 (Eastman Organic Chemicals). The midpoint potentials here are those given by Clark (16) and are referred to the normal hydrogen electrode.

The oxidation-reduction potentials of the cytochromes were determined by simultaneous measurement of the redox potential of the mitochondrial suspension under strictly anaerobic conditions and of the absorbance changes accompanying cytochrome oxidation or reduction. The potential was measured with a platinum electrode (Radiometer P101) and a calomel reference electrode (Radiometer K401) immersed in the suspension and connected to a Radiometer (Copenhagen) combination pH meter/millivoltmeter. The absorbance changes were measured with a dual wavelength spectrophotometer (8). The reaction cuvette had a working volume of 5 ml and an optical path length of 1 cm. The mitochondrial suspension in the cuvette was continuously stirred with a side-mounted magnetic stirrer and was maintained under a stream of argon (ultrahigh purity, $O_2 < 1$ μl/liter; Matheson Co.) at slight positive pressure. Small additions of reagents were made by means of a 10-μl Hamilton syringe through a septum which effectively prevented entry of air in the cuvette.

Mung bean mitochondria contain sufficient endogenous substrate that they act effectively as oxygen scavengers. The experiment was started by flushing the air out of the cuvette with the argon stream. The stirred suspension under argon usually became anaerobic within 5 to 10 min; anaerobiosis could be readily detected by a drop in the potential registered by the platinum electrode to about 250 mv from 325 mv. Ferricyanide was then added to make the measured potential more positive, and the cytochromes were allowed to become reduced slowly in anaerobiosis with reducing equivalents from endogenous substrates. The absorbance change resulting from cytochrome reduction was measured wtih the dual wavelength spectrophotometer and was recorded on a strip chart recorder. The following wavelength pairs were used for the various carriers: 445 to 455 nm for $a + a_3$; 549 to 540 nm for c_{547}; 552 to 540 nm for c_{549}; 556 to 540 nm for b_{553}, 560 to 570 nm for b_{557}; and 565 to 570 nm for b_{562}. (Subscripts give reduced-minus-oxidized difference absorbance maxima in spectra obtained at 77 K. Reference 25 provides details concerning this nomenclature.) Potentials were read directly from the millivoltmeter and the readings marked directly on the trace of the absorbance change. The record obtained is the absorbance change as a function of time with the potential reading corresponding to a given absorbance change marked at the appropriate point. Reduction of the cytochromes was completed by addition of about 20 μM NADH which these mitochondria use as substrate (21). This procedure was required in order to obtain the total absorbance change characteristic for the transition of a given cytochrome from complete oxidation to complete reduction. Reversibility of the potential readings was checked by reoxidation of the reduced cytochrome with ferricyanide.

The fraction of cytochrome reduced at a given potential was calculated from the absorbance change at that potential and from the total absorbance change. The fraction oxidized

was calculated on the assumption that only one component was present, and the ratio of (fraction oxidized):(fraction reduced) was plotted as the logarithm versus the potential on semilogarithmic graph paper. The data in this paper are presented in this manner with the potential E_h on a linear scale as the ordinate and $\log_{10}$ (oxidized-reduced) on a logarithmic scale as the abscissa. A single component with but one midpoint potential gives a straight line on such a plot, according to the equation given by Clark (16):

$$E_{hx} = E_{mx} + \frac{RT}{nF} \ln \frac{(ox)}{(red)}$$

where E_{hx} is the measured and E_{mx} the midpoint potential at pH x, F is the Faraday constant, and n is the number of electrons involved. Multiple components with differing midpoint potentials give sigmoidal curves on such plots, but these can be resolved into a series of linear plots by separating the components at the inflection points of curves. All the potential measurements in this study were carried out at pH 7.2. The potentials reported are referred to the normal hydrogen electrode; we use the sign convention recommended by Clark (16), in which the half-cell reaction for a redox dye D is written $ne + D_{ox} \rightleftarrows D_R$. By this convention, the more positive the midpoint potential of substance D, the better an oxidant is D_{ox} and the worse a reductant is D_R.

RESULTS

Cytochromes a and a_3. The plot of observed potential E_h against the logarithm of fraction oxidized/fraction reduced is shown in Figure 1 for cytochrome $a + a_3$, the redox state of which was monitored by the absorbance change at 445 to 455 nm. The mitochondria were depleted by aerobic incubation with ADP, P_i, and the uncoupler 1799 prior to the experiment. The data as obtained yield a series of points describing a sigmoidal curve with the high potential limb not fully developed. Such a
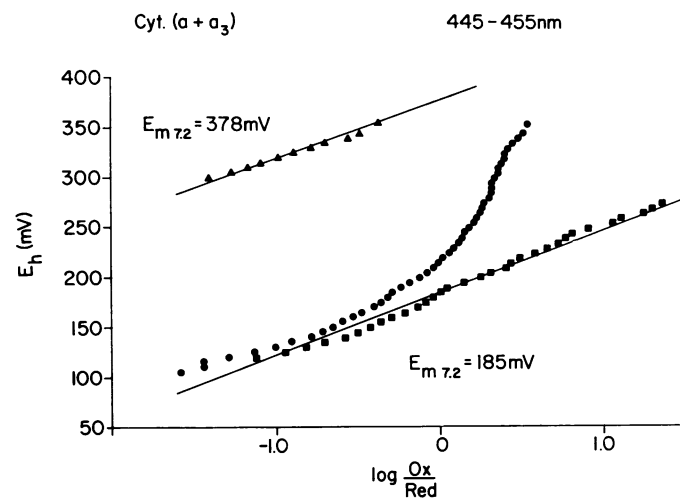

Cyt. $(a + a_3)$ 445–455nm

FIG. 1. Plot of E_h versus $\log_{10}$ (fraction oxidized)/(fraction reduced) for cytochromes a and a_3 in mung bean mitochondria. The redox state of the cytochromes was monitored with the wavelength pair 445 to 455 nm. The mitochondria were suspended in medium TP at 7.7 mg of protein/ml and depleted aerobically with 1.0 mM ADP and 20 μM 1799. The suspension contained 60 μM TMPD and 40 μM PMS as redox mediators; 0.5 mM ferricyanide was added after anaerobiosis to reoxidize the cytochromes. A blank run without mitochondria was carried out with ascorbate as reductant in order to correct the absorbance change for absorbance changes solely due to the redox mediators. The potential range covered in the experiment was +370 to +70 mv.

curve indicates that two components with different midpoint potentials are being measured at 445 to 455 nm; the two components can be resolved by splitting the total absorbance change into two parts at the inflection point of the curve. Replotting the two components gives the two set of points falling close to the lines shown in Figure 1. These theoretical lines correspond to a one-electron redox reaction. The line which gives the midpoint potential of +378 mv corresponds to cytochrome a_3 and accounts for 38% of the absorbance change at 445 nm, in good agreement with the value of 40% deduced from the low temperature spectophotometric studies of skunk cabbage (*Symplocarpus foetidus*) mitochondria by Bendall and Bonner (1) and the value of 41% obtained in recent kinetic studies with mung bean mitochondria (27). The line which gives the midpoint potential of +185 mv corresponds to cytochrome a. Since the absorbance measurement was stopped at a potential of +70 mv, there was negligible interference from the cytochromes b at this wavelength pair.

Measurement of the potentials for cytochromes a and a_3 is complicated by the endogenous substrate in mung bean
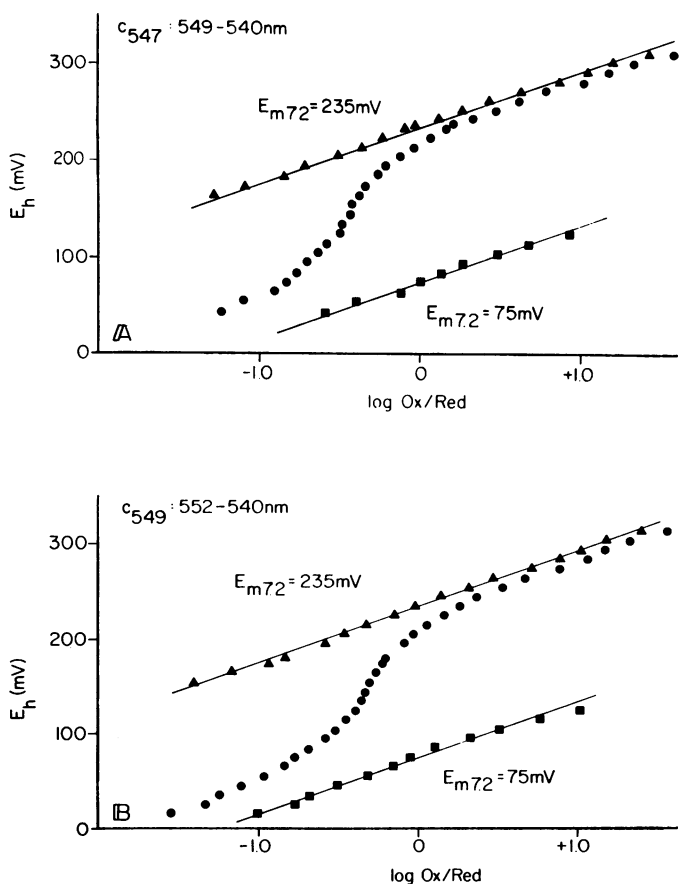

FIG. 2. A: Plot of E_h versus $\log_{10}$ (fraction oxidized)/(fraction reduced) for c_{547} in mung bean mitochondria. The redox state of the cytochrome was monitored with the wavelength pair 549 to 540 nm. The mitochondria were suspended in medium TP at 5.2 mg of protein/ml and depleted aerobically with 0.8 mM ADP and 16 μM 1799. The suspension contained 0.45 mM ferricyanide, 15 μM DAD, 17 μM PMS, and 17 μM PES as mediators. The potential range was +369 to −15 mv. B: Plot of E_h versus $\log_{10}$ (fraction oxidized)/(fraction reduced) for c_{549}, obtained under essentially the same conditions as in A, by using the wavelength pair 552 to 540 nm. Redox mediators were 25 μM DAD, 15 μM PMS, and 15 μM PES. The potential range was +360 to −15 mv. No absorbance corrections for the mediators were required with these wavelength pairs.

mitochondria. Although the oxidation rate of endogenous substrate is very low compared to the rate of succinate oxidation as measured with an oxygen electrode, it still provides reducing equivalents at a rate fast enough under steady state conditions to reduce some 90% of cytochrome a and 60% of the cytochromes c in aerobic mitochondria treated with cyanide (26). It is also rapid enough to consume ferricyanide added to an anaerobic suspension of these mitochondria at a rate which outpaces the response time of the platinum electrode, unless concentrations up to 0.5 mM are added. The absorbance change at 445 to 455 nm accompanying ferricyanide reduction at these concentrations interferes seriously with the observation of the cytochrome a_3 absorbance change, and must be corrected by means of blank runs with mitochondria omitted. As a result of these difficulties, the midpoint potentials for cytochromes a and a_3 were somewhat less reproducible than were those for the b and c cytochromes. The average values obtained from several experiments are $E_{m7.2} = 380 \pm 10$ mv for cytochrome a_3 and $E_{m7.2} = 190 \pm 10$ mv for cytochrome a. These values are nearly identical to those of 390 and 205 mv (33) for cytochromes a_3 and a, respectively, in rat liver mitochondria, and to values of 365 and 205 mv (18) for these components in submitochondrial particles obtained by sonication of beef heart mitochondria.

One curious—and somewhat unsettling—finding emerged from these measurements when we attempted to check the value for cytochrome a with the wavelength pair 603 to 620 nm. This wavelength pair appears to be quite specific to cytochrome a with little contribution from a_3, both from low temperature spectrophotometry (1) and from kinetic studies (27). Yet there seem to be at least two components in the potential range +350 to +150 mv, and at more negative potentials, an absorbance *decrease* at 603 nm is observed, which would normally be interpreted as an *oxidation* of cytochrome a. This cannot be the case, and it appears that some other component with a midpoint potential between 0 and +100 mv undergoes reduction with an absorbance change opposite to that of cytochrome a at these wavelengths. The problem is currently under investigation.

Wilson and Dutton (33) reported that the midpoint potential of cytochrome a_3 in coupled rat liver mitochondria becomes more negative when ATP is added in the absence of P_i to generate a high phosphate potential. This modified potential is about +300 mv. The value for cytochrome a is unchanged by addition of ATP under the same conditions. Qualitatively, the same effect is observed with mung bean mitochondria. The $E_{m7.2}$ value of cytochrome a is not affected, whereas that of cytochrome a_3 becomes more negative when ATP is added to fresh, coupled mitochondria suspended in medium T which lacks P_i. But consistent values for $E_{m7.2}$ of cytochrome a_3 under these conditions have not been obtained, in part due to the experimental difficulties described above, and we have as yet been unable to quantitate this effect.

Cytochromes c_{547} and c_{549}. These two cytochromes cannot be resolved spectrophotometrically except under special condtions at 77 K (23), but they can be resolved kinetically at room temperature (25). A plot of E_h against fraction oxidized/fraction reduced as measured at 549 to 540 nm, wavelengths suitable to c_{547}, is shown in Figure 2A, whereas that obtained with 552 to 540 nm, wavelengths suitable to c_{549}, is shown in Figure 2B. The data are readily resolved into two lines corresponding to one component with $E_{m7.2} = +235$ mv and the other with $E_{m7.2} = +75$ mv. Both lines are drawn through the points with a slope corresponding to n = 1, and the fit is satisfactory. The higher potential component accounts for 75% of absorbance change at 549 to 540 nm and 70% at 552 to 540 nm. These are the same percentages attributable to the
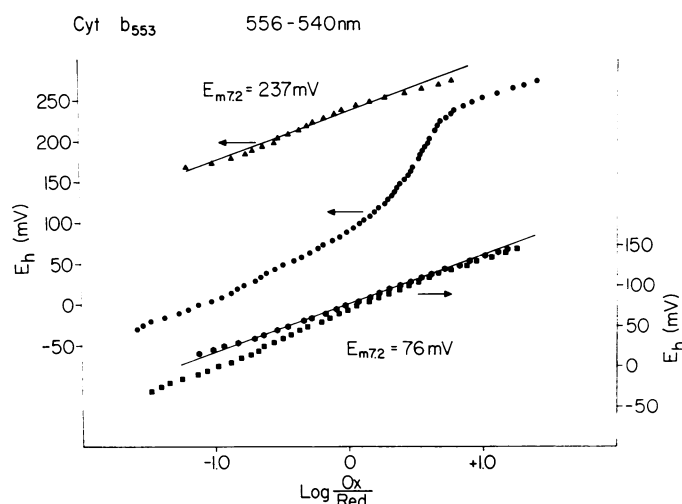
FIG. 3. Plot of E_h versus $\log_{10}$ (fraction oxidized)/(fraction reduced) for cytochrome b_{553} in mung bean mitochondria. The redox state of the cytochrome was monitored with the wavelength pair 556 to 540 nm. The mitochondria were suspended in medium TP at 14 mg of protein/ml and were depleted aerobically with 1 mM ADP and 20 μM 1799. The suspension contained as redox mediators: 50 μM PMS, 40 μM PES, 40 μM DAD, 3 μM pyocyanine, 0.1 mM DQ, and 0.4 mM ferricyanide. Blank runs showed no interference from mediator absorbance changes with this wavelength pair. The potential range was +325 mv to −80 mv.

rapidly oxidized components at these wavelength pairs, corresponding to c_{547} and c_{549}, which were observed in an earlier kinetic study (25). The high potential component is in fact the two c cytochromes which apparently cannot be resolved on the basis of their midpoint potentials; both must have $E_{m7.2}$ values close to the common value of +235 mv. The cytochrome c potentials were readily measured and reproducible; from several experiments the value of 235 ± 5 mv is obtained. This value is identical to the $E_{m7.2}$ listed by Dutton, Wilson, and Lee (18) for cytochrome c in intact pigeon heart mitochondria and for soluble cytochrome c bound inside submitochondrial particles, and only 10 mv more positive than that listed for cytochrome c in beef heart submitochondrial particles.

The contribution to the absorbance changes at 549 to 540 nm and 552 to 540 nm made by the lower potential component is the same as that of a slowly oxidized component with absorbance maximum at 552 nm found by kinetic methods (25). This component has an oxidation half-time at 18 C of 0.5 sec, which is the half-time observed for the oxidation of cytochrome b_{553}. It also has the same midpoint potential as b_{553}, as shown below. It remains fully oxidized in the aerobic steady state with succinate in the presence of antimycin A, however, and thus must be a c cytochrome. Whether this component is actually a small portion of the c_{549} pool which has been altered in some manner, or whether it is truly functional in the respiratory chain cannot be ascertained at present.

Cytochrome b_{553}. The redox state of this cytochrome was monitored during the potential measurement with the wavelength pair 556 to 540 nm. This wavelength pair also records absorbance changes attributable to cytochrome c_{549} and to cytochrome b_{557}. As a result, the data obtained fall on a complex curve, as shown in Figure 3 for an experiment carried out with depleted mitochondria. The high potential limb of the curve can resolved into a component well fit by a line of slope corresponding to n = 1, which has $E_{m7.2}$ = +237 mv and accounts for 26% of the absorbance change. This component is c_{549}. Subtracting the absorbance contribution of c_{549} still does

not yield a good straight line, as shown by the lowermost set of points in Figure 3. If all the absorbance change at potentials more negative than 0 mv, amounting to 7% of the total, is attributed solely to b_{557} and is also subtracted, the resulting points fall on the line indicated, which is drawn with slope corresponding to n = 1. This component accounts for 67% of the total absorbance change, has a midpoint potential of 76 mv, and is b_{553}. The results from several experiments at these wavelengths give 75 ± 5 mv as the value for $E_{m7.2}$ of b_{553}. The same value is obtained with depleted mitochondria suspended in medium TP, or with coupled mitochondria suspended in medium T and treated with 1 to 3 mM ATP.

Cytochromes b_{557} and b_{562}. The results from one set of absorbance-potential measurements carried out with depleted mitochondria with the wavelength pair 560 to 570 nm is shown in Figure 4A, and one set carried out with the pair 565 to 570 nm is shown in Figure 4B. The data give similar curves which are readily separable into two straight lines with slopes corresponding to n = 1. The higher potential component contributes 78% to the total absorbance change observed at 560 to 570 nm and 43% to the change observed at 565 to 570 nm,
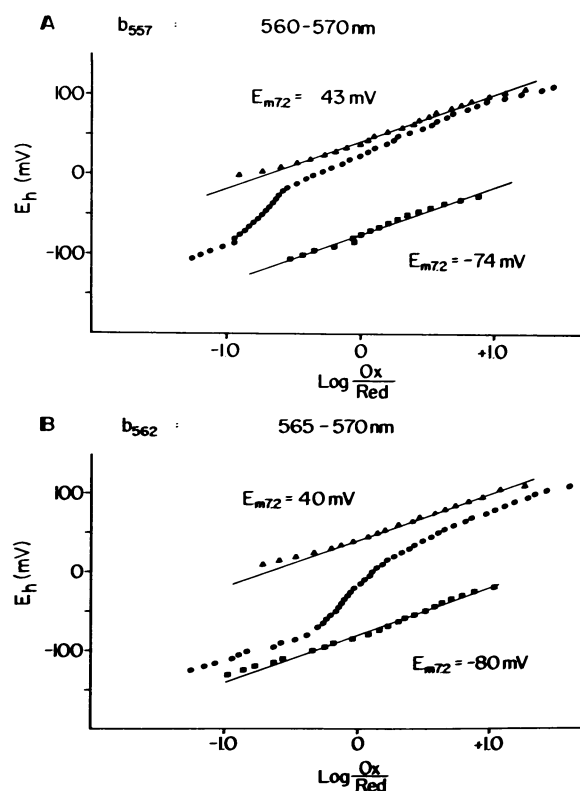

FIG. 4. A: Plot of E_h versus $\log_{10}$ (fraction oxidized)/(fraction reduced) for cytochrome b_{557} in mung bean mitochondria. The redox state of the cytochrome was monitored with the wavelength pair 560 to 570 nm. The mitochondria were suspended in medium TP at 9 mg of protein/ml and were depleted aerobically with 0.9 mM ADP and 19 μM 1799. The suspension contained as redox mediators: 17 μM DAD, 15 μM PMS, 15 μM PES, 60 μM DQ, 1.5 μM pyocyanine, 30 μM 2-hydroxy-1,4-naphthoquinone, and 0.12 mM ferricyanide. The potential range was +275 to −140 mv. B: Plot of E_h versus $\log_{10}$ (fraction oxidized)/(fraction reduced) for cytochrome b_{562} was performed under essentially the same experimental conditions as in A by using the wavelength pair 565 to 570 nm. The mitochondrial suspension contained 11 mg of protein/ml and the following redox mediators: 15 μM DAD, 15 μM PMS, 15 μM PES, 0.1 mM DQ, 3 μM pyocyanine, 25 μM 2-hydroxy-1,4-naphthoquinone, and 0.15 mM ferricyanide. The potential range was +250 to −160 mv.

and so is b_{557}; the lower potential component must be b_{562}. There is negligible interference from b_{553} at these wavelengths. From several experiments at both sets of wavelength pairs, the value of $E_{m7.2} = +42 \pm 5$ mv is obtained for b_{557}, and $E_{m7.2} = -77$ ± 5 mv for b_{562}. As with b_{553}, the same $E_{m7.2}$ values for b_{557} and b_{562} are obtained with depleted mitochondria or with mitochondria energized by addition of ATP.

Reverse Electron Transport. The redox state of an electron transport carrier of the respiratory chain may be changed upon energization of the chain by ATP as a result of reverse electron transport (9, 10, 12, 22). A striking example of this has been described by Chance and Maitra (14) with sulfide-inhibited pigeon heart mitochondria, in which reverse electron transport from exogenous and endogenous cytochrome c to endogenous pyridine nucleotide was induced by addition of ATP. The extent of cytochrome c oxidation and of pyridine nucleotide reduction was correlated with the phosphate potential, and, at the appropriate phosphate potential, cytochrome c and pyridine nucleotide both exhibit the same extent of oxidation-reduction. From this, one might naively conclude that ATP had shifted the E_{m7} of cytochrome c from $+230$ mv to -320 mv, or conversely, that E_{m7} for pyridine nucleotide had
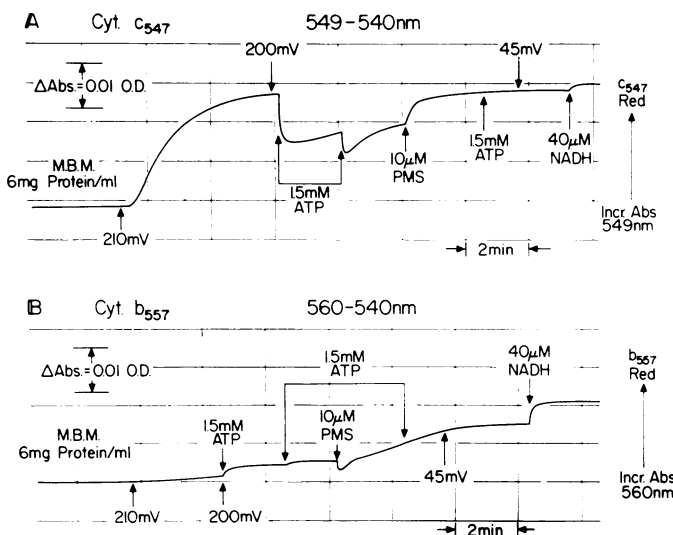

FIG. 5. Reverse electron transport upon addition of ATP to anaerobic mung bean mitochondria (MBM). The experiments in A and B were performed under the same experimental conditions as the potential measurements in Figures 1 to 4 but in the absence of redox mediators. A: Upon anaerobiosis, there is an increase in absorbance at 549 relative to 540 nm, corresponding to cytochrome c reduction. The anaerobic point is given approximately by a potential reading of $+210$ which, in the absence of mediators, is not a true potential reading as discussed in the text. Cytochrome c reduction is essentially complete at a potential reading of $+200$ mv. The ATP solution added at the points indicated had been bubbled with argon to remove dissolved oxygen in order to prevent reoxidation of the cytochrome c by oxygen rather than by reverse electron transport. The PMS solution was also bubbled with argon to remove oxygen. The reading of $+45$ mv indicated on the trace is a true potential reading, since PMS is present as mediator. Subsequent addition of NADH to give full reduction gives a very small absorbance change at this wavelength pair. B: This experiment was carried out with the same mitochondrial suspension under conditions identical to those used in A but with the wavelength pair 560 to 540 nm, suitable for monitoring the redox state of b_{557}. ATP was added at the point where the potential reading was $+200$ mv, corresponding to near complete c reduction as shown in the experimental record of A, and reduction of b_{557} is observed, as shown by an upward deflection of the trace. Addition of PMS causes reoxidation, followed by reduction. Addition of NADH completes the reduction.

shifted from -320 mv to $+230$ mv. This experiment was carried out under near-equilibrium conditions, but pyridine nucleotide and cytochrome c could equilibrate only through coupling sites I and II; no other pathway was available. In all the potential measurements reported here and elsewhere (15, 18, 33, 34), redox dyes were present to act as mediators between the platinum electrode and membrane-bound cytochromes. These dyes interact at various points in the respiratory chain, react rapidly with each other, and provide an external pathway for equilibration of the redox state of the electron transport carriers. This equilibration is rapid compared to the rate of reverse electron transport induced by ATP. Any change in redox state of a carrier caused by reverse electron transport is rapidly restored by equilibration with the mediators. Of these mediators, PMS is one of the most effective and one of the most useful for the measurement of cytochrome b midpoint potentials. The fact that two forms of b_T with different midpoint potentials in the unenergized and ATP-energized states can be observed in the presence of PMS (34) is good evidence that two forms do in fact exist, and that the observation is not an artifact caused by reverse electron transport. But the cytochromes b in mung bean mitochondria show no apparent midpoint potential change on addition of ATP present. In this case, it becomes necessary to show that these mitochondria are fully coupled and have the capacity for reverse electron transport in the absence of mediators in order to conclude that indifference to ATP is not an artifact caused by some defect in the energy coupling mechanism, but is an intrinsic redox property of the b cytochromes.

The experiment showing that these mitochondria are indeed coupled under the conditions of the potential measurements is presented in Figure 5. The same experimental set-up was used as for the other absorbance-potential measurements, but no mediator dyes were present. The mitochondria were suspended in medium T and allowed to go anaerobic under a stream of argon. At the relatively high protein concentration used in these experiments, enough soluble material of unknown nature is released by the mitochondria to react with the platinum electrode and gives a potential reading even in the absence of added dyes, but the reaction is too slow for the reading to be accurate. The reading is useful as a reference point between experiments, however. The result obtained with wavelengths appropriate to c_{547} is shown in Figure 5A. Addition of 1.5 mM ATP after reduction of the cytochrome by endogenous substrate in anaerobiosis causes reoxidation. A second addition causes some further oxidation, followed by a slow reduction, indicating a slow consumption of ATP. Addition of 10 μM PMS causes a rapid reduction of c_{547} and subsequent addition of 1.5 mM ATP has no effect. The absorbance changes observed upon repeating this experiment with wavelengths appropriate to b_{557} are shown in Figure 5B. Addition of 1.5 mM ATP at the point indicated, where the potential reading is the same as that at the ATP addition point in the cytochrome c_{547} experiment (Fig. 5A), gives partial reduction of the b cytochrome. A second addition of ATP gives a very slight further reduction. Addition of 10 μM PMS causes oxidation of b_{557}, and this is followed by reduction as PMS shuttles reducing equivalents to this cytochrome from regions of the respiratory chain with more negative potentials. Addition of ATP now has no effect. This experiment shows that the coupling mechanism of these mitochondria is intact, that reverse electron transport from the cytochromes c to cytochrome b_{557} can occur in the presence of ATP, but that the cytochromes b and c become equilibrated in the presence of PMS which abolishes the ATP effect. The absence of an ATP effect with the cytochromes b of mung bean mitochondria analogous to that observed for b_T (18) shows that these cytochromes act only as electron carriers

to the second coupling site and do not participate in the coupling site reactions. This constitutes yet another difference in the respiratory chain of plant mitochondria as compared to that of animal mitochondria.

DISCUSSION

The values of $E_{m7.2}$ determined for the various cytochromes in mung bean mitichondria are collected in Table I. There is a potential span of nearly 200 mv between the midpoint potentials of cytochrome a and a_3. This strongly suggests that the third site of energy conservation lies between these two cytochromes in plant mitochondria, as proposed by Bonner and Plesnicar (5) from application of the crossover theorem (13) to experiments carried out with mitochondria from etiolated seedlings of Black Valentine bean ($P.$ $vulgaris$). Site III in rat liver mitochondria also appears to lie between cytochrome a and a_3 from application of the crossover theorem (13) to the experiments of Wilson and Chance (32), and from the potential measurements of Wilson and Dutton (33).

The midpoint potential of cytochrome a is some 40 mv more negative than that of the c cytochromes in mung bean mitochondria. This is also true for rat liver and pigeon heart mitochondria, and for beef heart submitochondrial particles (18) although in these the difference is 20 to 30 mv. The value of $E_{m7.2} = +190$ mv is in good agreement with that of $+170$ mv predicted for the lower potential electron transport carrier for site III by the chemical hypothesis for mitochondrial energy conservation based on thiol-disulfide chemistry, which was recently proposed and developed in some detail (29).

There is also a potential span of about 200 mv between the midpoint potentials of b_{557} and the two cytochromes c, again suggesting that the second site of energy conservation lies between these carriers. This suggestion is also in agreement with the location for this site proposed by Bonner and Plesnicar (5) although they did not specify the particular b cytochrome. The $E_{m7.2}$ value of $+75$ mv obtained for b_{553} is close to the E_{m7} value of $+66$ mv for ubiquinone, obtained by Urban and Klingenberg (31), as predicted by previous kinetic studies (19, 28) of the oxidation and reduction rates of this cytochrome. It is also sufficiently close to the $E_{m7.2}$ for b_{557} that b_{553} and b_{557} most probably act together as the low potential carriers of site II. These potential measurements locate the coupling site enzyme for site II between b_{553}/b_{557} and c_{549}/c_{547}, and the coupling site enzyme for site III between a and a_3. Cytochrome c_{547}, which is the c cytochrome readily extractable with salt (23), is assumed to shuttle electrons between c_{549} and a, as has been demonstrated for rat liver mitochondria by Wohlrab (35).

The low $E_{m7.2}$ of -77 mv observed for b_{562} is expected in view of the energy requirement for its reduction by succinate (11, 25). Its function in the respiratory chain is still obscure, but it appears to play a role in mediating reverse electron transport from succinate to endogenous pyridine nucleotide. There is one aspect of the ease of reducibility of cytochrome b_{562} which seems curious for an electron transport carrier with $E_{m7.2} = -77$ mv. In the partially depleted state, there is some reduction of this cytochrome by succinate, but little, if any, reduction by exogenous NADH (28). Since the succinate-fumarate couple has $E_{m7} = +30$ mv (6), while the NADH/NAD$^+$ has $E_{m7} = -30$ mv (7), the reduction of b_{562} by succinate under these conditions depends on kinetic rather than thermodynamic factors.

The observation that the b cytochromes in plant mitochondria are strictly electron transport carriers, while one b cytochrome of animal mitochondria appears to have been incorporated into the site II coupling site enzyme (15, 18), emphasizes further the differences between mitochondria from the two

Table I. *Midpoint Potentials at pH 7.2 for the Cytochromes of Mung Bean Mitochondria*

Details of the experimental procedures are described in the text. The values for cytochromes a and a_3 are ±10 mv; the other values are ±5 mv.

Cytochrome	$E_{m7.2}$
	mv
a	$+190$
a_3	$+380$
b_{553}	$+75$
b_{557}	$+42$
b_{562}	-77
c_{547}	$+235$
c_{549}	$+235$

sources in this region of the respiratory chain, which includes the flavoproteins and ubiquinone. It also demonstrates that the coupling site enzymes at sites II and III need not be hemoproteins. Indeed, the chemistry recently proposed (29) for these coupling sites assumed that they were not hemoproteins. The fact that cytochrome a_3 in plant mitochondria, as well as in animal mitochondria, does show an ATP effect may not be relevant to this point inasmuch as there is as yet no evidence that any of the redox dyes used as mediators in the potential measurements actually do interact with a_3. In this case, the ATP effect on a_3 may be due to reversed electron transport rather than to an energized form of a_3 with a more negative midpoint potential. Further resolution of this problem must await further experiments.

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