The Respiratory Chain of Plant Mitochondria

X. OXIDATION-REDUCTION POTENTIALS OF THE FLAVOPROTEINS OF SKUNK CABBAGE MITOCHONDRIA

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ABSTRACT

The oxidation-reduction potentials of the flavoproteins of skunk cabbage (*Symplocarpus foetidus*) mitochondria have been measured under anaerobic conditions by means of a combined spectrophotometric or fluorimetric-potentiometric method. Five components were resolved whose oxidation-reduction reactions corresponded to two-electron changes, as expected for flavoproteins. The midpoint potentials at pH 7.2 are as follows, listed in order of increasingly negative potential: +170 millivolts, +110 millivolts, +20 millivolts, −70 millivolts, and −155 millivolts. The most negative component was highly fluorescent; the other components could only be identified by their characteristic absorbance changes. In addition to these components, which are mitochondrial, variable amounts of a very highly fluorescent flavoprotein with a midpoint potential of −215 millivolts was found. This component appears to be extra-mitochondrial. The same midpoint potential values at pH 7.2 were obtained with mitochondria in the uncoupled state as in mitochondria energized with ATP in the absence of phosphate.

The flavoproteins of the mitochondrial respiratory chain play a prominent role in the redox[1] reactions of electron transport and energy conservation which occur during oxidation of substrate. While the changes in redox state of the mitochondrial flavoproteins as a group can be followed spectrophotometrically (10), all the components have essentially the same difference spectrum, and individual components cannot be clearly resolved. Chance et al. (7) demonstrated that certain of these flavoproteins fluoresce to varying degrees in the oxidized state, and that this fluorescence is lost on reduction. The different ratios of fluorescence to absorbance changes, designated FA ratios, which are observed in the presence of selected substrate and inhibitors, can be used to differentiate the different flavoproteins which constitute the mitochondrial "flavoprotein chain" (6). A total of six flavoproteins could be distinguished in mammalian and avian mitochondria by means of this method (7, 9, 19). Mitochondria from plant sources do not respond to many of the respiratory chain inhibitors—e.g., rotenone (20)—which proved so useful in differentiating the flavoproteins of animal mitochondria. However, it proved possible to define operationally in terms of FA ratios four flavoprotein components in mitochondria from mung bean (*Phaseolus aureus*) hypocotyls (26) and from skunk cabbage (*Symplocarpus foetidus*) spadices (16). Two components reducible by succinate in mitochondria depleted of energy were designated Fp_{ha} and Fp_{hf}. These were classified qualitatively as high potential flavoproteins, with Fp_{ha} showing absorbance but no detectable fluorescence changes between the oxidized and reduced form and Fp_{hf} showing both types of changes. Both of the reduced components showed rapid oxidation rates upon reaction of anaerobic mitochondria with oxygen, but oxidized Fp_{hf} was reduced very slowly in comparison with oxidized Fp_{ha}. Addition of malate to energy-depleted mitochondria resulted in an absorbance change due to flavoprotein reduction, but no detectable fluorescence change: this component was designated Fp_M. A highly fluorescent component could be reduced by succinate in coupled mitochondria: this was designated Fp_{lf}. Both Fp_M and Fp_{lf} were classified qualitatively as low potential flavoproteins. The flavoprotein complement of plant mitochondria appears to differ from that of mammalian mitochondria and, as was pointed out in the previous report (26), is less well resolved because electron transport inhibitors specific to these components in plant mitochondria have as yet not been found.

The development by Dutton (13) of a combined spectrophotometric-potentiometric method for measuring the midpoint potentials of membrane-bound electron transport enzymes by using redox mediators under strictly anaerobic conditions provides another, independent method for differentiating the flavoproteins by quantitative determination of their midpoint potentials. This method has proved most fruitful when applied to the cytochromes of animal mitochondria (15, 17, 31, 32) and more recently to those of plant mitochondria (14). Further, the method can be readily adapted to measurement of fluorescence changes and FA ratios can thus be obtained. Erecinska et al. (17) found three flavoproteins in pigeon heart mitochondria with midpoint potentials of −45 mv, −160 mv, and −220 mv by this method. The last component is highly fluorescent and may consist of two components. A complete correlation with the components differentiated by means of FA ratios using selected substrate-inhibitor combinations was not made, but the power of the independent method was well demonstrated. When applied to yeast mitochondria and submitochondrial particles derived therefrom, a total of six flavoproteins with midpoint potentials ranging from +50 mv to −320 mv could be distinguished (18).

In this paper, the midpoint potentials of the flavoprotein components in skunk cabbage mitochondria are reported.

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[1] Abbreviations: redox: reduction-oxidation; FA: fluorescence to absorbance ratio; mCLAM: *m*-chlorobenzhydroamic acid; DAD: diaminodurene (1,4-diamino-2,3,5,6-tetramethyl benzene); 1799: *bis* (hexafluoroacetonyl) acetone; E_h: measured potential referred to the normal hydrogen electrode; $E_{m7.2}$: midpoint potential at pH 7.2.

These mitochondria contain the same operationally defined flavoprotein components as do mung bean mitochondria, but they are present in greater amounts with resultant increase in sensitivity in the measurement of both absorbance and fluorescence changes. The use of these mitochondria thus provides higher resolution in differentiating the separate flavoprotein components. In addition, skunk cabbage mitochondrial preparations contain only small, variable amounts of the highly fluorescent flavoprotein found in mung bean mitochondria which is reducible only by dithionite and not by substrate (26). Interference with the mitochondrial fluorescent flavoproteins by this material, whose presence probably indicates some peroxisomal contamination (23), is therefore minimal and readily accounted for.

MATERIALS AND METHODS

Skunk cabbage (*Symplocarpus foetidus*) flowers were collected from selected marshy areas adjacent to the Wissahickon Creek in Whitemarsh Township, Pennsylvania, and stored at 4 C. Mitochondria were prepared from the excised spadices by the method of Bonner (3) as modified by Storey and Bahr (29). Respiratory activity of the mitochondrial preparations was determined polarigraphically with a Clark electrode (Yellow Springs Instrument Co.) in a medium containing 0.3 M mannitol, 10 mM TES, and 5 mM phosphate adjusted to pH 7.2 with KOH. This is designated medium TP; the same medium with phosphate omitted is designated medium T. All mitochondrial preparations used in the potential measurements showed some respiratory control in the presence of 2 mM mCLAM (24), indicating that the mitochondria were intact. The protein content of each mitochondrial preparation was determined by a modified Lowry method (22).

The oxidation-reduction potentials of the flavoprotein components were determined under anaerobic conditions in a manner essentially identical to that described by Dutton and Storey (14) for determining the midpoint potentials of the cytochromes in mung bean mitochondria. The redox mediators added to the mitochondrial suspension to act between the membrane bound flavoproteins and the platinum electrode were the following, with midpoint potentials at pH 7 referred to the normal hydrogen electrode and number of electrons, n, involved in the redox reaction as given by Clark (12): potassium ferricyanide, +430 mv, n = 1 (Baker Chemical Co.); diaminodurene, +240 mv, n = 2 (generous gift from Dr. P. L. Dutton); pyocyanine, −43 mv, n = 2 (K and K Laboratories); sodium anthraquinone-β-sulfonate, −225 mv, n = 2 (Fisher Scientific Co.).

Absorbance changes corresponding to flavoprotein reduction or oxidation were recorded with the dual wavelength spectrophotometer (5) with a compensation circuit to reduce noise from light source fluctuations (8), using the wavelength pairs 464 to 492 nm or 468 to 492 nm (26, 29). The same results were obtained with either wavelength pair. The spectral band width of the light beam at each of the two wavelengths was held between 2 and 3 nm for the absorbance measurement. The mitochondrial protein content of these suspensions varied between 2 and 6 mg protein/ml.

The fluorescence changes corresponding to flavoprotein reduction or oxidation were determined with the same dual wavelength spectrophotometer modified to detect differential fluorescence rather than absorbance changes. Fluorescence was excited at the two wavelengths 464 and 492 nm, those used for the absorbance measurements. The former wavelength gives maximal excitation while the latter one gives somewhat less than 20% of maximal excitation, as calculated from the excitation spectrum for fluorescence emission for the flavoproteins of skunk cabbage mitochondria (16). The fluorescence emission, which has a maximum at 525 nm, was detected by means of a photomultiplier tube protected from the exciting light by a Wratten No. 77 guard filter. This filter has a broad transmittance maximum of 76% at 550 nm, but a transmittance of less than 0.1% at 500 nm. Fluorescence emission was detected from the front face of the cuvette. In order to provide light of sufficient intensity to excite a detectable fluorescence signal, the spectral band width at each wavelength was set at about 10 nm, so that a small amount of the light at the nominal wavelength of 492 nm passed through the guard filter. In practice, this light "leak" plus the fluorescence excited at 492 produced a signal approximately equal that from the fluorescence excited at 464 nm; the two signals were matched exactly at the beginning of the experiment by means of a continuously variable gain control. The differential change in fluorescence could then be detected and amplified with the same circuitry used for the dual wavelength spectrophotometer (8). Protein concentration varied between 5 and 14 mg protein/ml.

The fraction of total flavoprotein reduced at a given potential was calculated from the absorbance or fluorescence change at that potential relative to the fully oxidized state, and the total absorbance or fluorescence change obtained with NADH and dithionite as reductants. The ratio of (fraction oxidized)/(fraction reduced) for the total change treated as one component is plotted as the logarithm *versus* the potential E_h, referred to the normal hydrogen electrode, on semilogarithmic paper for convenient presentation of the data. The resulting complex sigmoidal curve, which actually derives from several components, was resolved into straight lines for each component by the arithmetic subtraction technique described by Wilson and Dutton (31, 32), Dutton *et al.* (15), Erecinska *et al.* (17, 18), and Dutton and Storey (14).

RESULTS

A plot of potential E_h as a function of the logarithm of the ratio (fraction oxidized)/(fraction reduced) obtained from the total absorbance change recorded at 464 to 492 nm is shown in Figure 1. The absorbance change spans the potential range +200 to −220 mv and, from the complexity of the curve, evidently represents the contributions from a number of components. Resolution of this curve yields the five sets of points shown in Figure 2. These fall close to the lines drawn through them with a slope of 30 mv per logarithmic decade, corresponding to a two electron redox reaction. The value of n = 2 is that expected for the oxidation and reduction of mitochondrial flavoproteins since only the fully oxidized or fully reduced forms are detected spectrophotometrically; the semiquinone form corresponding to a one electron transfer occurs fleetingly, if at all. The value of n = 2 is also that observed with the flavoproteins of pigeon heart and yeast mitochondria (17, 18). For the mitochondrial preparation from which the results of Figures 1 and 2 were obtained, the percentages of the components were as follows, listed by midpoint potential: $E_{m7.2} = +170$ mv, 6%, $E_{m7.2} = +110$mv, 55%; $E_{m7.2} = +18$ mv, 22%; $E_{m7.2} = -73$ mv, 19%; $E_{m7.2} = -157$ mv, 7%. The highest potential component was variable in amount in different samples, ranging from 6% to 12%, as was the lowest potential one which ranged from 6% to 10%. Average figures for the other components in the order listed are 55%, 20%, and 10%.

The differential fluorescence change corresponding to oxidation and reduction of flavoprotein in skunk cabbage mitochondria is plotted as the logarithm of the ratio (fraction oxidized)/(fraction reduced) *versus* E_h in Figure 3. The simple

sigmoid curve is readily resolved into two sets of points falling close to the lines drawn with a slope corresponding to n = 2. The component with $E_{m7.2} = -154$ mv accounts for 75% of the total fluorescence change in this mitochondrial preparation, and corresponds to the absorbing component with $E_{m7.2} = -157$ mv in Figure 2. The component with $E_{m7.2} = -212$ mv has no detectable absorbance change associated with it, and is evidently the highly fluorescent flavoprotein not associated with the mitochondria. The amount of this material varied considerably in different samples and was below the limit of detection in some. Average value obtained for $E_{m7.2}$ of this component was -215 mv. Whereas no attempt was made at quantitating the impression, it did appear that there was less nonmitochondrial fluorescent flavoprotein and more mitochondrial fluorescent flavoprotein in mitochondrial preparations made from spadices harvested early in the season.

The values of $E_{m7.2}$ for the various flavoprotein components of skunk cabbage mitochondria are listed in Table I. These are averages from several experiments and are within ±10 mv. Values for the mitochondrial content of each component are given based on a total of 0.55 nmole total flavoprotein/mg protein, the average value obtained from a number of preparations of skunk cabbage mitochondria, using a difference extinction coefficient of $\Delta E' = 6$ mM^{-1} cm^{-1} at 468 to 492 nm. The FA ratio for the single mitochondrial fluorescent protein is calculated on the normalized basis described previously (26). In order to refer more easily to the various components, an operational system of nomenclature based on midpoint potentials has been adopted in Table I. The designations Fp_{ha} and Fp_{1f} are retained from the earlier nomenclature (26). The designations Fp_{ma} and Fp_{1a} indicate that the one with $E_{m7.2} = +20$ mv is a "middle" potential flavoprotein, while that of $E_{m7.2} = -70$ mv is a "low" potential one. The designation Fp_{rha} is used for the component with $E_{m7.2} = +170$ mv indicating that its midpoint potential is the highest observed.

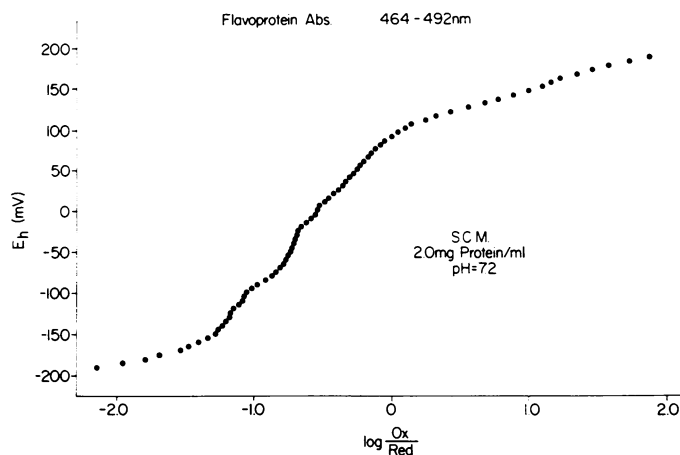The same values of $E_{m7.2}$ for the mitochondrial flavoproteins

FIG. 1. Plot of E_h versus log $_{10}$ (fraction oxidized)/(fraction reduced) for the flavoprotein complement of skunk cabbage mitochondria as determined by the differential absorbance change at 464 to 492 nm. The mitochondria were suspended at 2.0 mg protein/ml in medium TP containing 0.5 mM ADP and 10 μM 1799. The suspension contained 30 μM DAD, 30 μM pyocyanine, and 3 μM anthraquinone-β-sulfonate as redox mediators; 0.2 mM ferricyanide was added after anaerobiosis to reoxidize the flavoproteins. A blank was run without mitochondria with NADH as reductant to correct for the absorbance change due to the mediators alone. The potential range covered in the experiments was +340 mv to −300 mv. The reaction with the electrode is sluggish in the low potential region, and about 5 min was allowed for equilibration at each reading.
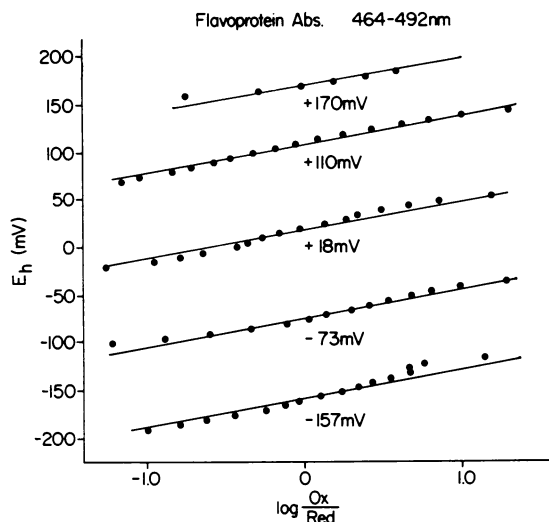

FIG. 2. Resolution of the absorbance change curve of Figure 1 into five components. The lines are drawn with a slope corresponding to n = 2 for a two electron redox reaction. The number beneath each line gives the value of $E_{m7.2}$ in millivolts, referred to the normal hydrogen electrode.
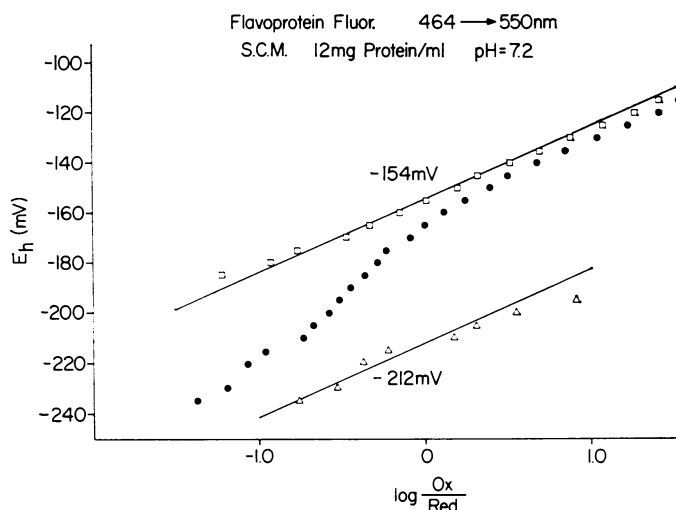

FIG. 3. Plot of E_h versus log $_{10}$ (fraction oxidized)/(fraction reduced) for the flavoprotein component of skunk cabbage mitochondria as determined by changes in fluorescence excited with 464 nm light and detected at 550 nm. The mitochondria were suspended at 12 mg protein/ml in medium TP; experimental conditions were otherwise the same as the experiment of Figure 1. The filled circles give the curve for the total fluorescence change. The open squares and triangles are for resolution of this curve into two components. The lines are drawn with a slope corresponding to n = 2. The number beneath each line gives the value of $E_{m7.2}$ in millivolts, referred to the normal hydrogen electrode.

are obtained in medium T plus ATP, a condition of high phosphate potential, and in medium TP plus ADP plus 1799, a condition of low phosphate potential and uncoupling. The energy state of the mitochondria does not affect the flavoprotein midpoint potentials, as was also observed for the midpoint potentials of the cytochromes (14).

DISCUSSION

Interference from Cytochromes. The wavelength pair 468 to 492 nm has been shown to be quite specific for monitoring

Table I. *Characteristics of Flavoprotein Components*

Flavoprotein Component	$E_{m7.2}$[1]	FA[2]	Content[3]
	mv		*nmole/mg protein*
Fp_{vha}	+170	0	0.04
Fp_{ha}	+110	0	0.30
Fp_{ma}	+20	0	0.11
Fp_{la}	−70	0	0.06
Fp_{lf}	−155	12	0.04

[1] Referred to normal hydrogen electrode.

[2] Ratio of fluorescence to absorbance calculated on the normalized basis previously described (26).

[3] Calculated on a total flavoprotein content of 0.55 nmole/mg protein. The percentage of each component is discussed in the text.

flavoprotein redox changes by kinetic means (26, 29). In this work, the same results were obtained with the pair 464 to 492 nm as with 468 to 492 nm. Any absorbance change due to a change in the redox state of the cytochromes would appear on a plot of E_h *versus* the logarithm of the ratio (fraction oxidized)-(fraction reduced) as a line with a slope corresponding to $n = 1$. This was not observed; all the points in Figure 2 fall close to lines with a slope corresponding to $n = 2$. The observed absorbance change at 464 nm to 492 nm may be safely attributed to the flavoproteins of these mitochondria. The same argument applies to the observed fluorescence changes; the points in Figure 3 fall close to lines with a slope corresponding to $n = 2$.

Flavoprotein Fp_{ha}. This component was defined operationally as a nonfluorescent flavoprotein which was readily reduced by succinate in energy-depleted mitochondria (26). It is completely reduced in the presence of cyanide by either succinate or NADH in the aerobic steady state. From kinetic studies of its reduction (27) and oxidation (16), it appears to act in concert with cytochrome b_{553}. Flavoprotein Fp_{ha} is evidently the component in Table I with $E_{m7.2} = +110$ mv. Its midpoint potential is close to +75 mv, the $E_{m7.2}$ of its partner cytochrome b_{553}. The amount of Fp_{ha} in skunk cabbage mitochondria calculated from the potential measurement is the same as that previously calculated from kinetic studies (16).

Flavoprotein Fp_{lf}. Only one fluorescent mitochondrial flavoprotein is found from the potential measurement, and this is evidently the low potential flavoprotein Fp_{lf}. This component has $E_{m7.2} = -155$ mv which is the same as that of the lowest potential flavoprotein found by absorbance change. It is interesting that the midpoint potential of Fp_{lf} is very close to that of −160 mv for a fluorescent flavoprotein in pigeon heart mitochondria (17). It is also close to $E_{m7} = -166$ mv for the malate-oxaloacetate couple (4). It is more positive than the $E_{m7} = -290$ mv for the dihydrolipoate-lipoate couple (21) and $E_{m7} = -320$ mv for the NADH/NAD couple (4), and thus is completely reduced even when the endogenous pyridine nucleotide is reduced to a very small extent (26).

Flavoprotein Fp_{hf}. The results of the potential measurements eliminate Fp_{hf} as single component, and show that the apparently synchronous fluorescence and absorbance changes, which led to its operational identification, should be assigned to a combination of flavoproteins. The fluorescence change must come from partial reduction of Fp_{hf}, even in mitochondria which have been depleted of energy to a degree sufficient to inhibit the reduction of endogenous pyridine nucleotide. This is understandable in terms of the midpoint potential of Fp_{hf}, which is some 160 mv more positive than that of

pyridine nucleotide. This store of mitochondrial energy—which is independent of the respiratory chain (28)—is still sufficient, even after prolonged aerobic incubation of the mitochondria with ADP plus uncoupler, to reduce some 10 to 15% of Fp_{lf}, albeit at a very slow rate. The absorbance changes with slow rates comparable to that for the partial reduction of Fp_{lf}, which led to the postulation of Fp_{hf} as a single component, must be due to the two flavoprotein components with $E_{m7.2} = +20$ mv and $E_{m7.2} = -70$ mv, plus a very small contribution from Fp_{lf} itself. In the potential measurements, the redox mediators added to the mitochondrial suspension interact at various points of the respiratory chain and serve to keep all the flavoproteins essentially in equilibrium with each other and with the measuring electrode. In experiments involving the selective reduction or oxidation with different combinations of substrates plus inhibitors, equilibration of the flavoprotein components may not occur, and their reduction rates may bear little relation to their midpoint potentials. It is therefore not possible *a priori* to assign the slow absorbance changes attributed to Fp_{hf} to the flavoprotein with $E_{m7.2} = -70$ mv; these absorbance changes may reflect contribution from both components. Evaluation of these contributions is currently in progress. Kinetic studies of the oxidation of the flavoproteins of skunk cabbage mitochondria revealed that mCLAM, a specific inhibitor of the alternate, cyanide-insensitive terminal oxidase of these mitochondria, inhibited the oxidation rate of what was then designated Fp_{hf}, now evidently Fp_{lf}. The alternate oxidase is known to connect with the respiratory chain between the first and second energy conservation sites (1, 2, 30). It may be that, when operative, the alternate oxidase can react rapidly with the low potential components associated with site I, which otherwise are oxidized more slowly by the cytochrome chain.

Flavoprotein Fp_M. This component was operationally defined by the rapid absorbance decrease corresponding to flavoprotein reduction, which was observed on addition of malate to energy-depleted mitochondria reduced with succinate. No accompanying fluorescence change was observed. The potential measurements reported here yield two candidates for Fp_m, the component with $E_{m7.2} = +20$ mv and that with $E_{m7.2} = -70$ mv. The latter appears to be the more logical candidate for Fp_M, but a decision between the two cannot be made at present on the basis of data at hand.

Summary of Flavoprotein Components. By providing an independent route to the differentiation of the flavoproteins of plant mitochondria, the potential measurements reported here have resolved this group of electron transport carriers into four, or possibly five, that are mitochondrial, as well as a sixth, highly fluorescent one which appears to be extra-mitochondrial. The components Fp_{hf} and Fp_{lf} identified earlier emerge from the potential measurements as single flavoproteins with $E_{m7.2} = +110$ mv and −155 mv, respectively. The existence of a second, high potential flavoprotein Fp_{hf} is gainsaid by the potential measurements; Fp_{lf} is the only fluorescent mitochondrial flavoprotein observed. The absorbance changes associated with the postulated Fp_{hf} are due to the two components with $E_{m7.2} = +20$ mv and −70 mv, respectively. One of these two may correspond to Fp_M. The very high potential flavoprotein with $E_{m7.2} = +170$ mv poses something of a mystery at present. Its inclusion in the mitochondrial group must be regarded as only tentative, since it is present in small amounts and is very difficult to extract from absorbance changes dominated by Fp_{ha}.

The flavoprotein complement of plant mitochondria is evidently quite different from that of animal mitochondria (17). There are three components somewhat similar to those found in yeast mitochondria (18) with values of $E_{m7.2} = +50$ mv, −35 mv, and −120 mv, respectively, but lack the very low potential flavoproteins found in these mitochondria. The flavo-

protein Fp_{ha}, the major component of the flavoprotein complement, has a midpoint potential far more positive than that found in mitochondria from these other sources.

The higher resolution of the flavoprotein complement has served to underline the complexity of the respiratory pigments of plant mitochondria. Three of these components: Fp_{ha}, Fp_{ma}, and Fp_{la} have $E_{m7.2}$ values quite close to those of cytochrome b_{553} (+75 mv), b_{557} (+42 mv), and b_{562} (−77 mv), respectively. Further, the $E_{m7.2}$ value of Ep_{vha} is remarkably close to that of cytochrome a (+190 mv). Whereas this suggests interactions between these components, it must be emphasized that only a close interaction between b_{553} and Fp_{ha} has been found experimentally (16, 27). Further, the identification of Fp_{vha} as a true mitochondrial flavoprotein is still tentative. A reasonable working hypothesis would be that each cytochrome b has associated with it one flavoprotein component, and this hypothesis is currently being tested.

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