The Respiratory Chain of Plant Mitochondria. II. Oxidative Phosphorylation in Skunk Cabbage Mitochondria

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Abstract. Mitochondria were prepared from the spadices of skunk cabbage (*Symplocarpus foetidus*) whose respiratory rate with succinate and malate showed 15 % to 30 % sensitivity to cyanide inhibition, and which showed respiratory control by added ADP. The observed respiratory control ratios ranged from 1.1 to 1.4. The change in pH of the mitochondrial suspension was recorded simultaneously with oxygen uptake: alkalinization of the medium, expected for phosphorylation of ADP, coincided with the period of acceleration in oxygen uptake caused by addition of an ADP aliquot. The ADP/O ratios obtained were 1.3 for succinate and 1.9 for malate. In the presence of 0.3 mM cyanide, the ADP/O ratio for succinate was zero, while that for malate was 0.7. These results are consistent with the existence of an alternate oxidase which interacts with the flavoprotein and pyridine nucleotide components of the respiratory chain and which, in the presence of cyanide, allows the first phosphorylation site to function with an efficiency of about 70 %. In the absence of respiratory inhibitors, the efficiency of each phosphorylation site is also about 70 %. This result implies that diversion of reducing equivalents through the alternate oxidase, thereby bypassing the 2 phosphorylation sites associated with the cytochrome components of these mitochondria, occurs to a negligible extent during the oxidative phosphorylation of ADP or State 3.

Addition of ADP or uncoupler to skunk cabbage mitochondria respiring in the controlled state or State 4, results in reduction of cytochrome *c* and the oxidation of the cytochromes *b*, ubiquinone and pyridine nucleotide. A site of interaction of ADP with the respiratory chain between cytochromes *b* and cytochrome *c* is thereby identified by means of the crossover theorem. Flavoprotein measured by fluorescence is also oxidized upon addition of ADP or uncoupler, but flavoprotein measured by optical absorbance changes becomes more reduced under these conditions. Depletion of the mitochondria by pretreatment with ADP and uncoupler prevents reduction of most of the fluorescent flavoprotein by succinate. These results indicate that skunk cabbage mitochondria contain both high and low potential flavoproteins characterized by different fluorescence/absorbance ratios similar to those demonstrated to be part of the respiratory chain in mitochondria from animal tissues.

Recent improvements in the techniques for isolating mitochondria from plant tissues (2) have made it possible to obtain preparations which meet the criteria for a high percentage of intact mitochondria (17). Chief among these criteria is control of respiratory rate by added ADP (9, 10). Such preparations have made it possible to measure ADP/O ratios for different substrates in plant mitochondria (16) and to locate points of interaction of ADP with the carriers of the plant respiratory chain (3), using the crossover theorem (8). Mitochondria isolated from the spadix of the skunk cabbage, which usually show a low degree of sensitivity to inhibition of respiratory rate by cyanide (4, 13, 14), are also usually loth to show control of respiratory rate by ADP. Hackett and Haas (14) investigated the phosphorylation of ADP in skunk cabbage mitochondria respiring with α-ketoglutarate as substrate by determining the disappearance of inorganic phosphate from the reaction mixture which contained glucose and hexokinase. They obtained P/O ratios by this method ranging between 2.6 and 3.6 for this substrate. The ratio was depressed by 0.1 mM DNP[1] to 0.3, but was 1.5 to 1.6 in the presence of either 0.5 mM cyanide or 10 mM azide. No measurements were reported for malate or succinate.

As part of a kinetic study of the electron transport system in skunk cabbage mitochondria, which is reported in an accompanying paper (18), we made mitochondrial preparations which regularly showed energy-linked reduction of pyridine nucleotide with succinate, using the method of Bonner (2) with some minor modifications. A number of these preparations showed respiratory control of oxygen uptake with ADP, particularly those made from the spadices of freshly collected skunk cabbage flowers which had not been subjected to sudden frosts during their period of growth. In this paper, we report ADP/O ratios for succinate and malate in the presence and absence of cyanide, the observation of a crossover point with ADP, and the effect of uncoupling on the steady state redox levels of pyridine nucleotide, flavoprotein, and ubiquinone.

Experimental Procedures

Mitochondria were prepared from excised spadices of skunk cabbage (*Symplocarpus foetidus*) flowers as described in our previous paper (18). Bis (hexafluoroacetonyl)-acetone, designated "1799", was the uncoupler generally used in this study: it was kindly supplied by Dr. P. Heytler of E. I. duPont de Nemours Company. Two other uncouplers were also used: FCCP and TCTFB. All 3 compounds have been shown in this laboratory to be effective uncouplers of plant mitochondria at a concentration of 6 μM.

Redox levels of the respiratory carriers were determined with dual wavelength spectrophotometer (5). The wavelength pair 549 to 540 mμ was used for cytochrome *c*, the pair 560 to 575 mμ for the cytochrome *b* complex, the pair 275 to 290 mμ or 282 to 295 mμ for ubiquinone, and the pair 468 to 488 mμ for flavoprotein. The selection of these wavelength pairs for monitoring the kinetics of the carriers is discussed in our previous paper (18). Changes in fluorescence of pyridine nucleotide and fluorescent flavoprotein were followed with an Eppendorf fluorimeter fitted with a Heraeus ST-75 mercury arc lamp, appropriate primary and secondary filters, and a scale expander constructed in these laboratories (11).

Simultaneous recording of changes in oxygen concentration, pyridine nucleotide fluorescence, and cytochromes *b* and *c* absorbance was carried out in stirred mitochondrial suspensions in a covered cuvette 4 cm wide, 2 cm deep, with a 1 cm optical path, attached to the dual wavelength spectrophotometer, kindly provided by Dr. L. Salganicoff of these laboratories. A Clark electrode was mounted on 1 side of the cuvette with a vibrating stirrer next to it; the stirrer was inserted through a narrow slit to minimize back diffusion of oxygen. The middle part of the cuvette was kept clear for absorbance measurements. The side of the cuvette away from the electrode was illuminated with a small mercury arc lamp, whose 366 mμ emission line was used to excite pyridine nucleotide fluorescence. This was detected by an adjacent photomultiplier tube equipped with a guard filter passing light between 400 mμ and 500 mμ. The photomultiplier tube of the spectrophotometer was protected with a filter transmitting between 500 mμ and 3000 mμ.

Simultaneous oxygen consumption rates and pH changes were measured in a stirred cuvette containing a Clark electrode and a standard glass electrode connected to a pH meter (Radiometer, Copenhagen) equipped with a scale expander.

[1] Abbreviations: DNP: 2,4-dinitrophenol; FCCP: *p*-trifluoromethoxy carbonyl cyanide phenylhydrazone; TCTFB: Tetrachlorotrifluoromethyl benzimidazole; 1799: bis-(hexafluoroacetonyl)-acetone.

Results

A simultaneous recording of the oxygen concentration and the pH in a suspension of skunk cabbage mitochondria with succinate as substrate is shown in figure 1. Upon addition of 0.13 mM ADP to the respiring mitochondria, there is an acceleration of oxygen consumption and, after an initial addition artifact, an alkalinization of the medium. Upon exhaustion of the added ADP, the respiratory rate reverts to its former value, and the alkalinization ceases. Respiratory control by added ADP is thus

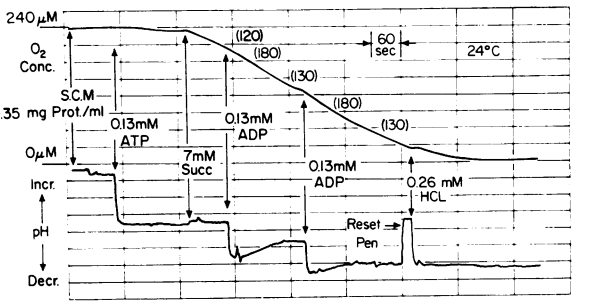

FIG. 1. Simultaneous recording of oxygen concentration and pH of a suspension of skunk cabbage mitochondria. The reaction volume is 3.9 ml, and the mitochondrial concentration is 0.35 mg protein/ml. Addition of 0.13 mM ATP causes a rapid downward deflection of the pH trace due to the acidity of the ATP solution. The rate at which the deflection occurs is a measure of the response time of the system; the time to 90 % deflection is about 5 sec. Rates of oxygen consumption in n atom O/min-mg protein are shown. At the point indicated on the lower trace, the recorder pen is reset to the middle of the chart, and 0.26 mM HCl is added to the suspension for calibration.

observed to be synchronous with the uptake of hydrogen ion from the medium expected for the phosphorylation of ADP to ATP. The respiratory control ratio of 1.4 is low compared to that observed with mitochondria from etiolated mung bean hypocotyls (16). The lack of acidification of the medium on anaerobiosis implies that this low ratio is not the result of ATPase activity. The ADP/O ratio of 1.3 with succinate is also low, but still quite comparable to the ratio of 1.6 observed with mung bean mitochondria (16). ADP/O ratios determined for both succinate and malate in skunk cabbage mitochondria by means of simultaneous oxygen consumption and alkalinization measurements are listed in table I. In the presence of cyanide, the ADP/O ratio with succinate falls to zero, but a value of 0.7 is obtained with malate. These results are consistent with the value of 1.6 obtained by Hackett and Haas for α-ketoglutarate in the presence of cyanide (14), since the substrate phosphorylation site should not be affected by cyanide.

A simultaneous recording of oxygen concentration, fluorescence of pyridine nucleotide, and differential absorbance of cytochrome *c* at 18° is presented

Table I. *Oxygen Consumption Rates, Respiratory Control Ratios and ADP/O Ratios Obtained With Succinate or Malate as Substrates, for Skunk Cabbage Mitochondria in the Presence and Absence of Cyanide*

Substrate (+ Inhibitor)	Oxygen uptake Rate, State 3	Resp. Control	ADP O
	μatom O/min-mg protein	*ratio*[1]	*ratio*[2]
7 mM Succinate	0.18	1.4	1.3
7 mM Succinate (+ 0.3 mM KCN)	0.11	0	0
25 mM Malate	0.15	1.3	1.9
25 mM Malate (+ 0.3 mM KCN)	0.11	1.1	0.7

[1] Rate in state 3 to that in state 4.

[2] Moles ADP phosphorylated to gram atoms O consumed.

in figure 2 for a suspension of skunk cabbage mitochondria showing respiratory control and 17 % inhibition of the state 4 respiratory rate by 0.3 mM KCN. Upon addition of succinate, there is a slow reduction of pyridine nucleotide to the state 4 steady state; cytochrome *c* is quite rapidly reduced, but then is slowly reoxidized to its state 4 steady state level. Addition of 0.26 mM ADP increases the rate of oxygen consumption by a factor of 1.2; pyridine nucleotide becomes more oxidized, but cytochrome *c* becomes more reduced. The effect with cytochrome *c* is small, but is consistently reproducible. Upon exhaustion of the ADP, the oxygen consumption rate reverts to its former value, and the pyridine nucleotide and cytochrome *c* resume their previous state 4 values. The ADP/O ratio in this experiment is 1.2. Upon anaerobiosis, both components become more reduced, cytochrome *c* more rapidly than pyridine nucleotide. Addition of malate in anaerobiosis has no effect on cytochrome *c*, outside of a pulse of oxidation brought about by the oxygen dissolved in the added malate solution. There is a further reduction of pyridine nucleotide, however, caused by the added malate. This "extra" pyridine nucleotide linked to malate, but not reducible in coupled mitochondria by succinate, is consistently observed with skunk cabbage mitochondria and makes up some 30 % to 40 % of the total reducible, fluorescent pyridine nucleotide. Addition of malate alone to coupled mitochondria gives full reduction and subsequent addition of succinate has no effect.

The companion record to that shown in figure 2 is shown for the cytochrome *b* complex in figure 3.

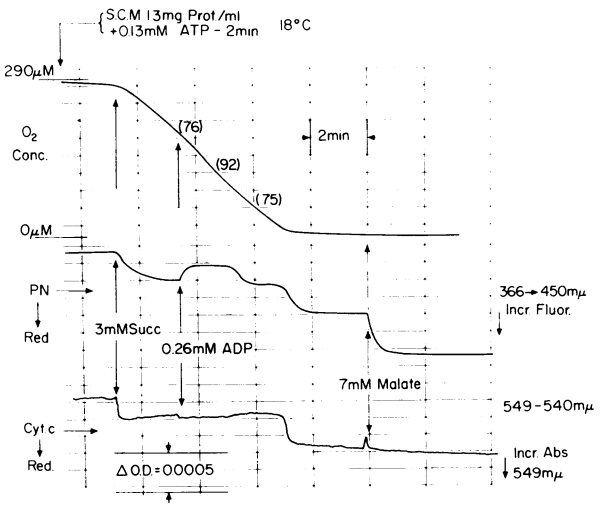

FIG. 2. Simultaneous recording of oxygen consumption, change in pyridine nucleotide fluorescence, and change in absorbance due to cytochrome *c* showing respiratory control with ADP in skunk cabbage mitochondria. Pyridine nucleotide fluorescence centering at 450 mμ is excited with light at 366 mμ; absorbance changes of cytochrome *c* are measured with the dual wavelength spectrophotometer at 549 to 540 mμ. Oxygen concentration is monitored with a Clark electrode. Further experimental details are given in the text. Oxygen rates in n atom O/min-mg protein are given by the figures in parentheses.

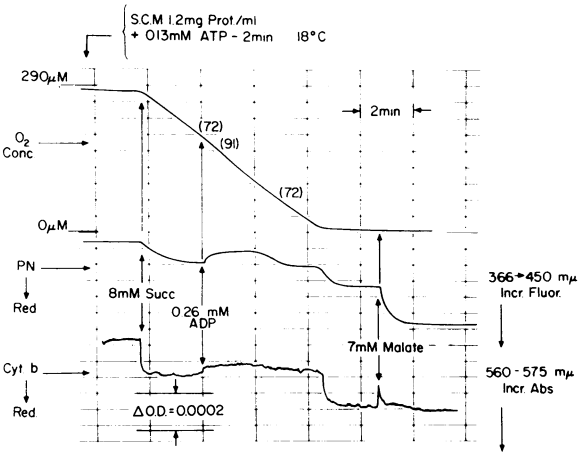

FIG. 3. Simultaneous recording of oxygen consumption, change in pyridine nucleotide fluorescence, and change in absorbance due to cytochrome *b* as measured at 560 to 575 mμ, showing respiratory control with ADP. Reaction conditions are identical to those in figure 2. Figures in parentheses give oxygen consumption rate in n atom O/min-mg protein.

In this experiment, the respiratory control ratio is 1.3 and the ADP/O ratio is 1.2. Addition of ADP produces an oxidation of cytochrome *b*, which, while not extensive, is always observed. Upon exhaustion of ADP, the oxygen consumption rate decreases to its original value; pyridine nucleotide becomes more reduced and returns to its state 4 redox level. The reduction of the cytochrome *b* complex to its state 4 level is slow and does not reach completion before the onset of anaerobiosis. The increase in extent of oxidation of the cytochrome *b* and decrease in extent of cytochrome *c* oxidation, which occur con-

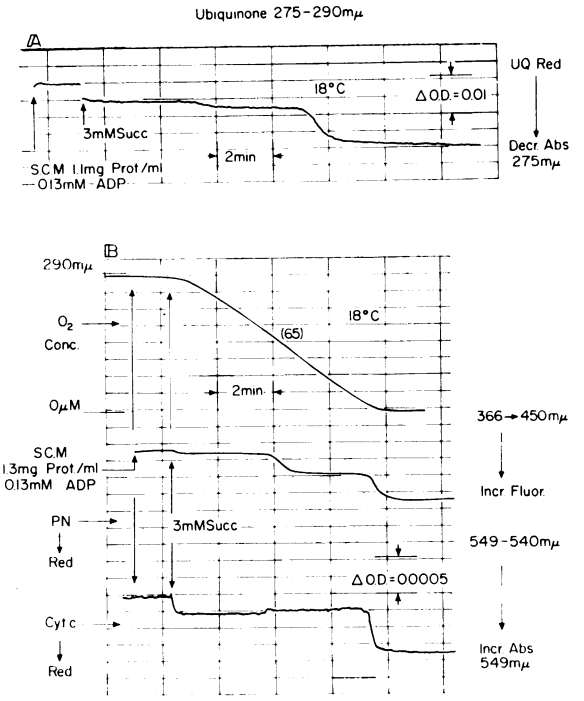

FIG. 4. A) Redox level of ubiquinone during the series of transitions from state 2 to state 3, then to state 4, and finally to state 5 (anaerobiosis) as monitored with the dual wavelength spectrophotometer using the wavelength pair 275 to 290 mμ. The light path is 0.5 cm. The mitochondrial suspension is treated with 0.13 mM instead of ATP prior to the addition of succinate. B) Simultaneous recording of oxygen consumption, change in pyridine nucleotide fluorescences, and change in absorbance due to cytochrome *c* as measured at 549 to 540 mμ. The mitochondrial suspension has been treated with 0.13 mM ADP instead of ATP prior to addition of succinate; otherwise, reaction conditions are those described in figure 2. Figures in parentheses give the oxygen consumption rate in n atom O/min-mg protein.

comitantly with an increase in oxygen uptake on addition of ADP, identify a point of interaction for ADP between the cytochromes *b* and cytochrome *c* of skunk cabbage mitochondria by means of the crossover theorem (8). The extent of the changes does not affect the predictions of this theorem, only the direction of those changes.

The redox level of ubiquinone in state 3 and state 4 cannot be ascertained in the experiments described above, in which ADP is added to the mitochondrial suspension after addition of succinate, because of the high absorbance of ADP below 300 mμ. If ADP is added to the suspension first, the 2 beams of the dual wavelength spectrophotometer can then be balanced in intensity, and the absorbance of ADP is thereby compensated. The mitochondria are then in state 2; addition of succinate then induces a state 2 to state 3 transition, followed by a state 3 to state 4 transition upon exhaustion of ADP. This effect is shown in figure 4A for ubiquinone; the state 3 to state 4 transition results in reduction of ubiquinone. A simultaneous recording of oxygen concentration and the redox levels of pyridine nucleotide and cytochrome *c* are shown in figure 4B for the parallel experiment to figure 4A. Pyridine nucleotide becomes reduced and cytochrome *c* becomes oxidized in the state 3 to state 4 transition, as expected from the record shown in figure 2. The redox state of ubiquinone follows that of cytochrome *b* and pyridine nucleotide in coupled skunk cabbage mitochondria. There is no initial increase in oxygen consumption rate upon addition of succinate to the mitochondrial suspension: the rate increases slowly and then remains constant at about the expected state 4 value. The state 3 induced by this order of addition of ADP and succinate corresponds to the "first state 3" observed by Ikuma and Bonner with mung bean mitochondria (16). The rate in this state is lower than that observed if ADP is added to these mitochondria pretreated with ATP followed by addition of succinate; this faster rate is also achieved on subsequent additions of ADP to mung bean mitochondria which have cycled through a "first state 3." In skunk cabbage mitochondria, the rate in the "first state 3" is apparently sufficiently less than that of the true state 3 that is indistinguishable from the rate observed in state 4. Under these conditions, the ADP/O ratio is only 0.6, about half that observed in the experiments of figure 2 and figure 3.

Addition of uncoupler after respiration with succinate has been initiated produces an effect similar to that produced by ADP, as shown in figure 5. The oxygen consumption rate increases, pyridine nucleotide becomes more oxidized, and cyto-

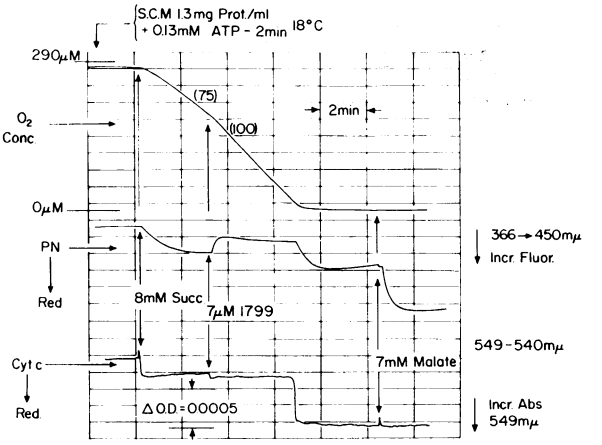

FIG. 5. Simultaneous recording of oxygen consumption, change in pyridine nucleotide fluorescence and change in absorbance due to cytochrome *c*, showing the effect of uncoupler added after substrate. Initial reaction conditions are identical to those in figure 2. Figures in parentheses give rates of oxygen consumption in n atom O/min-mg protein.

chrome *c* is more reduced in the uncoupled steady state. On anaerobiosis cytochrome *c* is rapidly reduced. Pyridine nucleotide is slowly reduced in anaerobiosis but, in the presence of uncoupler, a slow reoxidation is evidently also occurring. Addition of malate produces a further increase in pyridine nucleotide reduction but does not appear to suppress completely the slow reoxidation reaction.

The effect of adding uncoupler during the aerobic steady state with succinate on flavoprotein fluorescence, flavoprotein absorbance, and ubiquinone absorbance is shown in figure 6. As in the previous experiments, the mitochondria have been preincubated with ATP. While there is a slight decrease in absorbance at 468 mμ upon uncoupler addition, signifying flavoprotein reduction (fig 6A) there is a pronounced increase under the same conditions in the fluorescence emission appropriate to oxidized flavoprotein (fig 6B). Fluorescent flavoprotein is affected by uncoupler in the same way as is pyridine nucleotide; this would be expected if the bulk of the fluorescence emission comes from the flavoproteins connected with the substrate-linked NADH dehydrogenase and the first phosphorylation site (7, 15). Absorbing flavoprotein behaves in the opposite manner to fluorescent flavoprotein under these conditions: the differences between them 2 are discussed in more detail below. Ubiquinone becomes oxidized upon uncoupler addition (fig 6C), and in this respect behaves similarly to cytochrome *b*, pyridine nucleotide, and fluorescent flavoprotein under these same conditions.

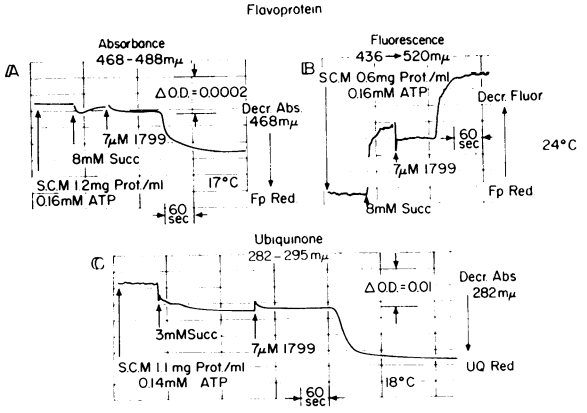

FIG. 6. Effect of adding uncoupler during the aerobic steady state with succinate on flavoprotein absorbance (A), flavoprotein fluorescence (B), and ubiquinone absorbance (C) in skunk cabbage mitochondria. The flavoprotein measurements were carried out on the same mitochondrial preparation at 17° for the absorbance change (A) and 24° for the fluorescence change (B). A different mitochondrial preparation was used for measurement of absorbance change due to ubiquinone shown in C. The light path for the flavoprotein absorbance measurement was 1.0 cm; that for the ubiquinone absorbance measurement was 0.5 cm.

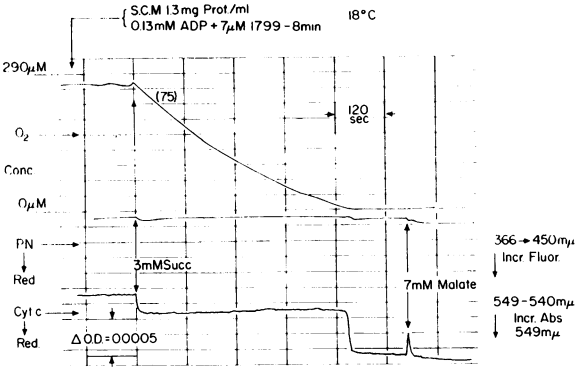

FIG. 7. Simultaneous recording of oxygen consumption, changes in pyridine nucleotide fluorescence, and change in absorbance due to cytochrome *c*, showing the effect of depletion by added ADP and uncoupler. At the point indicated on the records, the mitochondrial suspension has been incubated with 0.13 mM ADP and 10 μm 1799 for a total of 8 min. Otherwise, reaction conditions are identical to those described in figure 2. Figures in parentheses denote oxygen consumption as n atom O/min-mg protein. The pyridine nucleotide fluorescence is recorded at the same sensitivity as in figures 2, 3, and 4.

Incubation of the mitochondrial suspension with ADP and uncoupler for 6 to 10 min results in the depletion of energy conservation capacity. Oxidation of succinate under these conditions supports the reduction of pyridine nucleotide in skunk cabbage mitochondria to a very limited extent (18). This effect is shown in figure 7. Addition of succinate to a suspension of skunk cabbage mitochondria, which has been treated for 8 min with 130 μM ADP and 7 μM 1799, initiates the uptake of oxygen at a rate comparable to that obtained with these mitochondria in state 4. There is a fast, partial reduction of cytochrome *c*, and a very slight reduction of pyridine nucleotide. As the reaction proceeds, the rate of oxygen consumption decreases; the pyridine nucleotide slowly becomes more oxidized, and there is a perceptible drift to a higher degree of oxidation by cytochrome *c*. On anaerobiosis, cytochrome *c* becomes rapidly reduced, but pyridine nucleotide becomes reduced to less than 10 % of the extent achieved with succinate in coupled mitochondria. The addition of malate results in little further reduction of pyridine nucleotide.

The decrease in oxygen consumption rate observed in figure 7 is a good indication that depletion of the energy conservation capacity has been successful. Under conditions where no decrease in rate is observed after pretreatment of the mitochondria with ADP and uncoupler, up to 30 % of the endogenous pyridine nucleotide which can be reduced with succinate on anaerobiosis actually is reduced. While the length of time required for pretreatment to produce depletion varied somewhat from preparation to preparation, we found that at

least 6 to 8 min were required. The more extensive the depletion, the lower the percentage of pyridine nucleotide reduced on anaerobiosis. Depletion, as indicated by a decreasing oxygen uptake with succinate, was also accomplished with the uncouplers FCCP and TCTFB at 10 μM, as well as with 1799. The oxidation rate for malate responds similarly to the depletion process, but the inhibitory effect is much more pronounced than with succinate.

The changes in the redox level of flavoproteins as coupled or depleted skunk cabbage mitochondria pass from aerobiosis to anaerobiosis through the aerobic steady state with succinate are presented in figure 8 for the absorbing flavoproteins and fluorescent flavoproteins. These experiments were carried out in parallel on the same mitochondrial preparation, but in different locations at different temperatures. In the coupled condition, there is an absorbance change at 468 to 488 mμ between the aerobic and anaerobic states (fig 8A) corresponding to 0.67 nmole flavoprotein/mg protein, calculated on the basis of a millimolar difference extinction coefficient of 4 mM^{-1} cm^{-1} for this wavelength pair (18). The extent of reduction in the aerobic steady state is 17 %. The fluorescent flavoprotein, however, shows a steady state reduction level of 50 % (fig 8B). In depleted mitochondria, the steady state reduction level of absorbing flavoprotein is 19 % (fig 8C) while the absorbance change observed between aerobic and anaerobic states is 78 % of that observed in the same preparation of coupled mitochondria. Fluorescence change observed in depleted mitochondria is only 10 % of that observed in coupled mitochondria (fig 8D), but the extent of reduction in the aerobic steady state with succinate is still around 40 %. Addition of malate, after anaerobiosis has been achieved by the oxidation of succinate, gives little or no further change in absorbance or fluorescence with coupled skunk mitochondria, in contrast to the observed increase in fluorescence due to reduced pyridine nucleotide. There is also no further change in absorbance with depleted mitochondria on addition of malate, while a small, variable fluorescence decrease is observed under these conditions.

The diminution of flavoprotein fluorescence in the presence of succinate as a result of the depletion process is further evidence that a fluorescent flavoprotein of low potential, similar to the Fp_L plus Fp_{D1} in mammalian mitochondria described by Chance *et al.* (7) and Hassinen and Chance (15), also exists in plant mitochondria. If we assume that this flavoprotein complex remains completely oxidized in the

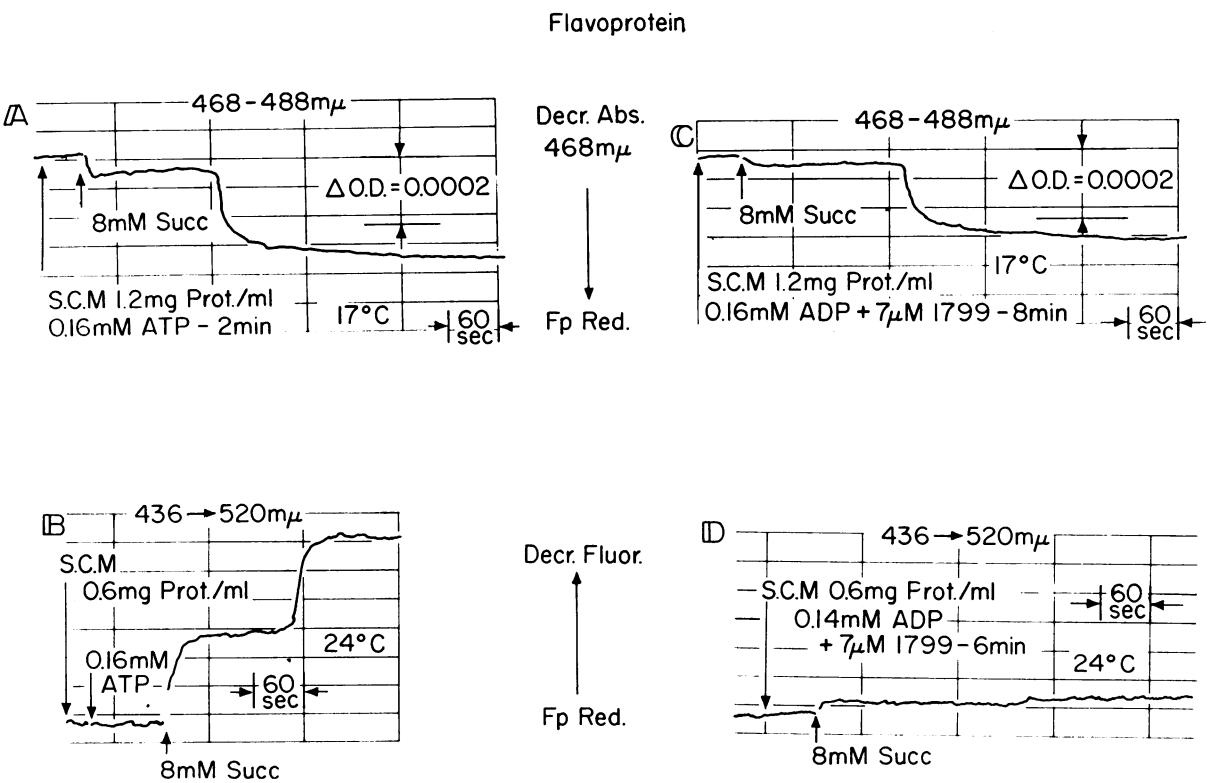

FIG. 8. Effect of depletion on absorbance and fluorescence changes due to flavoproteins in skunk cabbage mitochondria. Control experiments with coupled mitochondria are shown in A for absorbance and B for fluorescence changes; in these experiments the mitochondria are treated with 0.16 mM ATP for 2 to 3 min prior to the addition of succinate. Mitochondria were depleted by incubation with 0.16 mM ADP plus 7 μM 1799 for 7 to 9 min prior to the addition of succinate. The absorbance change mitochondria is shown in C and the corresponding fluorescence change is shown in D.

depleted state, then the 22 % loss in absorbance may be attributed to it. If we make the further assumption that the difference extinction coefficient at 468 to 488 mμ is the same for all the mitochondrial flavoproteins, then this flavoprotein would be present in skunk cabbage mitochondria at a level of 0.15 nmole/mg protein, which is the value observed in beef heart mitochondria (7). Unfortunately, the task of sorting out the other flavoproteins of the respiratory chain of skunk cabbage mitochondria has not yet been accomplished, and we cannot assign fluorescence/absorbance (f/a) ratios (7) to them. Most of the combinations of substrates and inhibitors used by Chance *et al.* (7) and Garland *et al.* (12) to make these assignments for mammalian mitochondria do not function or function differently in plant mitochondria. We have shown, however, in our kinetic study of the respiratory chain of these mitochondria, that both fluorescent and absorbing flavoprotein of depleted mitochondria reduced with succinate show the same kinetics of oxidation and reduction in a cycle initiated by an oxygen pulse (18). Since the degree of reduction of flavoprotein by succinate in the aerobic steady state is 40 % when measured by fluorescence (fig 8D) and 10 % when measured by absorbance (fig 8C) in depleted mitochondria, we conclude that there are 2, high potential flavoproteins which equilibrate fast enough to behave as a single kinetic entity. These 2 we term Fp_S and Fp_{D_2}, the flavoprotein of succinate dehydrogenase and the high potential flavoprotein of NADH dehydrogenase, by analogy with the mammalian system (7).

Ubiquinone is fully reduced by succinate in depleted as well as in coupled skunk cabbage mitochondria. In the depleted condition, these mitochondria show about 20 % to 30 % reduction of ubiquinone in the aerobic steady state with 3 mM succinate; under the same conditions the extent of reduction is about 50 % with mitochondria in the coupled condition. The kinetics of ubiquinone oxidation and reduction in the depleted state (18) are those expected for 1 pool of this respiratory carrier.

Discussion

The values of the ADP/O ratio obtained for oxidative phosphorylation with skunk cabbage mitochondria are in accord with 2 phosphorylation sites for succinate as substrate and 3 for malate, taking 0.6 to 0.7 as the ratio for each site. In the presence of cyanide, 1 site is still operative with malate as substrate, but there is no phosphorylation with succinate. This result is consistent with the idea put forward by Bendall, Bonner, and Plesnicar (1) that an alternate oxidase associated with the flavoprotein and pyridine nucleotide components of the respiratory chain functions in the presence of cyanide or antimycin A. Thus, the phosphorylation site associated with NADH-linked substrates continues to function in the presence of respiratory inhibitors, but the other 2 sites do not. The ADP/O ratio for α-ketoglutarate is calculated from our results to be 2.9 in the absence of inhibitor and 1.7 in the presence of cyanide, assuming 100 % efficiency for substrate phosphorylation, in good agreement with the data presented by Hackett and Haas (14).

With succinate, we observe a crossover point between the cytochromes *b* complex and cytochrome *c* upon addition of ADP. A similar result has been reported for mitochondria from white potatoes (3). One phosphorylation site between succinate and oxygen can be assigned to the region between the cytochromes *b* and cytochrome *c* in skunk cabbage mitochondria. We do not have enough data to assign 1 of the 3 cytochromes *b* to this site. The second phosphorylation site in these mitochondria between succinate and oxygen we place between cytochromes *a* and a_3, by analogy with such an assignment in black valentine bean mitochondria by Bonner and Plesnicar (3) and in rat liver mitochondria by Wilson and Chance (19).

The phosphorylation efficiency of the flavoprotein phosphorylation site should be given by the ADP/O ratio obtained with malate in the presence of cyanide, which is 0.7; the phosphorylation efficiency is then 70 %. If all sites have this efficiency, then the ADP/O ratio with succinate is calculated to be 1.4 and with malate to be 2.1 in the absence of cyanide. These ratios are close to the values observed. Indeed, if the efficiency is taken to be 65 % at each site, the agreement between calculated and observed ADP/O ratios is nearly exact. We conclude that electron transport through the alternate oxidase pathway, which would bypass the phosphorylation sites associated with the cytochromes, is negligible in skunk cabbage mitochondria in the absence of cyanide. Reducing equivalents, passing through the respiratory chain of plant mitochondria having a functional alternate oxidase, appear to select the cytochrome pathway to oxygen over the alternate oxidase pathway in state 3. The percentage of the total flux passing through the cytochrome pathway in the absence of cyanide we define as the "selectivity" of a branched respiratory chain with 2 terminal oxidases. In the case of skunk cabbage mitochondria in state 3, the selectivity appears to be 100 %. The cyanide "sensitivity" is defined as the percent inhibition of the total respiratory rate in state 3 by that inhibitor. Both these parameters are useful in describing the oxidative activity of plant mitochondria. We do not know as yet whether high selectivity in state 3 is a property characteristic of all plant mitochondria showing partial or nil cyanide sensitivity, or what degree of selectivity is shown in state 4.

The responses of the redox level of ubiquinone to ADP and uncouplers parallels those of pyridine nucleotide and highly fluorescent flavoprotein. This implies that ubiquinone participates in those energy conserving processes by which pyridine nucleotide

is reduced in coupled skunk cabbage mitochondria by the oxidation of succinate. One possibility is that reduced ubiquinone acts as the pool of reducing equivalents which then pass to pyridine nucleotide by an energy-linked process in reversed electron transport, as suggested by Chance (6). Ubiquinone could perform this function by interacting with Fp_{D_2} which, while in rapid equilibrium with Fp_S, is taken to be part of the first energy conserving site in conjunction with Fp_{D_1} (7). The results of our kinetic study showed that ubiquinone interacts on a sidepath with this pool of flavoprotein (18). Reversed electron transport from reduced ubiquinone would then occur through this site to Fp_{D_1} with the expenditure of energy, and thence to pyridine nucleotide.

A proposed scheme for electron transport in coupled skunk cabbage mitochondria is shown in figure 9. The pathway between succinate and oxygen is essentially that deduced from our kinetic study of uncoupled mitochondria (18), but we propose that the flow of reducing equivalents directly from the branch point Y to cytochrome *c* is severely inhibited or not functional in coupled mitochondria. Placement of the flavoprotein follows the scheme of Chance *et al.* (7) as discussed in the previous section of this paper, and ubiquinone is shown on a side path interacting with Fp_{D_2}. The additional NAD shown associated with malate dehydrogenase is to explain the further reduction of pyridine nucleotide by malate over that achieved by succinate in anaerobiosis, as shown in figure 2 and 3. Sites of energy conservation, and hence sites of oxidative phosphorylation, are designated by the traditional "squiggle". The designation of the cytochrome *b* complex by the single letter *b* is required by our present ignorance of how these components differ and how these differences contribute to their function.

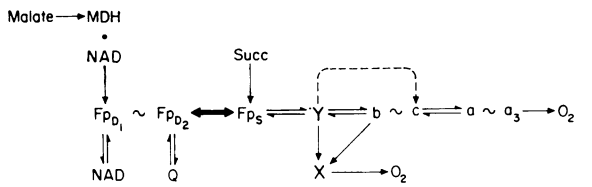

FIG. 9. Proposed scheme of electron transport in coupled skunk cabbage mitochondria. Sites of energy conservation are denoted by the symbol "~". The symbol Y is used for the branch point and the symbol X is used for the alternate oxidase. The dashed line between Y and cytochrome *c* denotes the path of electron transport which predominates in depleted mitochondria (18).

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