The Respiratory Chain of Plant Mitochondria

XI. ELECTRON TRANSPORT FROM SUCCINATE TO ENDOGENOUS PYRIDINE NUCLEOTIDE IN MUNG BEAN MITOCHONDRIA

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ABSTRACT

Energy-linked reverse electron transport from succinate to endogenous NAD in tightly coupled mung bean (*Phaseolus aureus*) mitochondria may be driven by ATP if the two terminal oxidases of these mitochondria are inhibited, or may be driven by the free energy of succinate oxidation. This reaction is specfic to the first site of energy conservation of the respiratory chain; it does not occur in the presence of uncoupler. If mung bean mitochondria become anaerobic during oxidation of succinate, their endogenous NAD becomes reduced in the presence of uncoupler, provided that both inorganic phosphate (P_i) and ATP are present. No reduction occurs in the absence of P_i, even in the presence of ATP added to provide a high phosphate potential. If fluorooxaloacetate is present in the uncoupled, aerobic steady state, no reduction of endogenous NAD occurs on anaerobiosis; this compound is an inhibitor of malate dehydrogenase. This result implies that endogenous NAD is reduced by malate formed from the fumarate generated during succinate oxidation. The source of free energy is most probably the endogenous energy stores in the form of acetyl CoA, or intermediates convertible to acetyl CoA, which removes the oxaloacetate formed from malate, thus driving the reaction towards reduction of NAD.

In the absence of P_i and presence of oligomycin, oxidation of succinate by the alternative cyanide-insensitive oxidase pathway, in the presence of sulfide to inhibit cytochrome oxidase, does not reduce endogenous NAD, either in the aerobic steady state or in anaerobiosis. Under these conditions, only the reversed electron transport pathway from succinate to endogenous NAD is active and ATP cannot interact with the respiratory chain. The source of energy for NAD reduction must come from the respiratory chain, and this result shows that oxidation of succinate through the alternate pathway does not provide this energy.

The reduction of endogenous pyridine nucleotide in mitochondria by succinate by means of an energy-linked reaction utilizing the free energy of substrate oxidation or of ATP hydrolysis to drive reversed electron transport through the respiratory chain has been known for some years (8, 11, 12, 14, 15, 32) in mammalian mitochondria. Kidney and heart mitochondria have proved particularly useful because of their low content of NADP (13, 17). Plant mitochondria contain no detectable NADP (26, 28, 29) and appear to lack the energy-linked NAD-NADP transhydrogenase (27). They are thus pe-

culiarly well suited for such a study but, curiously enough, very little published material concerning this reaction in plant mitochondria has appeared. Early work by Bonner (4) indicated that ATP was required for endogenous NAD reduction by succinate even if free energy were available from oxidation of the succinate by coupled mung bean mitochondria, since oligomycin appeared to inhibit the reaction. Ikuma (29) reported that this reduction was enhanced not only by ATP, but also by ADP and oligomycin. He also reported that respiratory inhibitors, as well as uncouplers, inhibited the reaction. Wilson and Bonner (50) have recently reported that submitochondrial particles made from mung bean mitochondria by osmotic shock can utilize succinate to reduce added NAD by an energy-linked reduction which is driven either by ATP or by substrate oxidation. This observation is in accord with the earlier one of Lee and Ernster (35), who utilized submitochondrial particles from beef heart muscle. It would appear that plant mitochondria should carry out this reaction in much the same way as mitochondria from animal tissue. If this were so, then energy-linked NAD reduction could be used as a marker for energy conservation in the plant respiratory chain, in particular when the path of respiration is through the alternate, cyanide-insensitive oxidase (2, 6, 25, 47). But reduction of endogenous NAD in mung bean mitochondria by succinate in the presence of uncoupler has also been observed in this laboratory; the reduction is inhibited by aerobic incubation in the absence of substrate and presence of ADP and uncoupler (44). The work reported in this paper was undertaken to clarify the pathway of energy-linked NAD reduction and to find conditions under which the reaction could be used as an indicator of energy conservation in the plant respiratory chain.

MATERIALS AND METHODS

Mitochondria were prepared from the hypocotyls of 5- or 6-day-old etiolated seedlings of mung bean (*Phaseolus aureus*), using substantially the methods of Bonner (5) and Ikuma and Bonner (30), with modifications as previously described (46). The mitochondria were assayed for respiratory control (18) in a medium containing 0.3 M mannitol, 10 mM TES, 5 mM P_i, and brought to pH 7.2 with KOH. This medium is designated TP; the same medium without phosphate is designated T. Oxygen consumption by the mitochondrial suspension with succinate or malate as substrate was measured in a closed cuvet with a Clark electrode (Yellow Springs Instrument Co.) as described by Estabrook (24). Mitochondrial protein content was determined by a modified Lowry method (38).

Adenine nucleotides were obtained from Boehringer Mannheim Corp., succinic acid from Aldrich Co., and L-malic acid from Sigma, and were used without further purification. The

uncoupler 1799[1] was generously supplied by Dr. Peter Heytler of E. I. duPont de Nemours and Co., Inc., and a sample of FOAA was generously provided by Dr. Ernest Kun of the San Francisco Medical Center, University of California.

The redox level of the endogenous NAD was monitored fluorimetrically (11) with an Eppendorf fluorimeter equipped with a 366 nm primary filter to provide light for excitation and a secondary filter transmitting either between 460 nm and 3000 nm or between 490 nm and 3000 nm to pass the emitted light. Flavoprotein fluorescence changes were also monitored with the Eppendorf fluorimeter as previously described (43), utilizing primary filters with either 436 nm or 463 nm (20 nm band width) transmittance for the excitation light. Flavoprotein absorbance changes were recorded with a dual wavelength spectrophotometer (10), using the wavelength pair 468 to 493 nm (43).

RESULTS

Energy-linked Reduction by Added ATP. The results presented in Figure 1 are evidence that reduction of endogenous NAD in mung bean mitochondria can utilize the energy from ATP to drive the reaction with succinate as electron donor, as do mitochondria from animal tissues. In the experiment of Figure 1, oxygen consumption is inhibited by sulfide, which blocks electron transport through cytochrome oxidase, but does not uncouple (17), and by mCLAM which blocks electron transport through the alternate oxidase (41). In the record of Figure 1A, addition of succinate to the doubly inhibited mitochondria has little effect, but subsequent addition of ATP results in reduction of about 50% of the endogenous NAD. The reduction is followed by a slow oxidation as ATP is utilized. Addition of uncoupler causes complete reoxidation, and reduction to a level of 70% occurs on malate addition. The ATP-driven reduction is inhibited by oligomycin. The records of Figure 1, B and C, show that the same series of additions affects the redox state of the fluorescent flavoprotein, Fp_{1f} (43, 45), in much the same way as it does that of pyridine nucleotide. The flavoprotein is essentially fully reduced on addition of ATP, fully reoxidized by uncoupler, then fully reduced again by malate, as would be expected from its midpoint potential, $E_{m7.2} = -155$ mv (45), some 170 mv more positive than that of the $NAD^+/NADH$ couple (9). The record of Figure 1D shows that the higher potential, low fluorescence flavoproteins are reduced by succinate in the absence of ATP, and that the energy-linked reactions are accompanied by the small absorbance changes characteristic of Fp_{1f}.

The experiment of Figure 1 was carried out in the absence of added P_i to maximize the phosphate potential[2] achieved by addition of ATP (17). If the same experiment is carried out in the presence of 5 mM P_i, no energy-linked reduction of either NAD or Fp_{1f} is obtained, as shown in the experimental records of Figure 2. Addition of malate to the uncoupled mitochondria under these conditions does give nearly complete reduction of pyridine nucleotide, as shown in Figure 2A. While suppressing the energy-linked reduction driven by ATP through a lowered phosphate potential, P_i enhances reduction of endogenous NAD by added malate.

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[1] Abbreviations: 1799: bis-(hexafluoroacetonyl)acetone; FOAA: monofluorooxaloacetic acid; mCLAM: *m*-chlorobenzhydroxamic acid; PN: pyridine nucleotide.

[2] The phosphate potential is defined at RT ln $(ATP)/(ADP)(P_i)$; a full discussion of this quantity is given in ref. 17. As used in this paper, a high phosphate potential means that the ratio $(ATP)/(ADP)(P_i)$ is large.
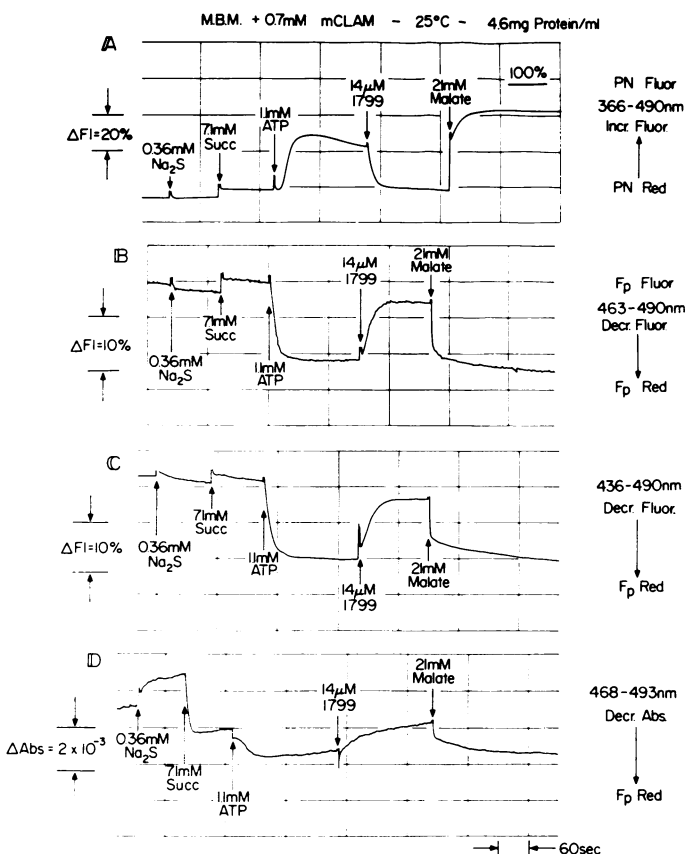

FIG. 1. Reversed electron transport from succinate to endogenous NAD in mung bean mitochondria in the absence of added P_i driven by added ATP. Respiration is blocked by sulfide which inhibits cytochrome oxidase and by mCLAM which inhibits the alternate oxidase (41). A: Fluorescence changes corresponding to reduction and oxidation of endogenous NAD. B and C: Fluorescence changes corresponding to reduction and oxidation of flavoprotein, using the more specific excitation wavelength of 463 nm (B) and the more intense excitation wavelength of 436 nm (C). D: Absorbance changes corresponding to reduction and oxidation of flavoprotein, monitored with the dual wavelength spectrophotometer at 468 to 493 nm.

Energy-linked Reduction by Substrate Oxidation. In the presence of P_i but absence of added ATP, reverse electron transport to endogenous NAD from succinate driven by the free energy of succinate oxidation is sluggish, resulting in a low degree of reduction during the aerobic steady state, and slow reduction on anaerobiosis (Fig. 3A). Addition of oligomycin at a concentration giving full inhibition of the mitochondrial ATPase (31) increases the reduction rate, resulting in some 70% reduction in the aerobic steady state after an initial slow reduction (Fig. 3B). This result agrees with Ikuma's report (29) of stimulation of this reaction by oligomycin. If ATP is added to the oligomycin-treated mitochondria, the rate of NAD reduction is markedly increased (Fig. 3C), the steady state extent of reduction is over 90% and complete reduction is attained rapidly on anaerobiosis. It is evident that the rate of succinate oxidation is more rapid in the experiment of Figure 3C than in those of Figure 3, A and B, since the duration of the aerobic steady state is some 40% shorter. This was verified by oxygen consumption measurements; the mitochondrial respiratory rate in the presence of ATP corresponds to the "second state 4" rate for succinate oxidation reported by Ikuma and Bonner (30). This rate is not affected by oligo-
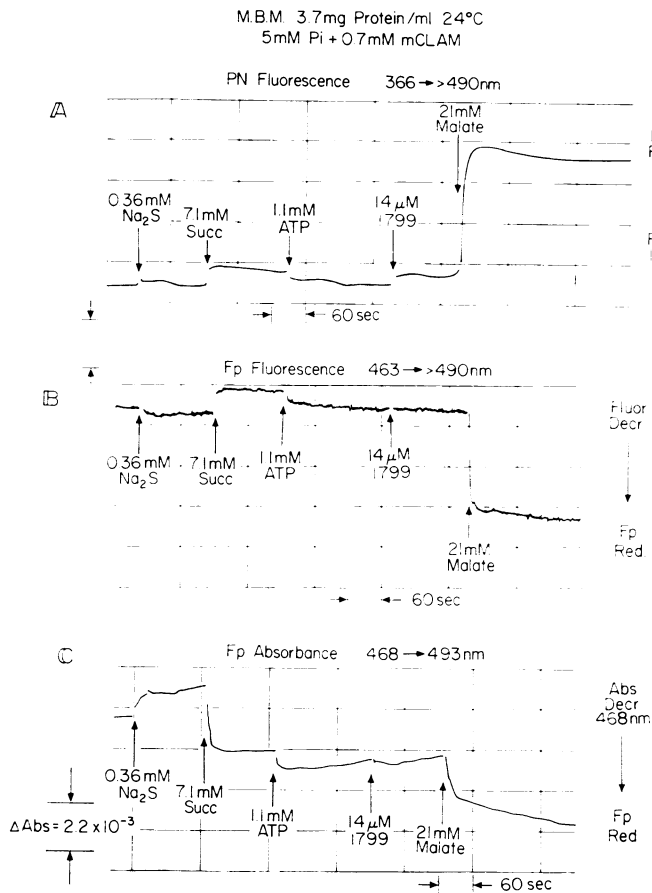
FIG. 2. The experiment of Figure 1 carried out in the presence of 5 mM P_i, giving a low phosphate potential compared to the conditions for Figure 1.

mycin at concentrations which prevent ATP from interacting with the respiratory chain.

If experiments analogous to those of Figure 3 are carried out in the absence of P_i, reduction of endogenous NAD is rapid in all cases, as shown by the records in Figure 4. In the absence of both P_i and oligomycin, the extent of NAD reduction is about 80% in the aerobic steady state (Fig. 4A). Comparison of the record in Figure 4A with that of Figure 3A indicates that the respiratory chain responds to the phosphate potential in the absence of oligomycin. In contrast with its stimulatory effect in the presence of P_i, oligomycin seems to slow down somewhat the rate of NAD reduction in the absence of P_i (Fig. 4B). If both ATP and oligomycin are present, NAD reduction is rapid and complete in both the presence (Fig. 3C) and absence (Fig. 4C) of P_i. Further, ATP stimulates the rate of succinate oxidation in the absence as well as in the presence of P_i, as shown by the shorter duration of the aerobic steady state in Figure 4C, and verified by oxygen consumption measurements.

It is evident from the foregoing experiments that energy-linked reversed electron transport from succinate to endogenous NAD occurs in mitochondria from plant tissues as readily as in animal mitochondria. The required free energy may derive from substrate oxidation, or it may derive from added ATP. Further, the reversed electron transport involves the first coupling site of the mitochondrial respiratory chain: the reaction is entirely specific to the endogenous pyridine nucleotides. Despite repeated attempts with fresh, tightly coupled mung bean mitochondria, no exogenous NAD^+ could be re-

duced via the NADH dehydrogenase which is located on the substrate side of the second site of energy conservation, and which allows these mitochondria to use NADH as substrate with respiratory control (30).

NAD Reduction not Involving the Respiratory Chain. One peculiarity of the reduction of endogenous NAD in mung bean and skunk cabbage (*Symplocarpus foetidus*) mitochondria is that it can occur, at least partially, with succinate as donor in anaerobiosis in the presence of an uncoupler (44, 46). The reduction no longer occurs if the mitochondria have been "depleted" of energy by aerobic incubation with ADP and uncoupler in the absence of added substrate (46). This observation suggests that there may be a second pathway by which reducing equivalents may be transferred from succinate to endogenous NAD, but which does not involve the first energy conservation site of the respiratory chain. The experimental records in Figure 5 show some of the characteristics of this reaction. Addition of succinate to mung bean mitochondria pretreated with ATP (Fig. 5A) gives nearly complete reduction of NAD in the aerobic steady state; reoxidation on addition of the uncoupler 1799 is also complete. On anaerobiosis, however, the endogenous NAD becomes reduced again, but at a slower rate. The reduction is complete since no further reduction occurs on addition of malate. If P_i is omitted from the reaction medium (Fig. 5B), the anaerobic reduction of NAD no longer occurs in the presence of uncoupler and subsequent NAD reduction by malate is slow. The anaerobic reduction pattern with regard to P_i of the endogenous NAD in Figure 5, A and B, is the opposite of that observed with the energy-linked
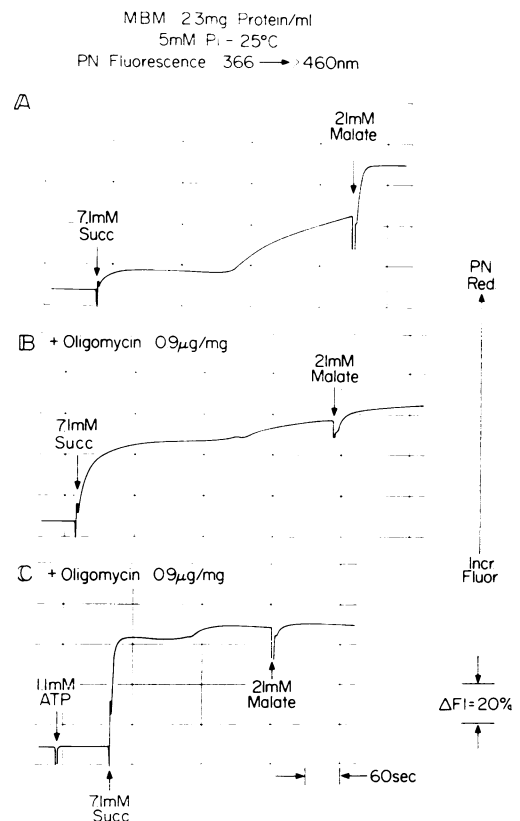

FIG. 3. Reversed electron transport from succinate to endogenous NAD, driven by the free energy of succinate oxidation, in the presence of 5 mM P_i. A: No additions prior to succinate; B: oligomycin added at 0.9 μg/mg mitochondrial protein; C: oligomycin added, followed by ATP at the point shown on the experimental record.

reductions shown in Figures 1 and 2. In the latter, the high phosphate potential attainable in the *absence* of added P_i is required for the reduction to occur. As the mitochondria age, the

FIG. 4. The experiment of Figure 3 carried out in the absence of added phosphate. Additions prior to succinate were as in Figure 3.

rate of the anaerobic, uncoupled reduction of NAD diminishes somewhat, and a lag between the transition to anaerobiosis and maximal reduction rate is observed (Fig. 5C). The reaction occurs equally well in the presence of oligomycin, but the duration of the lag period increases somewhat (Fig. 5D).

The effect of adding P_i in anaerobiosis on the uncoupled endogenous NAD reduction is shown in Figure 6C. The records of the control experiments, Figure 6, A and B, show the reduction in anaerobiosis in the absence and presence of oligomycin, respectively. Comparison of the two records shows the slight lag in reduction induced by oligomycin. Otherwise, the rates and extent of anaerobic reduction are nearly comparable, underlining the lack of participation of the respiratory chain energy conservation sites in this reaction. No reduction of endogenous NAD occurs in the absence of P_i (Fig. 6C) in the time required for complete reduction in its presence (Fig. 6A). Addition of 5 mM P_i in anaerobiosis does induce the reduction of endogenous PN, but only after a lag period during which the added P_i is presumably entering the mitochondria.

The requirement for ATP, or ATP precursor, in addition to P_i for uncoupled, anaerobic NAD reduction is evident from the experimental records of Figure 7. The relatively low extent of reduction in the aerobic steady state with succinate in the absence of ATP but presence of P_i is abolished by the addition of uncoupler, and no reduction occurs in anaerobiosis (Fig. 7A). Subsequent addition of malate gives rapid and complete reduction of the endogenous NAD. If ATP is added in anaerobiosis, as shown in Figure 7B, rapid and complete NAD reduction ensues after a short lag. If ADP is substituted for ATP as in Figure 7C, NAD reduction does occur, but more slowly and after a longer lag period. This effect is most probably due to generation of ATP, along with AMP, from the added ADP by the ATP-AMP kinase known to be present in plant mitochondria (37). It appears that some ATP is required for the reduction to take place, but that a high phosphate potential is not. The same effect is obtained with GTP, but again there is a lag which is somewhat longer, implying that GTP may serve to convert mitochondrial ADP to ATP.
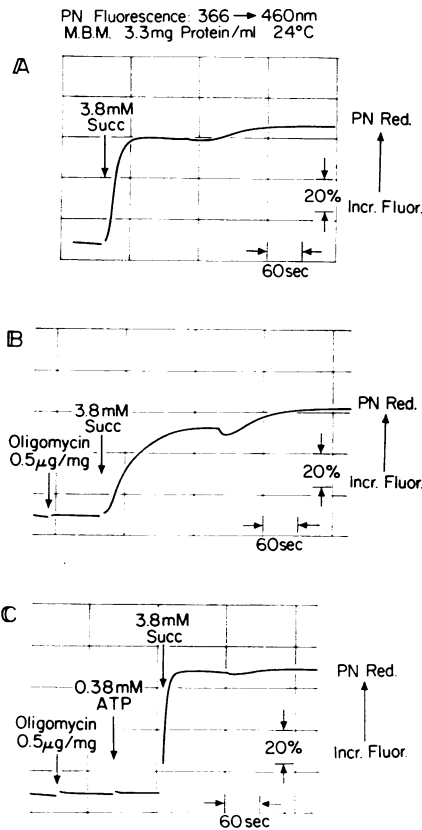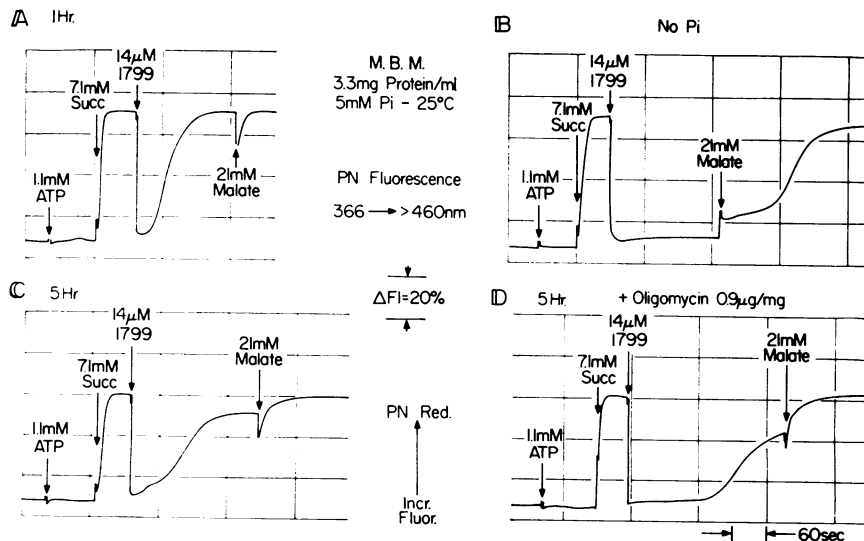

FIG. 5. Reduction of endogenous NAD in mung bean mitochondria in the presence of uncoupler on attaining anaerobiosis while oxidizing succinate. A: Freshly prepared mitochondria, 1 hr after isolation; the medium contains 5 mM P_i. Endogenous NAD is nearly completely reduced in state 4 with succinate; addition of the uncoupler 1799 causes complete reoxidation; reduction in anaerobiosis is slower but complete. B: Same freshly prepared mitochondria as in A, but no added P_i in the medium. No reduction of endogenous NAD is observed in the time span needed for complete reduction in A. Reduction with malate is slow. C: Same experiment as in A, but the mitochondria have been aged 5 hr. Note slight lag before start of NAD reduction in anaerobiosis. D: Experiment carried out with same aged preparation as in C, but the mitochondria were treated with oligomycin before starting the experiment.
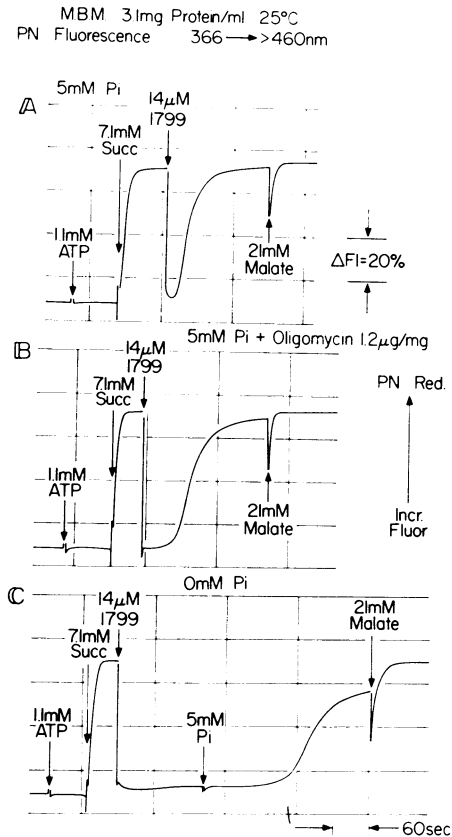
MBM 3.1mg Protein/ml 25°C
PN Fluorescence 366 ——▸ >460nm

FIG. 6. Requirement for P_i in NAD reduction in uncoupled mitochondria. A and B: Control experiments carried out in the presence of 5 mM P_i in the absence and presence of oligomycin, respectively. C: Reaction conditions as in A, but no P_i added to the medium at start of experiment. No reduction of endogenous NAD is observed in anaerobiosis over a time sufficient to obtain complete reduction in A. Addition of 5 mM P_i gives reduction after a short lag.

Another requirement for uncoupled, anaerobic NAD reduction is that some oxidation of succinate take place. When respiration is completely inhibited, as in Figures 1 and 2, the reaction does not occur. This observation indicates that the direct precursor to NAD reduction under these conditions is malate, derived from the fumarate formed by dehydrogenation of succinate.

In the absence of P_i, the energy-linked reduction of endogenous NAD by succinate proceeds only by reversed electron transport through the first energy conservation site of the respiratory chain. The free energy for the reaction, as shown by the records in Figures 1 and 4, can be provided by ATP or by succinate oxidation through the cytochrome pathway. If energy is also conserved during succinate oxidation through the alternate, cyanide or sulfide-insensitive pathway, it should be available for reduction of endogenous NAD. If no energy is conserved, there should be no reduction of endogenous NAD in the absence of P_i and presence of oligomycin. The experiments to distinguish between the two alternatives are shown in Figure 8. In the presence of ATP and absence of both P_i and oligomycin, the oxidation of succinate via the alternate oxidase pathway in sulfide-inhibited mung bean mitochondria is accompanied by about 15% reduction of endogenous NAD in the aerobic steady state (Fig. 8A). This rises to 50% reduction on anaerobiosis; reduction is complete on addition of malate. The 50% level of reduction in anaerobiosis with succinate is that expected from reverse electron transport driven

by ATP, by comparison with Figure 1A. If the experiment of Figure 8A is repeated with the same mitochondrial preparation, but in the presence of oligomycin, there is no reduction of endogenous NAD during the aerobic steady state, and a very slow, barely perceptible reduction in anaerobiosis (Fig. 8B). This experiment shows that oxidation of succinate through the alternate oxidase pathway by coupled mung bean mitochondria does not conserve the free energy of oxidation. There appears to be no energy conservation site between succinate dehydrogenase and the alternate oxidase, in agreement with results obtained with skunk cabbage mitochondria (47). If the experiments of Figure 8, A and B, are carried out in the presence of P_i, then endogenous PN is reduced by succinate, and the extent of reduction in anaerobiosis in the presence of oligomycin (Fig. 8D) is actually greater than in its absence (Fig. 8C). The extent of reduction in the aerobic steady state is essentially nil, and the rate of reduction in anaerobiosis is slow in both experiments, as would be expected if the reduction was taking place through the pathway not involving the respiratory chain. If reduction of endogenous NAD is to be used as a criterion for energy conservation in the respiratory chain of the plant mitochondria, it is evident that this second pathway must be suppressed by carrying out the experiments in the absence of added P_i.

DISCUSSION

The transfer of reducing equivalents from succinate to endogenous NAD is a reaction requiring input of free energy, since the midpoint potential of the NADH/NAD$^+$ couple is

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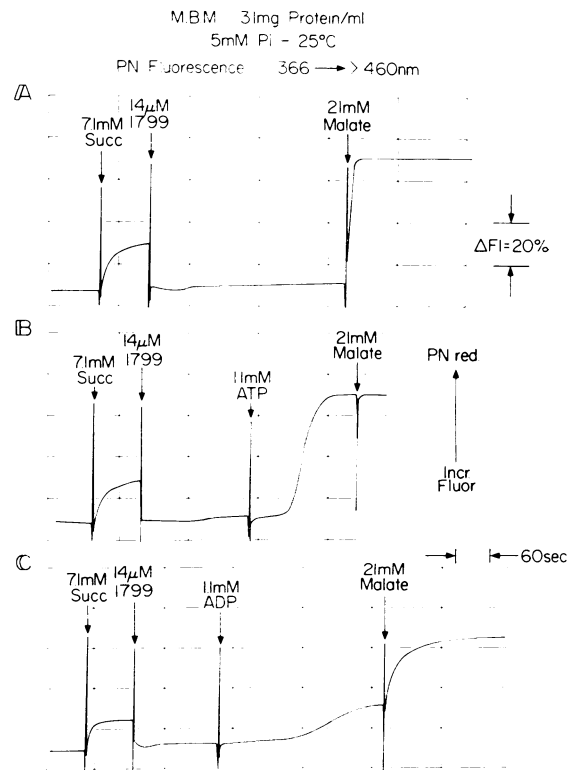5mM Pi - 25°C
PN Fluorescence 366 ——▸ >460nm

FIG. 7. Reaction conditions and mitochondrial preparation are the same as in Figure 6, but no ATP was added at the start of the experiments. A: Omission of ATP gives no reduction of endogenous NAD in anaerobiosis. B: Addition of ATP at point indicated gives rapid and complete reduction after slight lag. C: Addition of ADP at point indicated gives slow and incomplete reduction after a longer lag period.
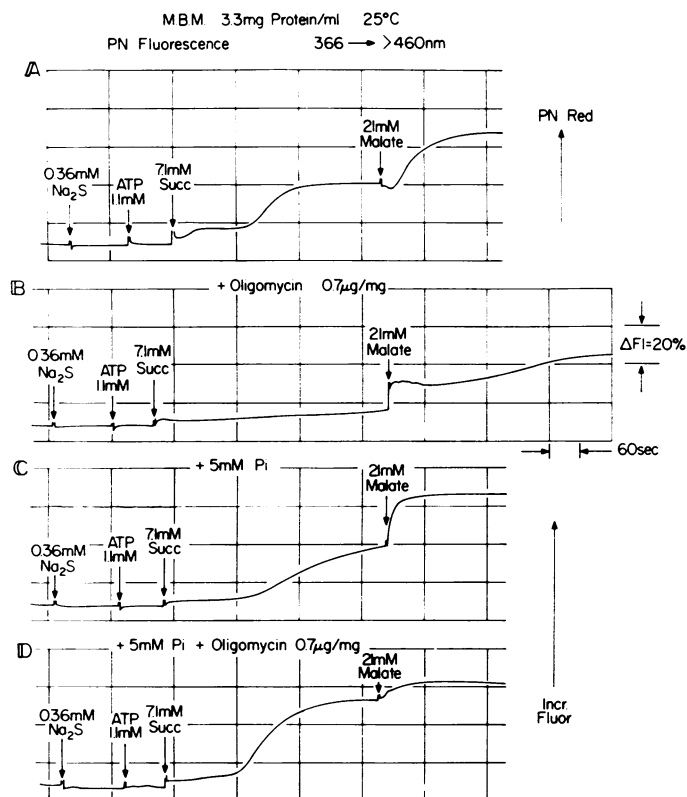
FIG. 8. Reduction of endogenous NAD in mung bean mitochondria oxidizing succinate in the presence of sulfide via the alternate oxidase pathway. A: No P_i or oligomycin added. B: No P_i added; mitochondria were treated with oligomycin at 0.7 μg/mg protein before start of the experiment. C: 5 mM P_i added, but no oligomycin. D: 5 mM P_i added; mitochondria were treated with oligomycin as in B.

some 350 mv more negative than that of the succinate/fumarate couple (7, 9, 22). It is evident from the results reported here that mung bean mitochondria can effect this transfer by reversed electron transport from succinate dehydrogenase through the first energy conservation site of the respiratory chain to the dehydrogenase for endogenous NADH. The energy may be supplied by ATP or by coupled oxidation of succinate. In this, they resemble all mitochondria from different sources which have the first energy conservation site fully functional.

It is also apparent that endogenous NAD may be reduced with reducing equivalents from succinate by another route which does not involve the respiratory chain, and which operates even in the presence of uncoupler. Free energy is needed, however, and this must come from endogenous energy stores, which can be depleted slowly by aerobic preincubation of the mitochondria with ADP and uncoupler. Two possible routes suggest themselves. One is dehydrogenation and decarboxylation to pyruvate of the malate produced from the fumarate of succinate oxidation, by means of a "malic" enzyme dependent on NAD, which McCrae (36) has shown to be present in a number of plant mitochondrial preparations. The enzyme from cauliflower buds has been characterized in detail by McCrae (36) and needs no P_i for activity. If operative in mung bean mitochondria, this enzyme should allow reduction of endogenous NAD by the malate formed from succinate in the absence as well as the presence of P_i. The midpoint potential of the (malate)/(pyruvate + CO_2) couple is −330 mv (33), which is slightly more negative than that of NADH/NAD+ (9). This

reaction would not seem to require input of free energy, and there appears to be no reason why depletion by anaerobic incubation with ADP and uncoupler should inhibit it.

This route would therefore appear improbable. The second route, suggested by the recent report on control of oxaloacetate content of mung bean mitochondria by Zimmerman and Ikuma (52), is dehydrogenation of the formed malate by malate dehydrogenase, followed by removal of the formed oxaloacetate to shift the otherwise unfavorable equilibrium (9) towards NAD reduction. This hypothesis may be tested experimentally, based on the finding of Kun (21, 34) that FOAA is a very potent inhibitor of malic dehydrogenase. The experiment is shown in Figure 9A. Addition of uncoupler and FOAA to mung bean mitochondria oxidizing succinate in the presence of ATP, P_i, and oligomycin, oxidizes the highly reduced endogenous NAD, which in turn remains oxidized in anaerobiosis. The control experiments are shown in Figures 9, B and C. If FOAA is added to the mitochondria before succinate (Fig. 9B) the reduction of endogenous NAD on subsequent addition of succinate is slowed somewhat, but the extent of reduction is nearly the same. This demonstrates that FOAA is not an uncoupler per se. Under these conditions, FOAA is reduced to fluoromalate since it is a substrate for malic dehydrogenase, albeit one which reacts slowly (21). Addition of uncoupler reoxidizes the endogenous NAD, which then does become rereduced in anaerobiosis. Omission of FOAA gives the experimental record in Figure 9C. Comparison of Figure 9, B and C shows that fluoromalate inhibits the malic dehydrogenase
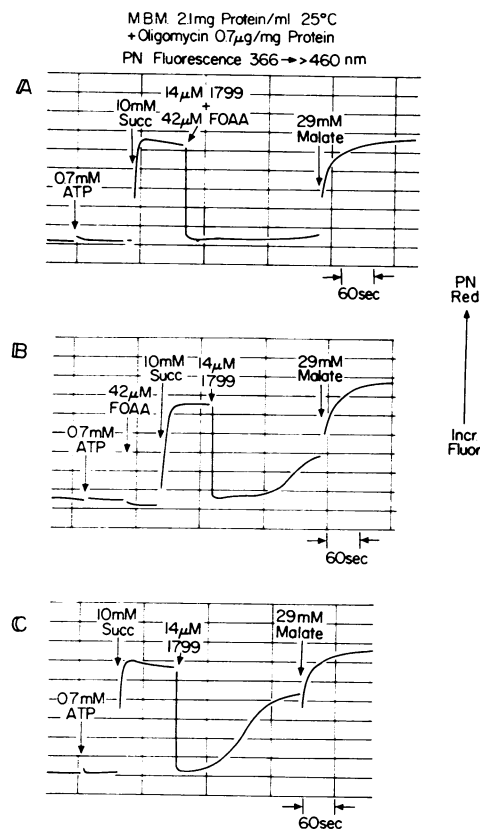

FIG. 9. Effect of FOAA on reduction of endogenous PN in uncoupled mung bean mitochondria in anaerobiosis. Reaction medium contains 5 mM P_i in all experiments; the mitochondria were treated with oligomycin at 0.7 μg/mg protein. A: FOAA added simultaneously with uncoupler 1799 in state 4; B: FOAA added prior to addition of succinate to produce state 1 to state 4 transition; C: control experiment without added FOAA.

partially, in accord with previous work (34), while FOAA inhibits it completely under these conditions. That the inhibition is competitive in this case, as described for the mammalian enzyme by Kun (21, 34), is shown by reversal of the inhibition on addition of a high concentration of malate.

The experiment with FOAA and the accompanying controls shown in Figure 9 verify the hypothesis that the anaerobic reduction by succinate of endogenous NAD in uncoupled mung bean mitochondria requires that malate dehydrogenase be active, and that the immediate source of reducing equivalents be malate derived via fumarase from succinate oxidation. If respiration is blocked by inhibition of both terminal oxidases, as in the experiment shown in Figure 2, no endogenous NAD is reduced in the presence of both phosphate and uncoupler by succinate. The requisite amount of free energy must still be supplied to achieve the high degree of NAD reduction observed in the reaction operating independently of the respiratory chain, since the reducing equivalents are being supplied by the succinate/fumarate redox couple. The free energy is evidently not supplied by the phosphate potential available to the mitochondrial matrix, since the reaction does *not* proceed in the absence of phosphate but presence of ATP, a condition of high phosphate potential, but does proceed at the lower phosphate potential obtaining in the presence of phosphate. It still requires some ATP, however. The most probable mechanism for the reduction in the presence of uncoupler is removal of oxaloacetate by condensation with intramitochondrial acetyl CoA, as suggested by Papa et al. (40) for stimulation of succinate oxidation in rat liver mitochondria, thereby driving the dehydrogenation of malate. This reaction evidently requires P_i, possibly for activation of the malate dehydrogenase by displacement of oxaloacetate from the enzyme. Since the malate is generated in the mitochondrial matrix space by succinate oxidation, P_i cannot be activating malate dehydrogenation by facilitation of malate entry into the mitochondrion via the malate/phosphate anion exchange carrier (19, 20).

This formulation implies that the free energy required for the reduction derives from intramitochondrial energy reserves as acetyl CoA, or as other esterified CoA derivatives convertible to acetyl CoA (39). These energy reserves would be subject to depletion by aerobic incubation of the mitochondria with uncoupler and ADP, as is indeed observed (43, 46, 47). The observed requirement for ATP, but not for high phosphate potential, may be rationalized by the proposal that it participates in the reactions by which other esterified CoA derivatives are converted to acetyl CoA. The existence of these energy reserves, which can be detected by the reduction of endogenous NAD in the presence of P_i and uncoupler on anaerobiosis from succinate oxidation, are a criterion of well prepared, tightly coupled mitochondria. Mung bean mitochondria which can carry out this reduction show a very rapid, sharp state 3 to state 4 transition (18) in oxygen uptake measurements, a criterion for tightly coupled plant mitochondria emphasized by Bonner (5). Conversely, those mitochondrial preparations, which have a slow transition from state 3 to state 4, do not show reduction of endogenous NAD by succinate on anaerobiosis in the presence of P_i and uncoupler.

Separation of the two routes of NAD reduction in mung bean mitochondria allows application of the energy-linked reaction of reverse electron transport through the respiratory chain as a probe for energy conservation in the respiratory pathway through the alternate oxidase. In the presence of oligomycin and sulfide, but absence of P_i which blocks the extra-respiratory chain pathway, there is no reduction of endogenous NAD by oxidation of succinate (Fig. 8B). This is strong support for the conclusion of Hackett et al. (25) that succinate oxidation through the alternate pathway occurs

with no energy conservation. It is also in accord with the observation that ADP is not phosphorylated by skunk cabbage mitochondria oxidizing succinate in the presence of cyanide (47). Wilson and Bonner (51) have demonstrated that certain guanidine inhibitors of energy transfer (50) in mung bean mitochondria do inhibit the oxidation rate of succinate in the presence of cyanide. From this they concluded that there might be energy conservation in the respiratory pathway from succinate to oxygen through the alternate oxidase. The results reported in this paper disagree with this conclusion, but offer a possible explanation for action of the guanidine inhibitors. It is evident from Figures 3 and 4 that ATP activates succinate oxidation in state 4, and that this activation is not affected by oligomycin. Hence, it is independent of the energy state of the respiratory chain. Since the effect of the guanidine inhibitors on succinate oxidation was observed in the presence of added ATP, it may well be that these inhibitors counteract the activating effect of ATP and thus produce apparent inhibition of the succinate oxidase rate. Whether the ATP effect is direct activation of succinate dehydrogenase or facilitation of succinate entry into the mitochondrion is as yet unclear.

The observation of energy conservation in succinate oxidation by the cyanide insensitive pathway in *Arum maculatum* by Bonner and Bendall (6) and by Wilson (48, 49) is not necessarily in disagreement with the results reported here for mung bean mitochondria and earlier for skunk cabbage mitochondria. Mitochondria from *Arum* contain cytochrome b_7 (1, 3), while those from mung bean and skunk cabbage do not (23, 42). The suggestion is here put forward for experimental verification that the presence of cytochrome b_7 in mitochondria from a given plant source implies an energy conservation site between succinate and the alternate, cyanide insensitive oxidase.

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