

Cooperation and Competition between Adenylate Kinase, Nucleoside Diphosphokinase, Electron Transport, and ATP Synthase in Plant Mitochondria Studied by ³¹P-Nuclear Magnetic Resonance¹

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Nucleotide metabolism in potato (*Solanum tuberosum*) mitochondria was studied using ³¹P-nuclear magnetic resonance spectroscopy and the O₂ electrode. Immediately following the addition of ADP, ATP synthesis exceeded the rate of oxidative phosphorylation, fueled by succinate oxidation, due to mitochondrial adenylate kinase (AK) activity two to four times the maximum activity of ATP synthase. Only when the AK reaction approached equilibrium was oxidative phosphorylation the primary mechanism for net ATP synthesis. A pool of sequestered ATP in mitochondria enabled AK and ATP synthase to convert AMP to ATP in the presence of exogenous inorganic phosphate. During this conversion, AK activity can indirectly influence rates of oxidation of both succinate and NADH via changes in mitochondrial ATP. Mitochondrial nucleoside diphosphokinase, in cooperation with ATP synthase, was found to facilitate phosphorylation of nucleoside diphosphates other than ADP at rates similar to the maximum rate of oxidative phosphorylation. These results demonstrate that plant mitochondria contain all of the machinery necessary to rapidly regenerate nucleoside triphosphates from AMP and nucleoside diphosphates made during cellular biosynthesis and that AK activity can affect both the amount of ADP available to ATP synthase and the level of ATP regulating electron transport.

Plant tissues exhibit significant AK activity in the mitochondrial intermembrane space (reviewed by Douce, 1985). In cells and mitochondria, AK appears to equilibrate adenylates (Bomsel and Pradet, 1968; Rusness and Still, 1973; Raymond et al., 1987; Hooks et al., 1994), and NDPK equilibrates nucleoside di- and triphosphates (Raymond et al., 1987; Hooks et al., 1989). The rate of nucleotide transformation by these enzymes may be higher than nucleotide fluxes through other enzymes, including respiratory ATP synthesis (Raymond et al., 1987). Use of the AK inhibitor

Ap₅A (Lienard and Secemski, 1973) has allowed the qualitative demonstration of AK's role in the conversion of AMP to ATP in mitochondria (Raymond et al., 1987; Fricaud et al., 1992; Busch and Ninnemann, 1996); yet the metabolic consequences of high phosphotransferase activities in plants have not been fully defined. Consider, for example, the fate of ADP generated by such processes as ion transport and biosynthesis in aerobic cells. AK might be expected to compete with the mitochondrial ATP synthase for ADP generated in the cytoplasm, with two concurrent consequences. First, AK would act to lower the ADP concentration experienced by the adenine nucleotide translocase, which delivers ADP to the ATP synthase. Second, AK would catalyze the synthesis of ATP independently of oxidative phosphorylation and mitochondrial adenylate transport. A separate and heretofore unanswered question concerns the role of mitochondria in the phosphorylation of nucleoside di- and monophosphates.

We address these metabolic possibilities by reporting ³¹P-NMR measurements of multiple nucleotide transformations in suspensions of highly purified intact potato (*Solanum tuberosum*) mitochondria. ³¹P-NMR spectroscopy can provide nondestructive quantitative information about the concentrations and physical states of nucleotides in complex mixtures. It has been widely used to study energy metabolism and mitochondrial function in living plant cells and tissues (Roberts, 1986, 1987). Our strategy in the present study included NMR measurement of phosphotransferase activities in mitochondria under conditions identical with those in the classical O₂ electrode. The O₂ electrode apparatus (Chance and Williams, 1956) has been the principal tool used to investigate mitochondrial metabolism and define respiratory states (for reviews, see Douce, 1985; Nichols and Ferguson, 1992). Our parallel measurements of respiratory activity and nucleotides enabled us to view nucleotide transformations with respect to mitochondrial physiological state. We describe the activities of nu-

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Abbreviations: AK, adenylate kinase; Ap₅A, P¹, P⁵-di(adenosine-5')pentaphosphate; NDP, nucleoside diphosphate; NDPK, nucleoside diphosphokinase; NMP, nucleoside monophosphate; PCA, perchloric acid; ppm, parts per million.

cleotide phosphotransferases relative to electron transport and ATP synthase in respiring mitochondria and discuss the physiological roles of these enzymes in plants.

MATERIALS AND METHODS

Intact mitochondria were isolated from 10 to 20 kg of potato (*Solanum tuberosum*) tubers using two successive Percoll gradients, according to the method of Neuburger et al. (1982). A thick slurry of mitochondria (80–100 μg^{-1} protein μL^{-1}) was stored on ice prior to experimentation. Then, 10 to 50 μL of mitochondria was suspended in 30 mL of reaction medium containing 10% $^2\text{H}_2\text{O}$, 0.3 M mannitol, 5 mM MgCl_2 , 20 mM phosphate buffer (pH 7.2), and 0.1% BSA. The medium was bubbled with pure O_2 prior to the addition of mitochondria to prolong respiratory activity prior to anoxia. Experiments were conducted at 21 to 22°C. Twenty-nine milliliters of the reaction mixture was placed in a 25-mm diameter NMR tube for measurement of nucleotide concentrations, and 1 mL was taken for measurement of O_2 consumption, as described by Neuburger et al. (1982). Mitochondrial physiological state is defined according to the convention of Chance and Williams (1956), which reflects the order of addition of reagents in a typical O_2 electrode experiment: state 1, mitochondria alone (in the presence of Pi and O_2); state 2, substrate added, respiration low due to absence of ADP; state 3, ADP added, respiration high; state 4, respiration low due to conversion of all ADP to ATP; state 5, anoxia.

Succinate was used as a respiratory substrate to eliminate any contribution from substrate-level phosphorylation (Douce, 1985). Nucleotide transformations due to mitochondrial AK activity were studied in the absence of contributions from respiration by omitting succinate from the reaction medium. ^{31}P -NMR spectroscopy of mitochondrial suspensions was performed in a Fourier transform spectrometer (model AMX 400, Bruker, Billerica, MA) operating at 161.93 MHz and equipped with a 25-mm-diameter probe. Spectra were accumulated in 5-min blocks using an interval between 90° pulses of 3 s and a sweep width of 9800 Hz; the signal was digitized using 8,000 data points zero-filled to 16,000. Proton irradiation using the Waltz sequence was applied at power levels of 2 W during data acquisition and 0.25 W during the delay period. Under these conditions the proportionality between signal intensities and concentrations for AMP, α - and β -ADP, and α - and γ -ATP signals were indistinguishable. Spectra were obtained with line broadening of 5 Hz to improve the signal-to-noise ratio. Chemical shifts were referenced to phosphoric acid at 0 ppm using the external reference methylene diphosphonate contained in a coaxial capillary. The purity of nucleotides was checked by NMR. Only in ADP were significant impurities found (approximately 8% AMP) in material purchased from Fluka and Boehringer Mannheim. Ap_5A was obtained from Sigma.

PCA extracts of mitochondria were prepared by adding 5% PCA to 3 mL of a mitochondrial suspension (400 mg of total protein). After the sample was centrifuged at 15,000g,

the supernatant was neutralized with 2 M KHCO_3 to a pH of approximately 5.2 and then centrifuged again at 10,000g. The resulting supernatant was lyophilized and stored in liquid nitrogen. The freeze-dried material was dissolved in 2.5 mL of water containing 10% $^2\text{H}_2\text{O}$. Divalent cations were chelated by the addition of sufficient amounts of 1,2-cyclohexylenedinitrilotetraacetic acid. The pH was adjusted to 7.5 in the presence of 50 mM Hepes and analyzed using a 10-mm-diameter NMR probe. Acquisition conditions utilized 15- μs pulses (70°) at 3.6-s intervals, 8,000 data points zero-filled to 16,000, and a sweep width of 6 kHz. Broad-band decoupling at 1 W during acquisition and 0.25 W during the delay was applied using the Waltz sequence. Chemical shifts were referenced to phosphoric acid at 0 ppm. The metabolites shown in Table I were quantified by the addition of known amounts of authentic nucleotides to the extracts. Reduced pyridine nucleotides were identified as oxidized derivatives (produced during PCA extraction) that resonated as a quartet to the left of the NAD/NADP quartet (which is stable in dilute, cold PCA). The assignment of the NADH/NADPH resonances was based on a comparison of spectra of authentic reduced pyridine nucleotides before and after PCA extraction. The quantity of NAD plus NADH in extracts was determined by subtracting the intensity of the downfield NADP/NADPH singlet from the sum of the NADH/NADPH and NAD/NADP quartets.

RESULTS AND DISCUSSION

Competition between AK and ATP Synthase for ADP in Respiring Mitochondria

ADP (1 mM) was added to a dilute, oxygenated suspension of potato mitochondria. Concentrations of adenylates were monitored by ^{31}P -NMR while the respiratory activity of a separate aliquot of the reaction mixture was followed using an O_2 electrode. O_2 electrode traces recorded classical respiratory control (Chance and Williams, 1956; Douce, 1985; Nichols and Ferguson, 1992) in which state 3 O_2 consumption continued at a linear rate for approximately

Table I. Quantities of low-molecular-weight ^{31}P metabolites in purified potato mitochondria

Determined from ^{31}P -NMR spectra of PCA extracts such as in Figure 4; similar results were obtained in analyses of two other mitochondrial suspensions.

Metabolite	Content
	<i>nmol mg⁻¹ protein</i>
AMP	0.05
ADP	0.19
ATP	0.4
Phosphorylcholine	0.55
Phosphorylethanolamine	0.69
Glycerophosphorylcholine	0.55
Glycerophosphorylethanolamine	0.17
NADP + NADPH	0.69
NAD + NADH	0.3
NADH + NADPH	0.72
NAD + NADP	0.25

30 min after ADP addition before decreasing to the state 4 rate (Fig. 1, top left). Immediately after ADP addition, levels of both ATP and AMP increased, with ATP accumulating at approximately twice the rate of AMP (Fig. 1), suggesting that a significant proportion of ATP synthesis during this period was due to AKs catalyzing the conversion of ADP to ATP and AMP. Thereafter, the rate of ATP accumulation decreased and O_2 consumption remained constant until ADP and AMP declined to zero and respiration returned to the state 4 rate (Fig. 1).

To determine the relative contributions of ATP synthase and AK to ATP synthesis in mitochondria given ADP, we

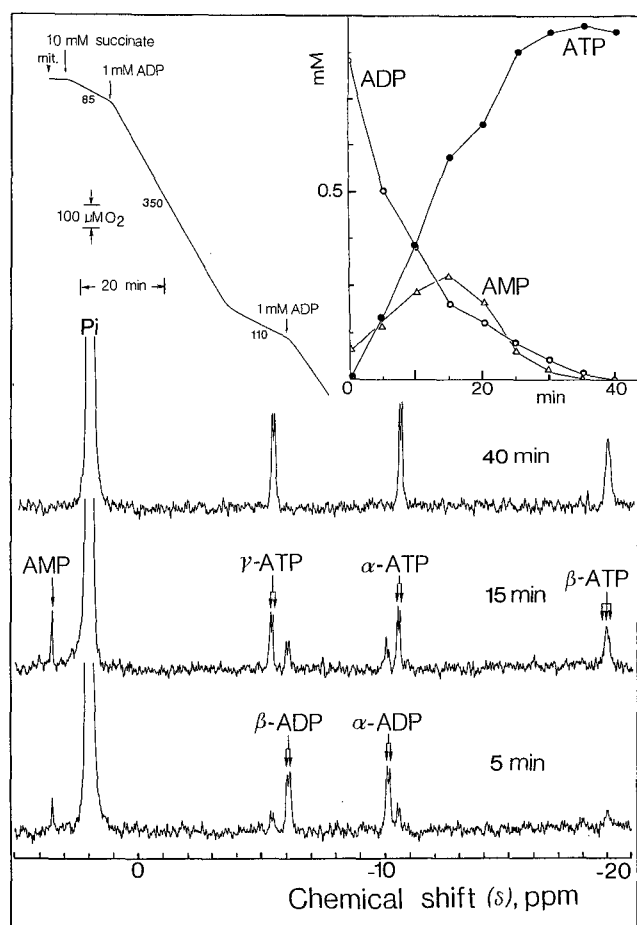


Figure 1. Accumulation of AMP in a respiring mitochondrial suspension ($110 \mu\text{g protein mL}^{-1}$) during ATP synthesis from ADP. ^{31}P -NMR spectra of adenylates in a suspension of mitochondria (mit.) following addition of ADP. The medium contained 20 mM succinate and was oxygenated prior to addition of mitochondria, as described in "Materials and Methods." Spectra were acquired over 5 min. The indicated time is the midpoint of acquisition for each spectrum, except the 0 time, which was collected prior to the addition of ADP. Top left, Effect of ADP on O_2 consumption in a mitochondrial suspension. The electrode trace was recorded using an aliquot of the mitochondrial suspension used to generate the NMR data, taken prior to addition of ADP. The values beside the trace correspond to $\text{nmol } O_2 \text{ consumed min}^{-1} \text{ mg}^{-1} \text{ protein}$. Top right, Changes in adenylate concentrations in the same mitochondrial suspension following addition of ADP, determined from ^{31}P -NMR spectra as shown. AMP present at time 0 is due to impurity of the added ADP.

measured the activities of these enzymes in separate assays. ATP synthase activity was measured one of two ways: (a) directly by ^{31}P -NMR in mitochondria in which adenylates (added as ADP) were first equilibrated via the AK reaction, then respiration was initiated by addition of succinate, and the initial rate of ATP accumulation was recorded in a similar fashion to that shown in Figure 1 or (b) indirectly estimated from O_2 electrode traces of state 3/state 4 transitions, which reflect the time required for conversion of defined quantities of ADP into ATP. Both approaches gave similar values for ATP synthase activity: 460 ± 170 (mean \pm SD) $\text{nmol min}^{-1} \text{ mg protein}^{-1}$. AK activity in mitochondria was determined by ^{31}P -NMR in suspensions lacking succinate, so that oxidative phosphorylation did not occur. Under these conditions, the mitochondria consumed no O_2 but were capable of rapid conversion of ADP to ATP plus AMP until concentrations of adenylates in accord with the equilibrium constant for the AK reaction were obtained (data not shown). The initial rates of conversion of ADP to AMP and ATP indicated that the activity of AK in mitochondria is 2 to 4 times that of ATP synthase.

These experiments demonstrate that ATP synthesis from ADP in respiring mitochondria does not occur in constant stoichiometry with electron transport. Immediately following the addition of ADP, AK and ATP synthase compete for this substrate and are responsible for comparable amounts of ATP synthesis, such that ATP accumulates faster than is possible by oxidative phosphorylation alone. Later, after AMP levels reach a maximum, the accumulation of ATP due to ATP synthase is slowed by the activity of AK, because adenylate mass action causes this enzyme to catalyze net conversion of AMP into ADP, which sustains ATP synthase activity. We note that adenine nucleotide translocase can control rates of oxidative phosphorylation (reviewed by Dry et al., 1987) and, therefore, may play a role in this interplay between AK and ATP synthase.

Cooperation of AK and ATP Synthase during Phosphorylation of AMP

O_2 consumption by succinate-fed mitochondria in the presence of ATP was immediately stimulated by AMP to the same extent as was observed with ADP (Fig. 2; compare with inset of Fig. 1). The phosphorylation of AMP by mitochondria has been shown to depend on AK activity, since this reaction is blocked in the presence of the AK inhibitor Ap_5A (Raymond et al., 1987; Fricaud et al., 1992). Using NMR spectroscopy we observed directly the action of AK without the use of an inhibitor. Immediately after 0.5 mM AMP was added, ADP was observed (Fig. 2), which reflects AK activity in excess of ATP synthase activity, as reported above. The greater initial AK activity relative to ATP synthase is in part due to the initial lack of ADP available for ATP synthesis. Note that in this experiment the net reaction catalyzed by AK is in the opposite direction of that which occurred initially in the experiments with ADP described in the previous section. The high AK activity in the direction of ADP formation was confirmed by direct measurements of rates of AMP and ATP conver-

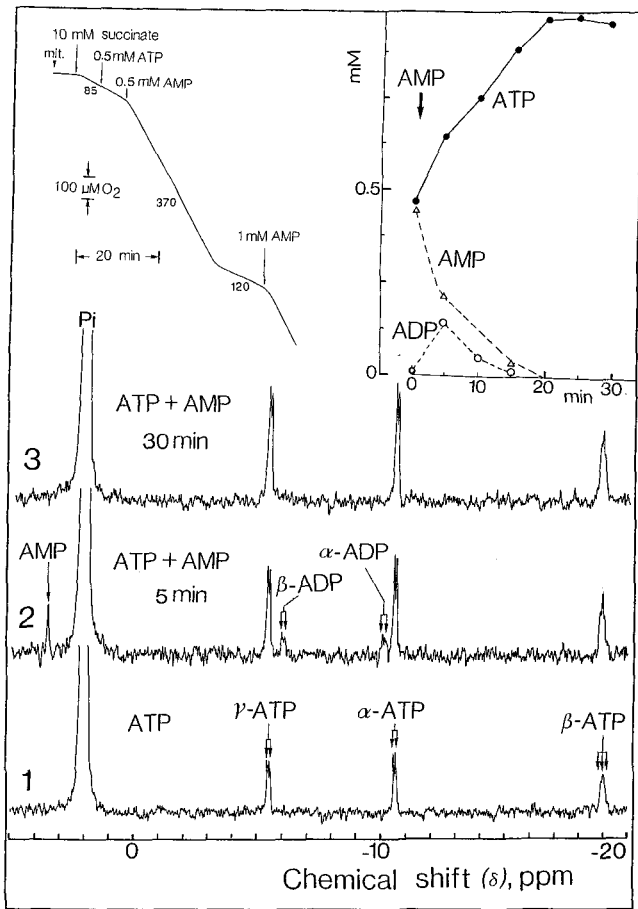


Figure 2. Accumulation of ADP in a respiring mitochondrial suspension ($120 \mu\text{g protein mL}^{-1}$) during ATP synthesis from AMP in the presence of ATP. ^{31}P -NMR spectra of adenylates in a suspension of mitochondria (mit.) following addition of AMP. The medium contained 20 mM succinate and 0.5 mM ATP and was oxygenated prior to the addition of mitochondria. Spectra were acquired over 5 min. The indicated time is the midpoint of acquisition for each spectrum, except the 0 time point, which was collected prior to addition of AMP. Top left, Effect of AMP/ATP mixture on O_2 consumption in a mitochondrial suspension. The electrode trace was recorded using an aliquot of the mitochondrial suspension used to generate the NMR data taken prior to the addition of AMP. The values beside the trace correspond to $\text{nmol O}_2 \text{ consumed min}^{-1} \text{ mg}^{-1} \text{ protein}$. Top right, Changes in adenylate concentrations in the same mitochondrial suspension following the addition of AMP, determined from ^{31}P -NMR spectra as shown.

sion to ADP in mitochondria lacking respiratory substrate to prevent ATP synthase activity (data not shown).

We were intrigued by the capacity of mitochondria to phosphorylate AMP in the absence of any added ATP or ADP (Fig. 3). This result suggested that mitochondria can retain, through extensive and prolonged steps of purification, a "catalytic" quantity of ATP that is somehow accessible to AK, enabling phosphorylation of AMP to ADP, which is in turn phosphorylated via ATP synthase. This possibility was confirmed by ^{31}P -NMR analysis of nucleotides in PCA extracts from large quantities of purified mitochondria (Fig. 4; Table I). These data show that mito-

chondria contain approximately $0.4 \text{ nmol ATP mg}^{-1} \text{ protein}$. The amount of adenylates found in these highly purified mitochondrial preparations was similar in magnitude to earlier measurements of preparations involving fewer purification steps (Vignais et al., 1976; Whitehouse et al., 1989), which suggests that this small pool of nucleotides is tightly sequestered in mitochondria.

The lag following AMP addition, before respiration reached the full state 3 rate and the rate of ATP synthesis was maximal (Fig. 3), may be postulated to result from limitation of AK activity by low ATP from rate-limiting transport of adenylates across the inner mitochondrial membrane or from inhibition of succinate dehydrogenase activity by low intramitochondrial ATP (Oestreicher et al., 1973; Singer et al., 1973). These possibilities are examined in the next section.

The quantities of reduced pyridine nucleotides evident in Table I and Figure 4 presumably reflect the action of dehydrogenases on small amounts of citric acid cycle intermediates contained in the mitochondria and depletion of O_2 in the thick mitochondrial suspension during prolonged

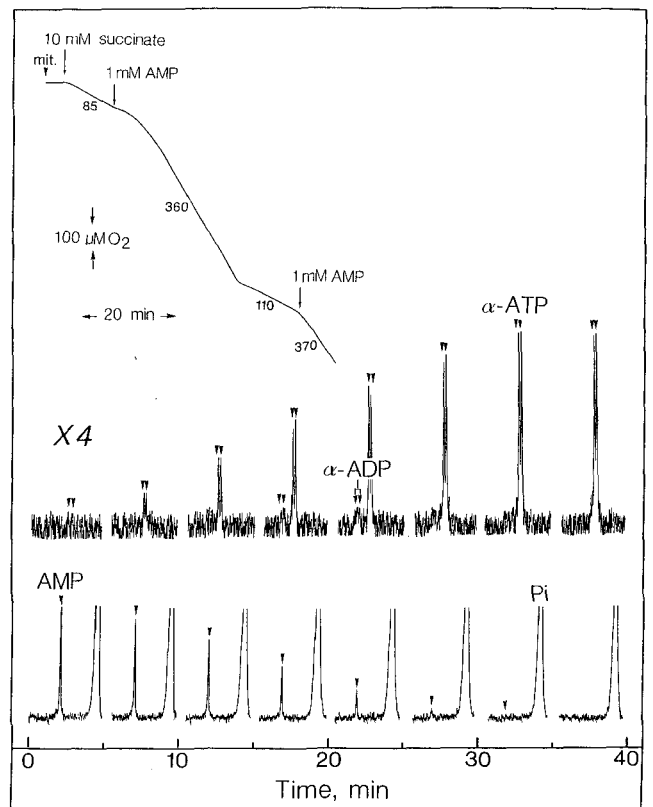


Figure 3. Phosphorylation of AMP in a respiring mitochondrial suspension ($115 \mu\text{g protein mL}^{-1}$) in the absence of added ATP or ADP. The medium contained 20 mM succinate and was oxygenated prior to the addition of mitochondria (mit.). Bottom, ^{31}P -NMR partial spectra showing selected adenylate resonances following the addition of 1 mM AMP to the mitochondrial suspension. Top left, Effect of AMP on O_2 consumption by mitochondria. The electrode trace was recorded with an aliquot of the reaction mixture used to obtain the NMR spectra, taken prior to addition of AMP. The values beside the trace correspond to $\text{nmol O}_2 \text{ consumed min}^{-1} \text{ mg}^{-1} \text{ protein}$.

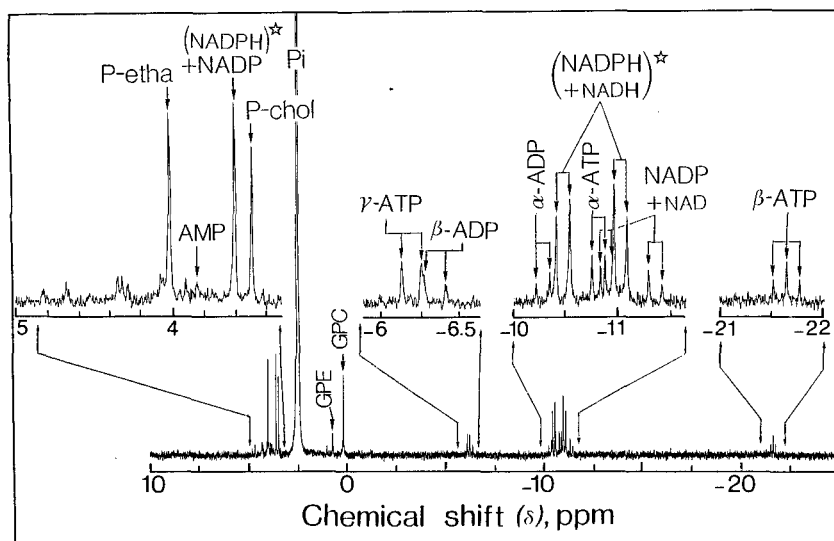


Figure 4. Proton-decoupled ^{31}P -NMR spectrum of a PCA extract of purified potato tuber mitochondria. The extract was prepared as described in "Materials and Methods" from 3 mL of a thick suspension of mitochondria (400 mg of protein). The spectrum was obtained in 8 h, with a 0.4-Hz line broadening. P-chol, Phosphorylcholine; P-etha, phosphorylethanolamine; GPC, glycerophosphocholine; and GPE, glycerophosphoethanolamine.

storage on ice. The presence of a pool of NADP(H) is not surprising because there are many NADP-dependent enzymes in plant mitochondria, including isocitrate dehydrogenase (Rasmuson and Møller, 1990) and dihydrofolate reductase (Neuburger et al., 1996). An additional physiological function for the NADPH pool has recently been suggested to be reduction of the alternative terminal oxidase of plant mitochondria to a more active form (Vanlerberghe et al., 1995). Finally, the small amounts of phosphorylcholine and phosphorylethanolamine that we found in purified mitochondria, together with their glycerol esters (Table I), could reflect polar lipid degradation during the course of mitochondrial isolation and storage or during PCA extraction.

The ^{31}P -NMR method allows total adenylate pool sizes and interconversion between different nucleotides to be monitored during the experiments described here. It is notable that at no point during our experiments did we detect any loss of adenylates or degradation products such as IMP. The inability of mitochondria to decrease the total adenylate pool is quite different from the behavior of adenylates in intact cells, where stresses such as anoxia cause increases in AMP and loss of total adenylates (Saglio et al., 1980). This observation indicates that the degradation of adenylates in plant cells is regulated outside mitochondria.

Communication between AK and the Mitochondrial Electron Transport Chain during Phosphorylation of AMP

The ability of mitochondria to phosphorylate AMP in the absence of exogenous ATP, described above (Fig. 3), immediately leads to questions about the location and physical state of the endogenous ATP pool in mitochondria (Table I) and the role of adenylate transport between the matrix and the intermembrane space. To investigate these possibilities we examined mitochondrial electron transport activity using the O_2 electrode under conditions optimized for observation of the lag between state 2 and state 3, which follows the addition of AMP (Fig. 3). We were able to

observe additional complexity in the respiratory response of succinate-fed mitochondria to AMP, in that AMP first caused inhibition of O_2 consumption (below the state 2 rate) before increasing to the full state 3 rate (Fig. 5C). This behavior was observed independently of whether the mitochondria were freshly prepared, stored as a thick pellet on ice for as long as 3 d, or diluted in buffered osmoticum for as long as 5 h at room temperature (data not shown). Both the inhibition of the state 2 rate and the eventual increase to state 3 were blocked by Ap_5A (Fig. 5D), demonstrating that both events require AK activity. Since it is established that succinate dehydrogenase, which faces the mitochondrial matrix, is activated by ATP, with half-maximal activation at micromolar concentrations of nucleotide (Oestreicher et al., 1973; Singer et al., 1973), we first postulated that AMP and AK inhibit succinate dehydrogenase by lowering mitochondrial ATP. However, we observed that in the presence of Ap_5A ATP inhibited respiration (Fig. 5D); therefore, we conclude that ATP activation of succinate dehydrogenase does not limit electron transport under these state 2 conditions. Rather, ATP directly or AMP and AK indirectly (via ATP) inhibit electron transport in state 2.

State 2 respiration fueled by exogenous NADH was also inhibited by AMP and ATP in a manner similar to succinate oxidation (Fig. 5). Together, the data in Figure 5 demonstrate that micromolar concentrations of ATP have a direct and rapid inhibitory effect on mitochondrial electron transport in state 2. The inhibition by ATP is rapidly reversed by ADP (state 3) (Fig. 5, D and G). Although the exact mechanism by which ATP regulates state 2 respiration is unclear, it appears reasonable to exclude the oxidases that feed electrons into the respiratory chain, because such similar behavior in both succinate dehydrogenase and the external NADH oxidase seems unlikely. This behavior is more readily explainable by adenylate regulation of downstream components of the inner mitochondrial membrane involved in electron and proton transport. Prominent among these are proteins such as ATP synthase, which are

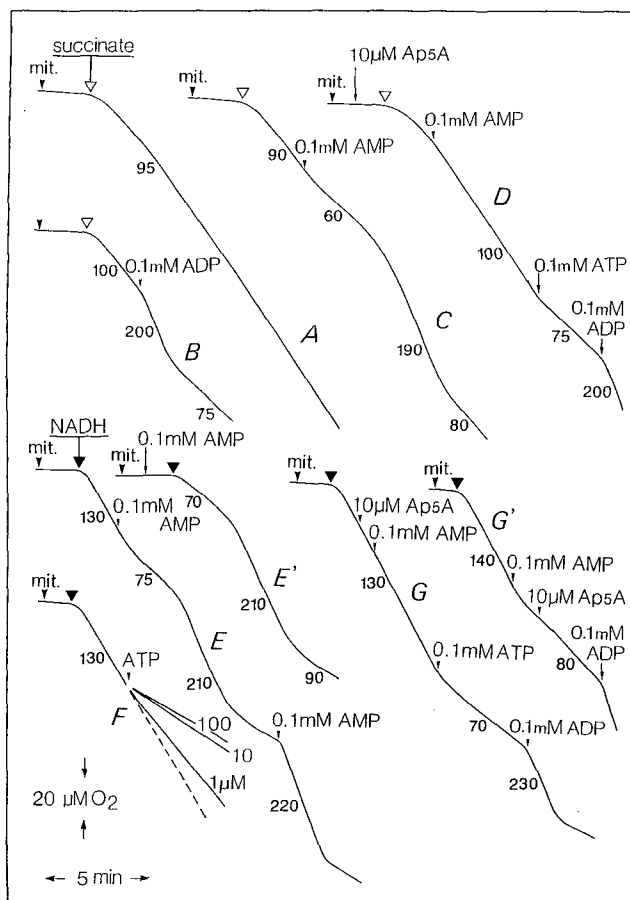


Figure 5. O_2 consumption by mitochondria (mit.) during phosphorylation of exogenous AMP. A, 20 mM succinate; B, succinate plus 0.1 mM ADP; C, succinate plus 0.1 mM AMP; D, succinate plus AMP in the presence of 10 μ M Ap_5A (identical results were observed with 0.5 mM Ap_5A), followed by 0.1 mM ATP, and then 0.1 mM ADP; E, 2 mM NADH plus 0.1 mM AMP; E', 0.1 mM AMP followed by 2 mM NADH; F, NADH plus 1 to 100 μ M ATP; G, NADH plus AMP in the presence of 10 μ M Ap_5A , followed by 0.1 mM ATP, and then 0.1 mM ADP; G', NADH plus 0.1 mM AMP, followed by 10 μ M Ap_5A , and then 0.1 mM ADP. The values beside the traces correspond to $nmol O_2$ consumed $min^{-1} mg^{-1}$ protein.

capable of dissipating transmembrane proton gradients (for a discussion, see Whitehouse et al., 1989); this possibility is illustrated in Figure 6. In this regard we observed that oligomycin, which interferes with ATP synthase, gave similar inhibition of state 2 respiration as AMP or ATP (data not shown). However, further investigation is clearly needed to evaluate the significance of this putative regulatory pathway and of other possible mechanisms involving modulation of proton fluxes (Whitehouse et al., 1989) and electron transport components by ATP (Oestreicher et al., 1973; Craig and Wallace, 1995). The inhibition of state 2 respiration by ATP in the range of 1 to 10 μ M (Fig. 5F) stands in contrast to estimated concentrations of ATP in the thick mitochondrial suspension of approximately 50 μ M (averaged over the entire volume of the suspension reported in Table I) or 130 μ M (assuming that all of the endogenous ATP is located in the mitochondrial matrix,

and the matrix volume is approximately 3 μ L mg^{-1} [Neuberger and Douce, 1980]), from which we infer that the great majority of ATP in mitochondria is protein-bound (compare Martins et al., 1992; Hooks et al., 1994). This inference is consistent with the capacity of mitochondria to retain ATP, as recorded in Table I, after washing in ATP-free medium during purification, noted above.

The tenacity with which mitochondria retain endogenous ATP makes it quite unlikely that simple diffusion of ATP from the matrix to AK in the intermembrane space plays a role in the phosphorylation of AMP (Fig. 3). Rather, an AMP-induced release of protein-bound ATP in the intermembrane space appears more likely, as illustrated in Figure 6. It is conceivable that sufficient molecules of AK could retain ATP during mitochondrial purification such that the process of conversion of AMP to ADP may begin immediately; in principle, a single molecule of ATP can prime the cycle of reactions shown in Figure 6, allowing all of the AMP to be phosphorylated.

The significant time required for AMP to stimulate respiration to the full state 3 level (Figs. 3 and 5) indicates that the threshold for inhibition of state 2 electron transport by ATP is exceeded before ADP levels become sufficient to stimulate respiration (state 3). The inhibitory state following AMP addition can be extended if AK is

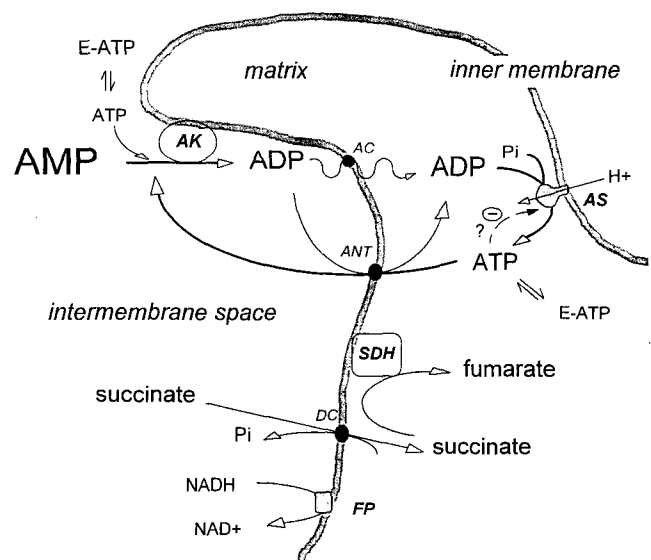


Figure 6. Postulated scheme describing biochemical events contributing to phosphorylation of exogenous AMP by mitochondria. AK catalyzes the transphosphorylation of AMP, initially using mitochondrial ATP released from intermembrane protein (E). ADP enters the mitochondria initially via the carrier of Abou-Khalil and Hanson (1973), labeled AC, and subsequently via the adenine nucleotide translocase (ANT) after matrix ATP builds up due to successive rounds of the AK-AC/ANT-AS-AC/ANT-AK cycle. The increase in ATP following AMP addition causes a transient inhibition of respiration, which we postulate occurs via ATP inhibition of proton entry into the matrix (see text for further discussion). ANT, Adenine nucleotide translocase; AS, ATP synthase; DC, dicarboxylate carrier; E-ATP, enzyme-bound ATP; FP, flavoprotein external NADH oxidase; SDH, succinate dehydrogenase.

inhibited by the subsequent addition of Ap₅A (Fig. 5G'), before significant synthesis of ADP occurs. The delay in attaining state 3 can be attributed to at least three processes. The first possibility is an AMP-induced inhibition of electron transport via mitochondrial ATP levels, discussed above. Second, activity of AK immediately following addition of AMP is undoubtedly limited by ATP, given the levels of endogenous free ATP, discussed above, which are low relative to the affinity of AK for ATP (Kuby et al., 1962; Busch and Ninnemann, 1996). Only after many cycles of AK/ATP-synthase/AK action, illustrated in Figure 6, will ATP build up to levels sufficient to saturate AK. Low initial ATP synthase activity due to limiting AK activity is consistent with the observation of much lower levels of ADP in Figure 3, compared with the levels seen when AMP is added in the presence of exogenous ATP (Fig. 2). A third possibility is that transport of adenylates also serves to delay attainment of state 3 following the addition of AMP.

Cooperation of NDPK and ATP Synthase

When mitochondria are given 1 mM UDP in the presence of 0.1 mM ATP and succinate, respiration occurs at rates similar to state 3 values observed with ADP (Fig. 7, top left). Similar results were observed with GDP, thymidine 5' diphosphate, and IDP (data not shown). ³¹P-NMR spectra of the reaction mixture given UDP show that the UDP is rapidly converted to UTP (Fig. 7), suggesting that mitochondria contain significant quantities of NDPK relative to ATP synthase. We tested for this possibility directly by monitoring interconversion of nucleotides in a mitochondrial suspension lacking the respiratory substrate succinate. When mitochondria were given 1 mM ATP and 1 mM UDP, a rapid accumulation of UTP and a concomitant decrease in ATP was observed (Fig. 8). The final concentrations of UDP, UTP, ADP, and ATP in the reaction mixture were essentially equal, demonstrating equilibration via NDPK. These results show that plant mitochondria contain sufficient NDPK activity to regenerate nucleoside triphosphate from NDP at a rate comparable to, or higher than, the maximum rate of respiration. In mammals, NDPK has been found between the inner and outer mitochondrial membranes (Brdiczka, 1991). If UDP and succinate were given to mitochondria in the absence of added ATP, O₂ uptake was barely stimulated, in contrast to the result shown in Figure 7. However, ³¹P-NMR did reveal a continuous synthesis of UTP, although at a rate 3 to 4 times slower than that shown in Figure 7. These results indicate that the pool of sequestered ATP found in purified mitochondria, which can serve to prime the synthesis of ADP from ATP via AK (Fig. 4; Table I), is also accessible to NDPK.

Unlike the response to NDPs, NMPs other than AMP were unable to enhance mitochondrial O₂ consumption in the presence of ATP and succinate and only small amounts of NMPs were phosphorylated, even after periods of several hours (data not shown), indicating that mitochondria contain little NMP.

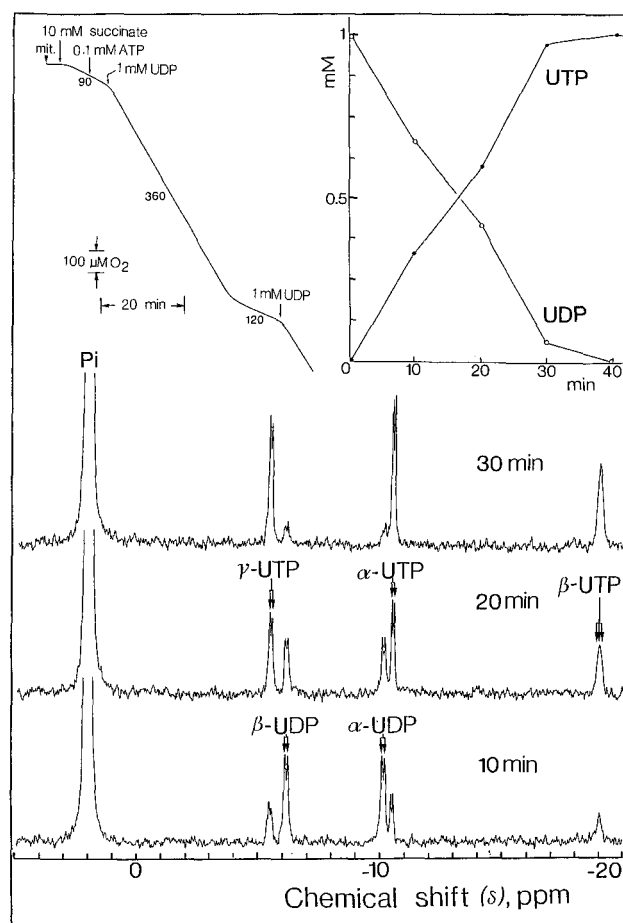
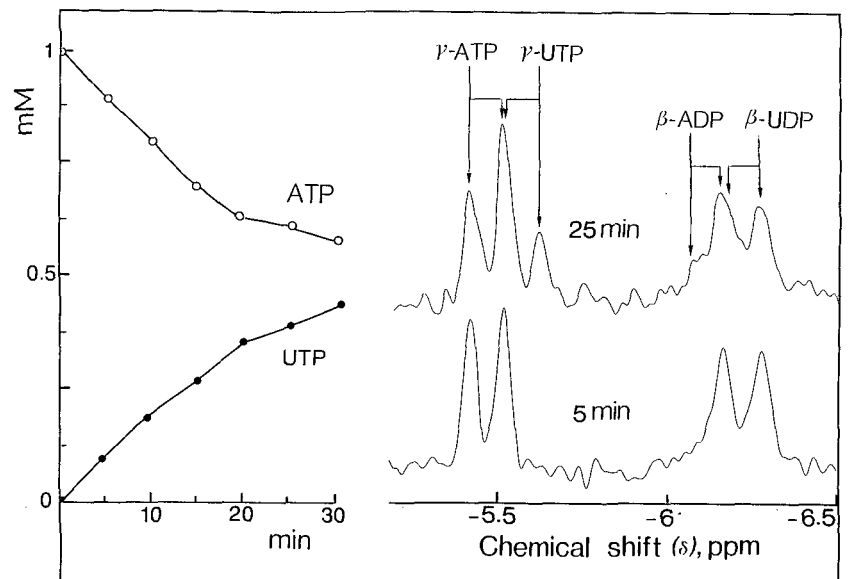


Figure 7. Synthesis of UTP from UDP by respiring mitochondria (mit.). ³¹P-NMR spectra of nucleotides in a suspension of mitochondria (110 μg protein mL⁻¹) following the addition of 1 mM UDP in the presence of 0.1 mM ATP. The medium contained 20 mM succinate and was oxygenated prior to the addition of mitochondria. Spectra were acquired over 5 min; the indicated time is the midpoint of acquisition for each spectrum. The small amount of ATP present during the reaction gives weak signals, one just evident as a small shoulder on the left side of the γ-UTP resonance (Fig. 5) and the other two overlapping with the α- and β-UTP resonances. Top left, Effect of UDP on O₂ consumption in a mitochondrial suspension. The electrode trace was recorded using an aliquot of the mitochondrial suspension used to generate the NMR data taken prior to addition of UDP. The values beside the trace correspond to nmol O₂ consumed min⁻¹ mg⁻¹ protein. Top right, Changes in nucleotide concentrations in the same mitochondrial suspension following the addition of UDP, determined from ³¹P-NMR spectra as shown; UTP values were obtained after subtraction of the small contribution from ATP signals.

CONCLUSIONS

Our experiments show that both AK and ATP synthase are important for mitochondrial synthesis of ATP from ADP, as well as from AMP. When ADP is generated in the cytoplasm, AK activity initially accounts for much of the ATP made, so that the net rate of ATP synthesis is initially greater than the maximum rate of oxidative phosphorylation. Only as the AK reaction approaches near-equilibrium does oxidative phosphorylation become the primary path-

Figure 8. Equilibration of adenine and uridine nucleotides by mitochondrial NDPK. Right, ^{31}P -NMR partial spectra of the β -NDP and γ -nucleoside triphosphate resonances in a suspension of mitochondria ($110 \mu\text{g protein mL}^{-1}$) following the simultaneous addition of 1 mM ATP and 1 mM UDP. Succinate was omitted from the medium, which was oxygenated prior to the addition of mitochondria. Spectra were acquired over 5 min; the indicated time is the midpoint of acquisition for each spectrum. Left, Changes in nucleoside triphosphate concentrations in the same mitochondrial suspension following the addition of ATP and UDP, determined from ^{31}P -NMR spectra as shown.



way for net ATP synthesis. One implication of these results for energy metabolism in plant cells is that when net ATP hydrolysis to ADP is stimulated, for example, in response to increased biosynthetic or membrane transport activity, potential increases in the concentration of cytoplasmic ADP will tend to be muted by the metabolism of ADP via AK, which we have shown can effectively lower the concentration of ADP available to the mitochondria for oxidative phosphorylation. Moreover, there are clear examples of the rate of mitochondrial respiration in aerobic plant cells being limited by low concentrations of ADP (reviewed by Douce, 1985; Dry et al., 1987). Consequently, the ability of AK to limit buildup of cytoplasmic ADP when net ADP production increases may necessarily serve to delay or reduce increases in respiratory activity. Any muting of the immediate respiratory response to an increased cellular demand for ATP is unlikely to be deleterious, since AK, in limiting accumulation of ADP, provides the cytoplasm with ATP. The stabilizing influence of AK we describe here has similarities to the roles of AK as a buffer and frequency filter of changes in adenylates found in theoretical studies of cells in which ATP consumption changes (Veuthey and Stucki, 1987). However, in cells in which metabolic fluxes that generate AMP and ADP are comparable, AK will not act as a simple buffer of ATP levels, since ATP synthesized by AK from ADP will be used to transphosphorylate AMP.

The pool of ATP in mitochondria, which cannot be dislodged even by extensive washing during organelle purification but whose release is triggered by exogenous AMP, can serve as an effective "catalytic" co-substrate for AK, priming the conversion of AMP to ADP, which can subsequently be used in oxidative phosphorylation (Fig. 5). We demonstrated that AK activity can influence respiration not only positively, by providing ADP for ATP synthase activity, but also negatively via changes in the concentration of mitochondrial ATP. The biochemical events depicted in Figure 6 have each been reported individually over the past three decades. We show here how these

separate events are integrated to regulate respiration in the intact mitochondrion. The biochemical sequence we have found in isolated mitochondria provided with exogenous AMP (Fig. 6) mirrors the metabolic transformation that takes place in plant tissues during emergence from dormancy and anoxia, when AMP can be the predominant nucleotide present initially, and is eventually converted primarily to ATP during germination or recovery following anoxic stress (Saglio et al., 1980; Standard et al., 1983; Raymond et al., 1987). Sequestration via protein-nucleotide interactions may protect and conserve the small mitochondrial pool of ATP during stress, dormancy, and experimental manipulations.

The activities of AK and NDPK in mitochondria enable the rate of phosphorylation of AMP and NDPs other than ADP to be phosphorylated at rates equal to the maximum rate of oxidative phosphorylation. In vivo the rates of production of AMP and NDP from reactions such as amino and fatty acid activation and polysaccharide biosynthesis are significant relative to the rate of ATP breakdown to ADP. The results presented here show that the mitochondrion can fully satisfy cellular needs for regeneration of all of these products of metabolism.

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