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The role(s) of adenylate kinase and the adenylate carrier in the regulation of plant mitochondrial respiratory activity

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The effect of inhibitors of the ATP synthase, the adenylate carrier and adenylate kinase on the respiratory rate, steady-state membrane potential and mitochondrial ATP levels have been investigated in potato and pea leaf mitochondria. Under ADP-limited conditions, it was found that the addition of oligomycin, aurovertin or efrapeptin increased the membrane potential and decreased the respiratory rate, implying that ATP synthesis was occurring prior to inhibition. Molybdate, NEM or vanadate had no effect on the ATP synthesised, suggesting that ADP-regeneration required for continued phosphorylation was not due to contaminating ATPases or phosphatases. ATP levels were significantly reduced by carboxyatractyloside (CAT) and increased by P^1,P^5-di(adenosine-5′) pentaphosphate (Ap_5A). The respiratory rate could be stimulated by the addition of AMP and the stimulated rate was sensitive to oligomycin and aurovertin. Preincubation with CAT or Ap_5A abolished AMP stimulation of NADH oxidation. It is suggested that respiration can sustain a limited but significant net formation of ATP, even in the absence of any added ADP. A model involving the combined activities of the adenylate carrier, adenylate kinase and the ATP synthase is proposed to account for the ATP synthesised under these conditions. Furthermore, it is suggested that the cycling of mitochondrial ADP and ATP via this model may represent a major regulatory influence on the activity of mitochondrial respiration under conditions of ADP-limitation – a condition likely to reflect the in vivo situation in plant cells.

Introduction

The reactions of the tricarboxylic acid cycle, the respiratory chain and the ATP synthase are all interconnected within the mitochondrial matrix and require the co-ordinated control of all of these processes for continued metabolism. The requirements of these different processes are met by the assorted activities of the various carriers, enzymes and exchange devices present in the mitochondrial system [1]. During respiration, substrates are oxidised and a protonmotive force (pmf) is generated across the inner mitochondrial membrane. The pmf is used as the driving force for the different carriers and exchangers as well as for the synthesis of ATP [2].

The use of the pmf by the ATP synthase during ATP synthesis induces an increase in the rate of respiration – a phenomenon known as 'respiratory control' [3]. The activity of the TCA cycle will thus depend partly on the rate of oxidation of substrates by the respiratory chain (for co-factor regeneration) and partly on the availability of ADP to the ATP synthase complex. If there were absolute or very tight coupling between all these activities, then the production of TCA intermediates for cellular metabolic requirements would be wholly dependent on the cellular requirement for ATP and hence on the availability of ADP to the synthase. In plants, these two requirements for intermediates and ATP are not necessarily balanced, since the supply of carbon for synthetic purposes, under certain conditions, may be more important than

Abbreviations: Ap_5A, P^1,P^5-di(adenosine-5′) pentaphosphate; CAT, carboxyatractyloside; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; NEM, *N*-ethylmaleimide; $TPMP^+$, methyltriphenylphosphonium; pmf, protonmotive force.

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the supply of ATP and hence it is likely that there is a mechanism(s) which will allow a relaxation of coupling between these metabolic events.

Plant mitochondria display rapid rates of substrate oxidation when in state 4 or state substrate (which is defined as the respiratory rate prior to addition of any ADP) in comparison to their mammalian counterpart [4,5]. These rapid respiratory responses have been variously attributed to the activity of an endogenous H^+/K^+ antiporter and/or to the engagement of non-phosphorylative pathways such as the alternative pathway and/or the rotenone-insensitive bypass [6]. Using cyanide-sensitive potato mitochondria it has been shown that at high membrane potentials there is an increase in the ionic conductance of the membrane compared to conductance at low membrane potentials and that the ATP synthase is active during the generation of the membrane potential [7], even under ADP-limited conditions. It was therefore of some importance to investigate the activity of the synthase during the steady state and to assess its role in facilitating rapid respiratory rates in the absence of added ADP.

In this current study we have used a $TPMP^+$-sensitive electrode to monitor the effects of inhibitors of the ATP synthase components (F_0 and F_1) upon the respiratory rate and membrane potential of purified potato and pea leaf mitochondria under ADP-limited conditions (state-substrate). It was found that the addition of oligomycin, aurovertin or efrapeptin increased the steady-state membrane potential and decreased the respiratory rate, implying that ATP synthesis was occurring prior to inhibition. When levels of ATP were measured in the mitochondrial suspensions before, during and after energisation it was found that, although ATP was synthesised under state-substrate conditions, it did not accumulate within the mitochondrial matrix. Since ADP was not added to the suspensions it must therefore be assumed that mitochondria contain an ATP hydrolytic activity which is capable of regenerating the ADP required for the synthesis of ATP under these conditions. The nature of the renewable source of ADP required for continued respiration and ATP synthesis in the steady state was therefore investigated. It was found, using inhibitor treatments, that the mitochondrial suspensions did not contain any contaminating F-, P- or V-type ATPases or phosphatases. Investigation into the possible role of the adenylate carrier and adenylate kinase in this process revealed that, during ATP synthesis, under state-substrate conditions, ATP was exported to the intermembrane space (via the adenylate carrier) where it was hydrolysed by the action of adenylate kinase and ADP re-imported into the mitochondrial matrix in exchange for ATP.

It is proposed that this cycling of mitochondrial ADP and ATP, involving the combined activities of the adenylate carrier and the mitochondrial adenylate kinase, represents a major regulatory influence on the activity of mitochondrial respiration under conditions of ADP limitation.

Materials and Methods

Fresh potato tubers (*Solanum tuberosum* L.) were obtained from the local market and stored at 4°C. Pea (*Pisum sativum* L., cv Feltham First) seedlings were grown in trays of soil for 12–14 d. TPMP bromide was from Aldrich (Gillingham, Dorset, UK). Efrapeptin and aurovertin B were kind gifts from Professor R.B. Beechey (University of Wales, Aberystwyth, UK). Luciferin and luciferase were from LKB (Wallac, Sweden.). All other chemicals, including caboxyatractyloside and Ap_5A, were from Sigma (Poole, Dorset, UK).

Potato tuber and pea leaf mitochondria were isolated and purified on continuous Percoll gradients as previously described [7,8]. Reactions were carried out at room temperature in a 2 ml reaction medium which contained 0.3 M mannitol, 10 mM KH_2PO_4, 1 mM $MgCl_2$, 10 mM KCl and 10 mM Mops (pH 7.4) in a specially constructed cell housing a Rank oxygen electrode, a $TPMP^+$-sensitive electrode and a magnetic stirrer.

Mitochondrial membrane potentials were continuously monitored with a $TPMP^+$-electrode as previously described [7]. The electrode was calibrated at the start of each experiment by successive additions of $TPMP^+$ to a final concentration of 2.5 μM and membrane potentials were calculated using the Nernst equation using a matrix volume of 1.4 μl/mg protein [9]. Corrections for binding of $TPMP^+$ to mitochondria and as a result of substrate addition were made as previously explained [7]. Due to the presence of a H^+/K^+ antiporter in plant mitochondria [6], the contribution of the pH component of the pmf is negligible and this was further ensured by the composition of the reaction medium such that $\Delta p = \Delta\psi$ [6].

Mitochondria (0.5–1 mg protein) were incubated in the electrode cell with $TPMP^+$ and energised either by the addition of 5 mM succinate or 1 mM NADH. Where appropriate, inhibitors were added either before energisation (in the case of Ap_5A and CAT) or during the steady state (in the cases of aurovertin, efrapeptin and oligomycin) as shown in the figures.

ATP levels in the reaction media were determined using the sensitive luciferin technique in an LKB 1250 Luminometer as previously described [7]. Briefly, mitochondria (0.5–1 mg protein) were incubated in a reaction medium and a sample was taken for ATP determination (typical endogenous ATP content was 0.45–0.6 nmol/mg protein). Succinate was added and a further sample was taken after 6 min (approx. 0.8–1.1 nmol ATP/mg protein). When the effect of vanadate,

molybdate or NEM was investigated, the inhibitor was added to the reaction mixture and a sample taken, followed by the addition of succinate (after 1 min of incubation with inhibitor) and a further sample was taken after 6 min. Appropriate blanks containing reagents, but not mitochondria, were performed for each sample.

Protein concentrations were determined as in Lowry et al. [10] using BSA as standard.

Results

Fig. 1 illustrates the respiratory state we have termed state-substrate (to distinguish it from state 4). In the absence of any added ADP, the addition of a substrate to potato mitochondria, results in a rapid rate of respiration. Note that in the case of succinate the respiratory rate is not, initially, linear, since succinate dehydrogenase normally requires ATP or ubiquinol to activate the enzyme complex [11]. The addition of ADP results in a stimulation of respiratory activity and, of particular interest, in a decrease in the respiratory rate following exhaustion of the ADP. Similar decreases in the state-substrate rate to that observed under true state 4 conditions can be elicited either by the addition of 250 μM ATP or 2 μg oligomycin/mg protein (Fig. 1A). It is apparent from Fig. 1 that state-substrate is not substrate (or tissue) specific since comparable decreases in the overall oxygen uptake rates can be observed when exogenous NADH is used as a substrate (Fig. 1B). Such differences in rate, either as a result of the phosphorylation of ADP or due to the addition of ATP or oligomycin, have been attributed to the mitochondrial ATP synthase acting as an ion-influx channel [7]. This is because state-substrate conditions favour the release of the inhibitor protein IF_1 from its inhibitory site on the F_1-ATP synthase, facilitating increased proton conductance [12]. True state 4 conditions favour the rebinding of IF_1 to F_1 and hence prevent the ATP synthase acting as a dissipative route for proton re-entry [12]. Oligomycin is presumed to inhibit the respiratory rate as a result of binding to F_0 [13,14]. An alternative possibility for such decreases in respiratory rates could be as a consequence of an increased ATP content within the mitochondrial matrix. Such conditions have also been demonstrated to favour the rebinding of the inhibitor protein [12].

In an attempt to determine whether the ATP synthase was acting merely as a dissipative route without concomitant synthesis of ATP, the effects of inhibitors of the ATP synthase complex upon the steady state membrane potential generated in the absence of exogenous ADP were investigated (Fig. 2). Energisation with succinate generated a membrane potential of 189 mV which increased to 201 mV upon subsequent addition of oligomycin [Fig. 2a]. Similar results were observed when either efrapeptin and aurovertin, both of which specifically inhibit the F_1-ATP synthase [13], replaced oligomycin (Figs. 2a and b). Thus, in every

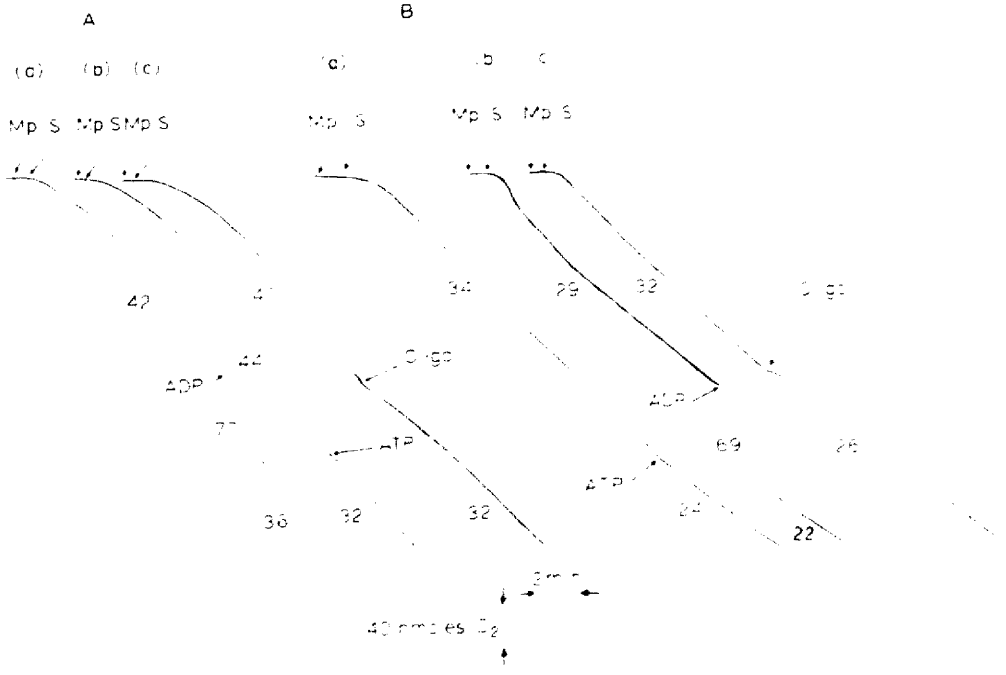

Fig. 1. Characteristics of state-substrate oxidation in potato mitochondria. Mitochondrial (0.7 mg protein) respiratory activity was measured as indicated in Materials and Methods and initiated by the addition of either 5 mM succinate (A) or 1 mM NADH (B). Further additions are as indicated with either 100 μM ADP, 250 μM ATP or 2 μg oligomycin/mg protein. Respiratory rates are expressed in nmol O_2/min per mg protein.

case, the addition of an ATP synthase inhibitor results in an increase in the steady-state membrane potential in the absence of added ADP. Fig. 2 also demonstrates that when oligomycin was added to mitochondria oxidising succinate in the presence of an F_1-ATP synthase inhibitor such as efrapeptin or aurovertin, it resulted in a further increase in the steady-state membrane potential. Such results strongly suggest that, before the addition of the inhibitors, there was an influx of protons through the synthase. This influx may be a passive flow but is far more likely to drive the synthesis of ATP. In support of this idea, we have previously demonstrated that both state-substrate and state 4 can sustain a limited but significant net formation of ATP, even in the absence of any added ADP [7]. Since ATP is synthesised during the steady-state without being accumulated, it must be hydrolysed to regenerate the ADP required for the synthesis of the ATP.

In order to determine whether the source(s) of renewable-ADP is within or outside the mitochondrial matrix during the steady-state, the role of the adenylate carrier was investigated. This carrier catalyses an electrogenic exchange of ADP for ATP across the inner mitochondrial membrane [15,16] and is specifically inhibited by (CAT) [17]. If the renewable source(s) of ADP is in the matrix then inhibition of the carrier should not have any effect on either ATP synthesis or the levels of ATP. The effect of CAT on the level of ATP in the mitochondrial suspension was determined once a steady state had been achieved following energisation. A comparison of Figs. 3a and 3b reveals that the level of ATP in the presence of CAT was 10% of that observed in its absence. The decrease in ATP content of the mitochondrial suspension in the presence of CAT is accompanied by an increased steady-state membrane potential (results not shown). The subsequent addition of oligomycin under these conditions had only a minimal effect on the established potential. In other words, the membrane potential generated in the presence of CAT was higher than in its absence and was not significantly increased by oligomycin. These finding strongly suggest that the ADP-regenerating system(s) was not located in the mitochondrial matrix.

It is possible that the ADP regenerated in our experimental system resulted from ATP hydrolytic activity associated with contaminating ATPases, even though highly purified mitochondria were used in these investigations. There are several possible sources of contaminating ATPases, such as those associated with the plasma and vacuolar membranes in addition to non-specific acid phosphatases, all of which can consume ATP and thus generate ADP. In an attempt to determine whether any of these were responsible for generating the ADP outside of the mitochondrion we investigated the effect of inhibitors of these various

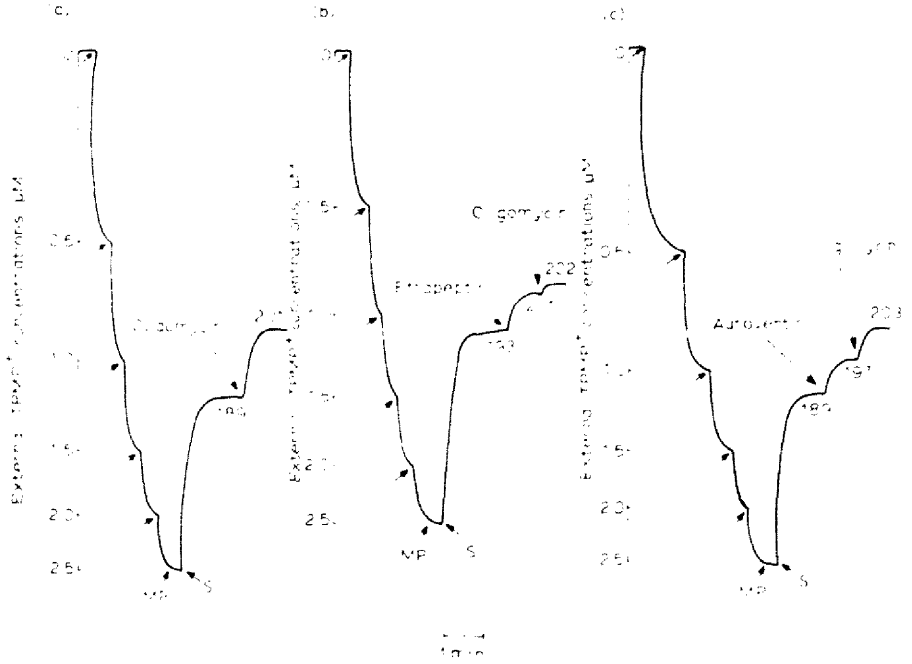

Fig. 2. The effect of ATP synthase inhibitors on the steady-state membrane potential in potato mitochondria. The TPMP$^+$-electrode was calibrated by a series of additions (represented by arrows) to a final concentration of 2.5 μM. Membrane potentials were measured as described in Materials and Methods in the presence of 0.75 mg mitochondrial protein (Mp) and 5 mM succinate (S). Once the steady-state membrane potential was established, inhibitors were added as indicated: (a) 2 μg oligomycin/mg, (b) 3 μg efrapeptin/mg followed by 2 μg oligomycin/mg and (c) 4 μg aurovertin/mg followed by 2 μg oligomycin/mg. Numbers refer to the membrane potential in mV.

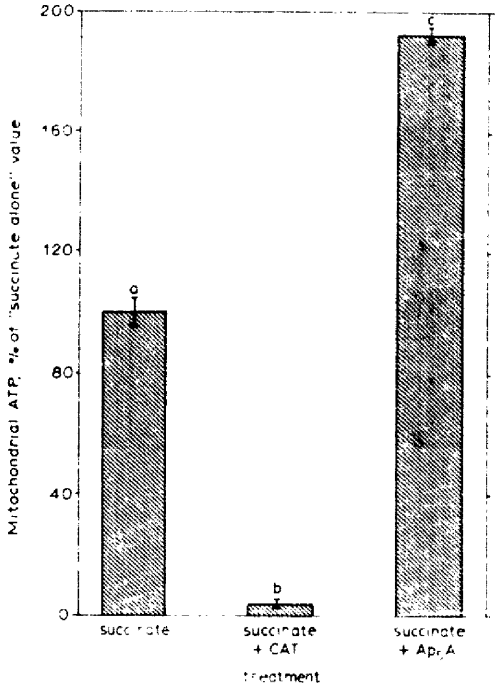

Fig. 3. The effect of CAT and Ap_5A on endogenous mitochondrial ATP levels. Potato mitochondria (2 mg protein) were incubated in 2 ml of reaction medium containing (a) 5 mM succinate, (b) 5 mM succinate and 2.5 μg CAT/mg or (c) 5 mM succinate and 10 μM Ap_5A. Samples were taken after 6 min incubation and the ATP content was analysed as indicated in Materials and Methods. Results are expressed as % of the ATP content in the sample incubated with substrate alone (100% = 0.85–1 nmol ATP) and represent means ± S.D. from four independent measurements.

ATP-consuming reactions [18–20] on the ATP content of the mitochondrial suspension during state-substrate oxidation.

Mitochondria were suspended in the presence (separately) of each of the inhibitors and ATP levels were determined both before and after energisation with succinate (see Fig. 4). The amounts of ATP synthesised in the presence of 1 mM molybdate and 100 μM vanadate were similar to those in the control lacking the inhibitor. It was therefore possible to eliminate contamination by acid phosphatases and vacuolar ATPases as being responsible for the regeneration of ADP. By contrast, treatment with NEM produced a small reduction in the amount of ATP synthesized. Although this result is suggestive of contamination by a plasma membrane ATPase [20], a similar decrease in the ATP content would also have been expected with the vanadate treatment [18]. NEM is, however, relatively unspecific and is known to also have an inhibitory effect on the adenylate and phosphate carriers [21,22] and it is therefore quite conceivable that the effect observed in Fig. 4 is related to this latter inhibitory action rather than to inhibition of a plasma membrane ATPase. These results suggest that ADP is not regenerated by the actions of any contaminating ATP-hydrolytic activities.

Since the results described in Figs. 3 and 4 suggest that ADP is regenerated by reaction(s) located outside the mitochondrial matrix and appears not to involve contaminant enzymes in the mitochondrial suspension, we investigated the role of adenylate kinase in the ADP-regeneration reaction. In plant tissues, data in the literature suggest that adenylate kinase is located in the intermembrane space being tightly bound to the inner membrane [23] and, similar to other systems, is potently inhibited by Ap_5A [24,25]. Mitochondria were incubated with 10 μM Ap_5A and samples for ATP determinations were taken before and after energisation with succinate. It can be seen from Fig. 3c that, in the presence of Ap_5A, the ATP content of the mitochondrial suspension was approximately double that found in the corresponding control. Furthermore, the inclusion of Ap_5A resulted in a slower generation of the membrane potential, compared to the control, but the final membrane potential was of a value similar to that observed in Fig. 2a (results not shown). These results implicate adenylate kinase in the extra-mito-

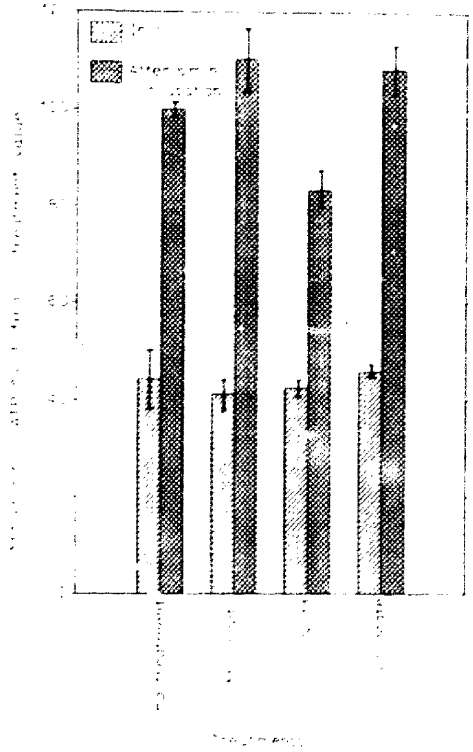

Fig. 4. The effect of molybdate, NEM and vanadate on endogenous mitochondrial ATP levels. Potato mitochondria (2 mg protein) were incubated in 2 ml reaction medium containing 5 mM succinate (no treatment) and either 1 mM molybdate, 1 mM NEM or 100 μM vanadate. Samples were taken before addition of succinate and following 6 min incubation and the ATP content analysed as indicated in Materials and Methods. Results are expressed as % of the ATP content in the sample incubated with substrate alone (as in Fig. 3) and represent means ± S.D. from three independent measurements.

chondrial renewal process for ADP. When the kinase activity is inhibited by Ap_5A, ATP is not hydrolysed extramitochondrially and will thus accumulate within the mitochondrial matrix (see Fig. 3). Further confirmation implicating a role of adenylate kinase in state-substrate oxidation is illustrated by the experiments depicted in Figs. 5 and 6. Purified pea leaf mitochondria were used in these experiments to demonstrate that the responses were not tissue-specific. The addition of aliquots of AMP to mitochondria oxidising NADH (via the external dehydrogenase) resulted in a stimulation of respiration which could be inhibited by either oligomycin or aurovertin. If the AMP is being phosphorylated to ADP by adenylate kinase, then 100 μM AMP will give rise to 200 μM ADP and hence yield a P/O ratio characteristic of two sites of phosphorylation. It can be seen from Fig. 5c that such a result was obtained. When mitochondria were preincubated with 10 μM Ap_5A for 5 min prior to addition of substrate, AMP did not result in any respiratory stimulation (confirming that there was no ADP contamination of the AMP solution), but respiratory control could still be observed upon subsequent addition of ADP, suggesting that Ap_5A does not inhibit the respiratory chain. Similarly, preincubation in the presence of CAT also prevented AMP stimulation confirming the involvement of the adenylate carrier in this process. It is also apparent from Fig. 6b that the initial respiratory rate, in the presence of CAT, is considerably lower than that observed in Fig. 5, consistent with the observed increase in membrane potential caused by the inclusion of this inhibitor (results not shown).

Discussion

In the present paper we have investigated the nature of state-substrate oxidation in plant mitochondria. A characteristic feature of plant mitochondria is that they display a rapid state 4 respiratory rate, in comparison to their mammalian counterpart, and furthermore are typified by the intial rate of respiration (prior to the addition of ADP) being faster than true state 4 (a condition we have termed state-substrate) [4,5]. Rapid respiratory rates under state 4 conditions, which are accompanied by the generation of high membrane potentials, have been attributed to the presence of an endogenous K^+/H^+ antiporter [6] and/or a high membrane ionic conductance [7]. Although the nature of the conductance pathway is unclear, a possible route for H^+-re-entry could be via the ATP synthase, either resulting in the synthesis of ATP or as a result of slippage without any net synthesis [26]. The results presented in this report, along with previous direct measurements on ATP formation [7], are however clearly consistent with a limited but significant amount of ATP being synthesised during the steady state, even in the absence of exogenous ADP. This notion is supported by the finding that inclusion of either an F_0 or an F_1 inhibitor increased the membrane potential

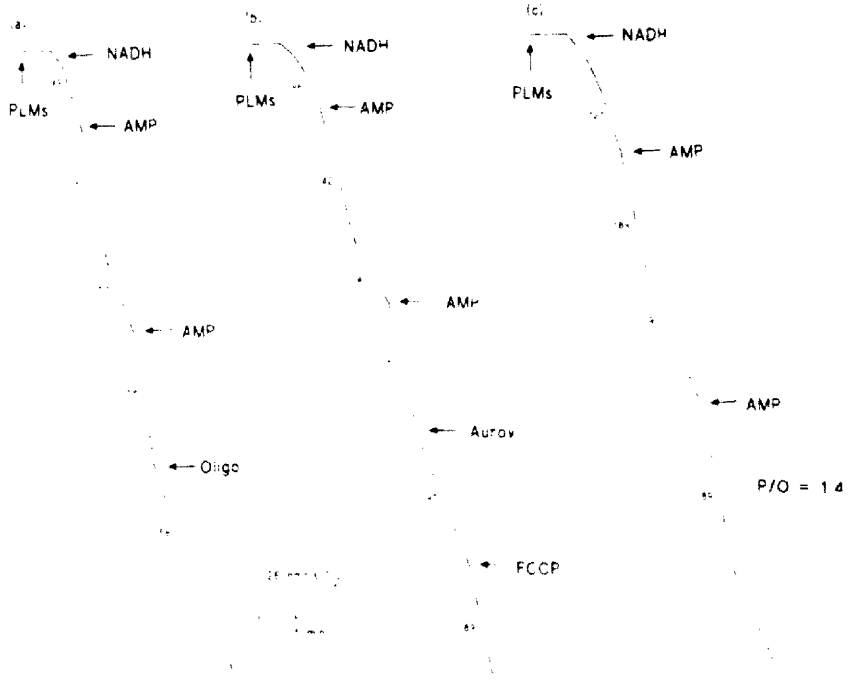

Fig. 5. The effect of AMP on exogenous NADH oxidation in pea leaf mitochondria. Mitochondrial (0.5 mg protein) respiratory activity was measured as described in Materials and Methods in the presence of 1 mM NADH and as indicated either 100 μM AMP, 2 μg oligomycin (Oligo) mg, 4 μg aurovertin (Aurov) mg or 1 μM FCCP. Rates are expressed as nmol O_2 · min per mg protein.

and decreased the respiratory rate (Figs. 1 and 2) and furthermore that ATP levels were significantly reduced by CAT but substantially increased by Ap_5A (Fig. 3). These latter results clearly implicate the involvement of the adenylate carrier and adenylate kinase in the ATP synthesis process during state-substrate oxidation. ATP synthesis in the absence of added ADP is not restricted to isolated plant mitochondria, however, since similar observations have been reported for rat liver mitochondria [27,28].

A model to account for such a system is illustrated in Fig. 7. In this model ATP is continuously synthesised in energised mitochondria, even when ADP is omitted from the reaction mixture. This is achieved because the newly synthesised ATP is exported, via the adenylate carrier, to the intermembrane space in exchange for ADP entry into the matrix where it is available for ATP synthesis. Such a cycling prevents ATP levels within the matrix from rising sufficiently high to cause IF_1 binding to the synthase and thereby inhibit the synthase. Once in the intermembrane space, the newly exported ATP would be available for cellular metabolism or, if demand for ATP is low, then hydrolysis by adenylate kinase to regenerate ADP, thereby completing the cycle. The source of AMP required for the adenylate kinase reaction may lie with the activity of acyl CoA synthetase proposed to reside on the outer mitochondrial membrane [29]. Indeed, preliminary results indicate that state-substrate respiratory rate can be stimulated by approx. 20% upon addition of CoA and, furthermore, such stimulations are not observed with mitoplasts (Moore, A.L. and Whitehouse, D.G., unpublished observations). Obviously, further work is required to substantiate these findings. Nonetheless, Figs. 5 and 6 clearly demonstrate the involvement of both AMP and adenylate kinase in the maintenance of state-substrate respiratory activity. A number of other reactions which might generate ADP (e.g. contaminating F-, P- or V-type ATPases and nonspecific phosphatases) have been shown (see Fig. 4) to be absent from the purified mitochondrial preparations used in this study. Equally, conditions conducive to the operation

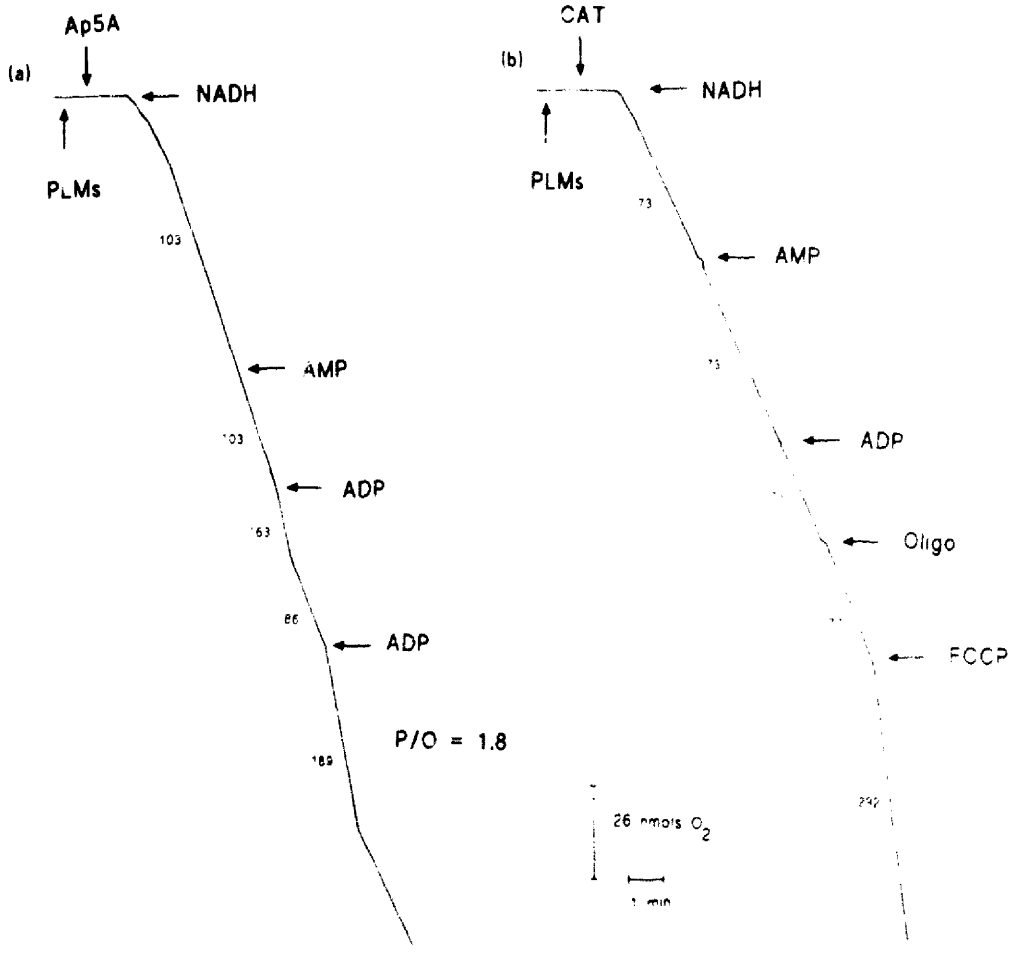

Fig. 6. The effect of Ap_5A and CAT on exogenous NADH oxidation in pea leaf mitochondria. Figure legend as in Fig. 5. Mitochondria (0.5 mg protein) were preincubated with either 10 μM Ap_5A or 2.5 μg CAT/mg for 2 min prior to the addition of 1 mM NADH and as indicated either 100 μM AMP, 100 μM ADP, 2 μg oligomycin (Oligo)/mg or 1 μM FCCP. Rates are expressed as nmol O_2 · min per mg protein.

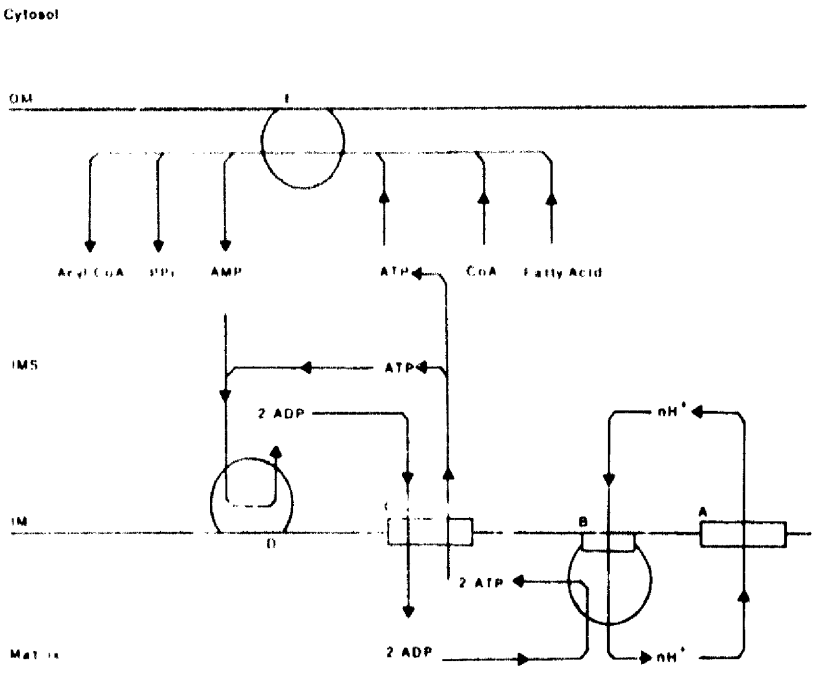

Fig. 1. Proposed scheme for the cycling of adenylates in plant mitochondria. OM, outer membrane; IMS, intermembrane space; IM, inner membrane; A, respiratory chain; B, ATP synthetase; C, adenylate translocator; D, adenylate kinase and E, acyl-CoA synthetase.

of a membrane-bound hexokinase [30] are lacking (exogenous hexoses being absent), ruling out the activity of this enzyme in the regeneration of ADP. Furthermore, the specific inhibition of this system by Ap_5A readily demonstrates the key role of adenylate kinase in the ADP-renewal process.

Although this cycle might be considered energetically wasteful, it may be important in vivo when cellular demand for ATP is low but demand for TCA cycle intermediates is high. The ADP generated by the proposed cycle would ensure a fast rate of respiration under conditions of ADP limitation – a condition considered to reflect the in vivo situation in plant cells [1,8]. Additionally the co-operative activities of the ATP synthase, adenylate carrier and adenylate kinase will increase membrane conductance at high membrane potentials and therefore facilitate rapid respiratory rates [7]. Respiration has long been regarded as a generator of the pmf to drive ATP synthesis (or ion movements) but these results reflect a growing viewpoint that respiration is also a generator of cellular intermediates (as suggested to be the role of non-phosphorylating pathways in plant mitochondria [4–6]) and this activity may continue at low physiological ADP concentrations by means of the proposed mitochondrial adenylate cycle.

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