

## MITOCHONDRIAL ARGININE KINASE IN THE MIDGUT OF THE TOBACCO HORNWORM (*MANDUCA SEXTA*)

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### Summary

Mitochondria isolated from the posterior midgut of the tobacco hornworm (*Manduca sexta*) contain arginine kinase. The distribution of mitochondrial and cytoplasmic marker enzymes indicates that the presence of mitochondrial arginine kinase is not due to cytoplasmic contamination. Arginine is not oxidized by the midgut mitochondria but, when metabolic substrates and ATP are present, respiration can be initiated by the addition of arginine. Under these conditions, there is no return to State 4 respiration, indicating regeneration of ADP by the arginine kinase reaction. Respiration can be blocked, however, by atractyloside, an inhibitor of the adenine nucleotide translocator. These results indicate that arginine kinase resides outside the matrix. Mitochondrial arginine kinase is specific to L-arginine since analogs of L-

arginine are ineffective in stimulating respiration in the presence of ATP. Coupling between the adenine nucleotide translocator and arginine kinase was investigated using kinetic and thermodynamic approaches. There were no differences in the activities of arginine kinase in respiring and non-respiring mitochondria when they were measured at different ATP or arginine concentrations. This result indicates that arginine kinase does not have preferential access to the ATP exported out of the matrix. A comparison of the apparent equilibrium constant and the mass action ratio of the arginine kinase reaction also confirms that there is no microcompartmentation of the reaction.

Key words: *Manduca sexta*, midgut, tobacco hornworm, arginine kinase, mitochondria, epithelia.

### Introduction

Phosphagen kinases catalyze the reversible phosphorylation of guanidine compounds, and several different phosphagen kinases have been identified in animals (Morrison, 1973). Of these phosphagen kinases, creatine kinase (CK; creatine + ATP  $\leftrightarrow$  creatine phosphate + ADP) from mammals is the best understood. Several roles in cellular energetics have been attributed to the CK system, three of which are (1) as a temporal energy buffer, (2) as a spatial energy buffer, and (3) to increase the efficiency of oxidative phosphorylation (reviewed by Wallimann *et al.* 1992). As a temporal energy buffer, the CK system stabilizes ATP levels at the expense of creatine phosphate under conditions when ATP use exceeds the ability of the mitochondria to produce ATP. Spatial energy buffering has been proposed for cells such as cardiac myocytes or spermatazoa, where there are cytosolic and mitochondrial forms of the enzyme. Through the action of mitochondrial creatine kinase, ATP produced at the mitochondria is converted to creatine phosphate, which diffuses to a cytosolic site of ATP utilization (e.g. myofibrillar ATPase). There, the cytosolic creatine kinase converts the creatine phosphate to ATP, and the creatine diffuses back to the mitochondria. Therefore, creatine and creatine phosphate, rather than ATP

and ADP, are shuttled between the site of ATP synthesis and its site of use. It has also been suggested that the presence of a mitochondrial creatine kinase may increase the efficiency of oxidative phosphorylation by maintaining a low concentration of ATP in the vicinity of ATP synthesis.

Arginine kinase (AK) is the phosphagen kinase found in insects. Schneider *et al.* (1989) demonstrated that AK and arginine phosphate served as a temporal energy buffer system in locust femoral muscle. At rest, the arginine phosphate concentration in femoral muscle is four times higher than that of ATP. When the locust jumps, ATP levels are stabilized at the expense of arginine phosphate. In contrast, the resting levels of ATP and arginine phosphate are nearly identical in the highly aerobic flight muscle. Upon initiation of flight, ATP levels fall and there is no change in arginine phosphate concentration, indicating that flight muscle AK plays little or no role in temporal energy buffering. Flight muscle, however, is not the only aerobic insect tissue that contains AK. High activities of this enzyme are found in ion-transporting epithelia such as locust hindgut (Chamberlin and Phillips, 1983) and lepidopteran midgut (Chamberlin, 1987; Gindling *et al.* 1995). The present study explores the possibility that the AK system

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in the midgut of the tobacco hornworm (*Manduca sexta*) might serve as a temporal energy buffer. This function is most effective if the arginine phosphate levels exceed ATP levels at rest. To determine whether the AK system could be involved in an ATP buffering system in the midgut, ATP, arginine and arginine phosphate levels were measured in this tissue.

If, like CK in vertebrates, AK is to play a role in increasing the efficiency of oxidative phosphorylation, it must be present in the mitochondria. Focusing on insect flight muscle, a tissue with a high mitochondrial density and high AK activity, several researchers (Ellington and Hines, 1991; Schneider *et al.* 1989; Wyss *et al.* 1995) have failed to detect a mitochondrial form of AK. Like the insect flight muscle, the tobacco hornworm midgut also has a high mitochondrial content, and the present study demonstrates the presence of AK in mitochondria isolated from this tissue.

If mitochondrial AK from the midgut acts in a manner analogous to that of CK in mammalian mitochondria, then there should be a functional coupling between AK and the adenine nucleotide translocase (ANT), the transporter that exchanges ATP and ADP across the inner mitochondrial membrane. A functional coupling between CK and ANT has been demonstrated in mammalian heart mitochondria using both kinetic and thermodynamic approaches. For example, the  $K_m$  for ATP of the creatine kinase reaction is over eight times lower in respiring mitochondria than in rotenone- and oligomycin-poisoned mitochondria because the CK reaction has preferred access to the ATP transported out of the mitochondria (Wyss *et al.* 1992). A comparison of the mass action ratio ( $[ATP][\text{creatine}]/[ADP][\text{creatine phosphate}]$ ) with the apparent equilibrium constant ( $K'_{eq}$ ) of the CK reaction in heart mitochondria (DeFuria *et al.* 1980) or mitoplasts (a preparation consisting of inner membrane and matrix only; Saks *et al.* 1985) revealed that creatine phosphate was synthesized even when the mass action ratio was less than  $K'_{eq}$ . This apparent violation of thermodynamics has been interpreted to mean that the CK reaction is not occurring in the bulk solution but in a microenvironment near ANT (Saks *et al.* 1985). In contrast to the CK system, Doumen and Ellington (1990b) failed to show any coupling between AK and ANT in mitochondria isolated from the horseshoe crab (*Limulus polyphemus*) heart. In the present study, kinetic and thermodynamic studies were undertaken to determine whether there is compartmentation of the AK reaction in mitochondria isolated from the tobacco hornworm midgut.

## Materials and methods

### Insects

*Manduca sexta* (L.) larvae were raised from eggs provided by the United States Department of Agriculture (USDA) or a colony derived from the USDA stock and maintained in the Biological Sciences Department at Ohio University. Larvae were fed a commercial diet (no. 9783, BioServ, Frenchtown, NJ, USA) and maintained at 25 °C on a 16 h:8 h L:D cycle. Fifth-instar larvae weighing 3–5.5 g were used in all studies.

### Metabolite analysis

Metabolite levels were measured in the midgut after it had been quickly dissected from the animal, rinsed and weighed in a cold saline identical to that described by Chamberlin (1994) except that it lacked arginine. The tissue was then frozen in liquid nitrogen and homogenized in 5 vols of 6% perchloric acid. The homogenate was centrifuged at 12000g for 1 min, and the supernatant removed and neutralized with 3 mol l<sup>-1</sup> K<sub>2</sub>CO<sub>3</sub>. The neutralized extract was then frozen in liquid nitrogen and stored at -80 °C until it was analyzed for arginine, arginine phosphate and ATP levels as described below. Preliminary studies revealed that this extraction procedure did not lead to acid hydrolysis of arginine phosphate.

Arginine, arginine phosphate, ATP and ADP levels were measured spectrophotometrically in a Gilford Response spectrophotometer. Arginine and arginine phosphate were measured according to a modified method of Gäde (1985). The assay medium contained the tissue or mitochondrial extract, 5 mmol l<sup>-1</sup> ADP, 0.4 mmol l<sup>-1</sup> NADH, 2.5 mmol l<sup>-1</sup> pyruvate and 5 mmol l<sup>-1</sup> MgCl<sub>2</sub> in 100 mmol l<sup>-1</sup> imidazole, pH 7.2. The arginine concentration was determined by measuring the change in NADH concentration upon the addition of 1 unit ml<sup>-1</sup> octopine dehydrogenase (1 unit = 1 μmol product min<sup>-1</sup>). Once the octopine dehydrogenase reaction had reached equilibrium, AK (5 units ml<sup>-1</sup>) was added to determine the arginine phosphate concentration in the extract.

ATP was determined by monitoring the increase in NADPH when hexokinase (2 units ml<sup>-1</sup>) was added to the assay medium containing tissue or mitochondrial extract, 0.4 mmol l<sup>-1</sup> NADP, 5 mmol l<sup>-1</sup> MgCl<sub>2</sub>, 50 mmol l<sup>-1</sup> glucose and excess glucose-6-phosphate dehydrogenase (1 unit ml<sup>-1</sup>) in 100 mmol l<sup>-1</sup> imidazole, pH 7.2.

ADP was determined by monitoring the change in NADH absorbance when pyruvate kinase (2 units ml<sup>-1</sup>) was added to the assay medium containing mitochondrial extract, 100 mmol l<sup>-1</sup> KCl, 10 mmol l<sup>-1</sup> MgCl<sub>2</sub>, 2 mmol l<sup>-1</sup> phosphoenolpyruvate, 0.3 mmol l<sup>-1</sup> NADH and lactate dehydrogenase (2.5 units ml<sup>-1</sup>) in 100 mmol l<sup>-1</sup> imidazole, pH 7.2.

### Isolation of the mitochondria

Mitochondria were isolated using the method described in Gibellato and Chamberlin (1994). Briefly, this entails gently homogenizing the posterior midguts in isolation medium and separating the mitochondria by differential centrifugation. To assess the cytoplasmic contamination of the mitochondrial preparation, the activities of AK, citrate synthase (mitochondrial enzyme) and phosphoglucoisomerase (cytoplasmic enzyme) were measured in the homogenates and isolated mitochondria. Prior to the measurement of these enzyme activities, the initial tissue homogenate used to prepare mitochondria as well as the final mitochondrial suspension were sonicated after the addition of Triton X-100 (final concentration 0.1%). Arginine kinase, citrate synthase and phosphoglucoisomerase were assayed according to the methods described in Chamberlin (1994) and Chamberlin *et al.*

(1997). Homogenate and mitochondrial protein concentrations were measured by the method of Gornall *et al.* (1949).

#### Mitochondrial respiration

Respiration of isolated mitochondria was measured using a Clark-type electrode in a temperature-controlled (25 °C) chamber. One part mitochondrial suspension was added to nine parts reaction medium (1% bovine serum albumin, 120 mmol l<sup>-1</sup> KCl, 50 mmol l<sup>-1</sup> sucrose, 5.55 mmol l<sup>-1</sup> MgCl<sub>2</sub>, 0.55 mmol l<sup>-1</sup> sodium malate, 0.11 mmol l<sup>-1</sup> palmitoyl carnitine, 10 mmol l<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub>, 10 mmol l<sup>-1</sup> Hepes, pH 7.2). Respiration was stimulated by the addition of ADP (State 3 rate) or ATP plus arginine. The concentrations of the stock solutions of ADP, ATP and arginine were measured spectrophotometrically. Data were recorded using a computer data-acquisition system (Sable Datacan V).

#### Arginine kinase activity in respiring and non-respiring mitochondria

In order to use arginine-stimulated respiration as a measure of AK activity in respiring mitochondria, the AK reaction must be rate-limiting for oxygen consumption. If this is the case, then the arginine phosphate:O<sub>2</sub> ratio will not exceed the ADP:O<sub>2</sub> ratio. The arginine phosphate:O<sub>2</sub> ratio was determined by measuring the amount of arginine phosphate produced and oxygen consumed after 5 mmol l<sup>-1</sup> arginine had been added to the mitochondria in reaction medium containing various concentrations of ATP. After the addition of arginine, the oxygen consumption was monitored for 3–5 min. The chamber was then opened and a 250 µl sample of the suspension was added to 90 µl of 20% perchloric acid. The acidified sample was then neutralized, frozen, and subsequently analyzed for arginine phosphate as described above. The ADP:O<sub>2</sub> ratio was determined in each preparation by measuring the amount of oxygen consumed after a sample of known ADP concentration had been added to the respiration chamber.

The velocities of the AK reaction at different ATP or arginine concentrations were determined in respiring and non-respiring mitochondria. For respiring mitochondria, the organelles were suspended in reaction medium containing various concentrations of ATP, and respiration was stimulated by the addition of 4.6 mmol l<sup>-1</sup> arginine. The arginine phosphate:O<sub>2</sub> ratio did not differ from the ADP:O<sub>2</sub> ratio (see Results) and, therefore, AK activity could be calculated from the arginine-stimulated rate of respiration. Because there is a respiration rate in the presence of ATP alone, it was necessary to subtract this ATP-dependent rate from the total stimulated rate (with ATP and arginine present). This corrected rate of respiration (the arginine-stimulated rate) was converted to an enzymatic activity by multiplying by the ADP:O<sub>2</sub> ratio determined in the same mitochondrial preparations. The activity of AK in non-respiring mitochondria was measured spectrophotometrically. The assay solution consisted of mitochondrial reaction medium to which was added 2 mmol l<sup>-1</sup> phosphoenolpyruvate, 0.2 mmol l<sup>-1</sup> NADH, 2 µg ml<sup>-1</sup> oligomycin, 10 µmol l<sup>-1</sup> atractyloside, excess pyruvate kinase

and excess lactate dehydrogenase. The change of absorbance at 340 nm was monitored after the sequential addition of mitochondria, various concentrations of ATP and 4.6 mmol l<sup>-1</sup> arginine.

The arginine-dependence of the AK reaction was determined in respiring mitochondria by suspending mitochondria in reaction medium containing 0.88 mmol l<sup>-1</sup> ATP. Respiration was stimulated by the addition of different concentrations of arginine. The assay for non-respiring mitochondria was similar to that described above except that 3 mmol l<sup>-1</sup> ATP was present and arginine concentrations were varied.

#### Mass action ratio

To determine whether the AK reaction in isolated mitochondria is displaced from equilibrium in respiring mitochondria, the mass action ratio ([ATP][arginine]/[ADP][arginine phosphate]) was compared with the  $K'_{eq}$  determined in the same mitochondrial preparations. Mitochondria were prepared as described above, but were diluted fivefold in isolation medium before the beginning of the experiment. Reaction medium containing 1 mmol l<sup>-1</sup> ATP, 0.5 mmol l<sup>-1</sup> ADP, 5 mmol l<sup>-1</sup> arginine and 1 mmol l<sup>-1</sup> arginine phosphate was equilibrated to 25 °C, and the mitochondrial suspension was added so that the mitochondria were diluted a further tenfold. 1, 5 and 10 min after the addition of the mitochondria, a sample of the suspension was acidified, neutralized and frozen as described above.  $K'_{eq}$  was determined by adding mitochondria to reaction medium containing 2 µg ml<sup>-1</sup> oligomycin and 10 µmol l<sup>-1</sup> atractyloside, and samples were taken 1, 5 and 10 min after the addition of mitochondria. Furthermore, 12 min after the addition of the mitochondria, 1 mmol l<sup>-1</sup> arginine phosphate was added, and samples were taken 1, 5 and 10 min later. ATP, ADP, arginine phosphate and arginine levels were measured as described above. Preliminary studies that involved pelleting the mitochondria and removing the supernatant prior to perchloric acid extraction showed insignificant concentrations of ATP, ADP, arginine and arginine phosphate in the mitochondria.

#### Calculations and statistics

Values are expressed as means ± S.E.M., with *N* indicating the number of midguts or mitochondrial preparations measured. Statistical analyses were conducted using analysis of variance (ANOVA) and, where appropriate, a Tukey's *post-hoc* test.  $P \leq 0.05$  was considered to represent a significant difference.

## Results

ATP, arginine and arginine phosphate levels in the midgut were 1.26 ± 0.25 µmol g<sup>-1</sup> (*N*=9), 3.76 ± 0.48 µmol g<sup>-1</sup> (*N*=13) and 0.45 ± 0.07 µmol g<sup>-1</sup> (*N*=13), respectively. The same, low arginine phosphate:arginine ratio was detected in preliminary studies in which midguts were incubated in oxygenated saline

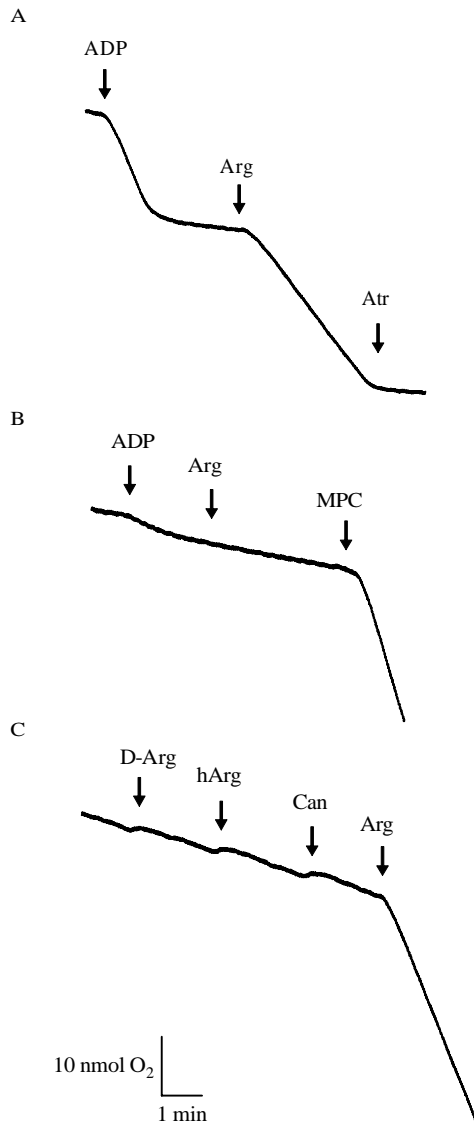


Fig. 1. Respiration of isolated midgut mitochondria ( $0.38 \text{ mg protein ml}^{-1}$ ). (A) State 3 respiration was initiated by the addition of  $84 \text{ nmol}$  of ADP to mitochondria suspended in reaction medium (see text for composition). After State 4 respiration had been achieved,  $5 \text{ mmol l}^{-1}$  L-arginine (Arg) was added and respiration was subsequently inhibited by the addition of  $10 \mu\text{mol l}^{-1}$  atractyloside (Atr). (B) The effects of adding  $168 \text{ nmol}$  of ADP to mitochondria suspended in modified reaction medium, which lacked palmitoyl carnitine and malate.  $5 \text{ mmol l}^{-1}$  L-arginine and  $5 \text{ mmol l}^{-1}$  malate plus  $100 \mu\text{mol l}^{-1}$  palmitoyl carnitine (MPC) were subsequently added. (C) The effects of adding  $5 \text{ mmol l}^{-1}$  D-arginine (D-Arg), homoarginine (hArg), canavanine (Can) and L-arginine on the respiration of mitochondria suspended in reaction medium containing  $0.85 \text{ mmol l}^{-1}$  ATP.

prior to freezing and extraction (data not shown). This indicates that the measured low level of arginine phosphate in freshly dissected midguts is not caused by anoxic conditions, which might arise during the dissection process.

The addition of ADP initiates a rapid increase in State 3

Table 1. Enzyme activities in mitochondria and homogenates of posterior midguts

	Activity ( $\text{nmol min}^{-1} \text{ mg}^{-1}$ homogenate)		Ratio (mitochondria/ homogenate)
	Mitochondria	Homogenate	
Arginine kinase	$716.1 \pm 13.6$	$1099.0 \pm 50.0$	$0.65 \pm 0.026$
Citrate synthase	$619.0 \pm 25.1$	$135.9 \pm 7.2$	$4.57 \pm 0.18$
Phosphoglucosomerase	$9.3 \pm 0.4$	$108.3 \pm 4.1$	$0.087 \pm 0.0069$

Values are means (S.E.M.,  $N=4$ ).

respiration (Fig. 1A), which returns to a low rate of oxygen consumption when all the ADP has been phosphorylated (State 4; Fig. 1A). The State 3 respiration rate of mitochondria in reaction medium is  $125.9 \pm 6.0 \text{ nmol O}_2 \text{ min}^{-1} \text{ mg}^{-1}$  protein ( $N=24$ ) and the midgut mitochondria are well coupled, as indicated by a high respiratory control ratio (State 3/State 4) of  $22.6 \pm 2.2$  ( $N=22$ ). When arginine is added to mitochondria that have synthesized ATP, an increase in respiration rate is observed (Fig. 1A), but the respiration rate does not return to the State 4 rate because ADP is regenerated by the mitochondrial AK reaction. The addition of atractyloside, an inhibitor of the ANT, inhibits the arginine-stimulated respiration (Fig. 1A). These results indicate that there is AK in the mitochondria and that the enzyme is outside the matrix. Arginine itself is not oxidized (Fig. 1B), and analogs of L-arginine (homoarginine, canavanine, D-arginine) do not stimulate mitochondrial respiration in the presence of ATP (Fig. 1C). The presence of mitochondrial AK is confirmed by the data presented in Table 1 in which the enzymatic activity of AK is detected in isolated mitochondria. The presence of AK in the mitochondria is not due to cytosolic contamination, as indicated by the extremely low activity of the cytosolic enzyme phosphoglucosomerase in the mitochondrial preparation (Table 1).

The ATP-dependent respiration rate was the same at all ATP concentrations used (Fig. 2A). Both the total stimulated rate (the ATP-dependent rate plus that produced by the addition of arginine) and the arginine-stimulated respiration rate (the total stimulated rate minus the ATP-dependent rate) saturate at ATP concentrations at or above  $0.8 \text{ mmol l}^{-1}$  (Fig. 2A). The total stimulated rate never reaches the State 3 respiration rate (Fig. 2B). There is no difference between the arginine phosphate: $\text{O}_2$  ratio and the ADP: $\text{O}_2$  ratio (Fig. 3). The results presented in Figs 2 and 3 indicate that the rate of ADP production by the AK reaction does not exceed the rate of ADP transport by the adenine nucleotide translocator. Therefore, it was valid to use the arginine-stimulated respiration rate to calculate AK activity in respiring mitochondria as described in Materials and methods. The activity of AK in respiring and non-respiring mitochondria does not differ at several ATP (Fig. 4) or arginine (Fig. 5) concentrations.

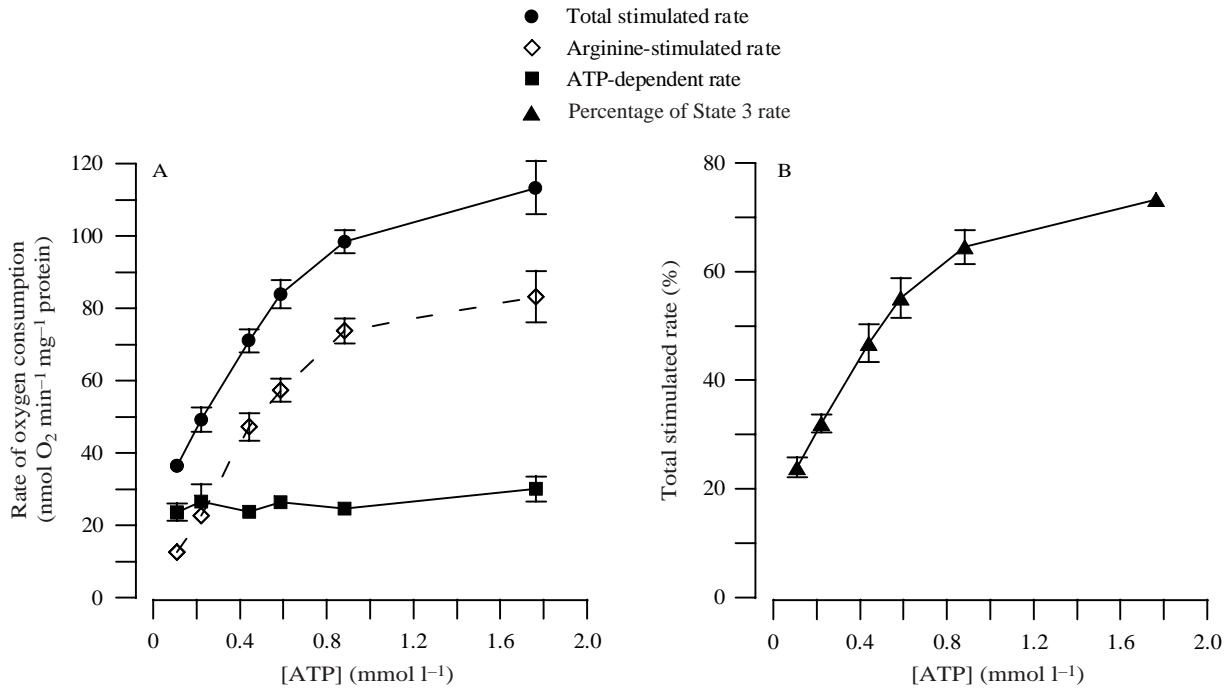


Fig. 2. (A) Rate of oxygen consumption after 4.6 mmol l<sup>-1</sup> arginine had been added to mitochondria in reaction medium containing ATP (total stimulated rate). The arginine-stimulated rate was calculated by subtracting the ATP-dependent rate from the total stimulated rate. (B) The total stimulated rate as a percentage of the State 3 rate determined for the same mitochondrial preparations. Values are means  $\pm$  S.E.M. ( $N=6$ ).

Ten minutes after the addition of mitochondria to reaction medium containing oligomycin and atractyloside,  $K'_{eq}$  is  $17.5 \pm 0.7$  ( $N=6$ ). When arginine phosphate is added to displace the equilibrium, the mass action ratio moves towards the  $K'_{eq}$  (Fig. 6A). The mass action ratio in respiring mitochondria is

higher than the  $K'_{eq}$ . Under respiring conditions, arginine phosphate continues to be produced (Fig. 6B), as would be expected if the AK reaction were driven by the relative substrate concentrations in bulk solution.

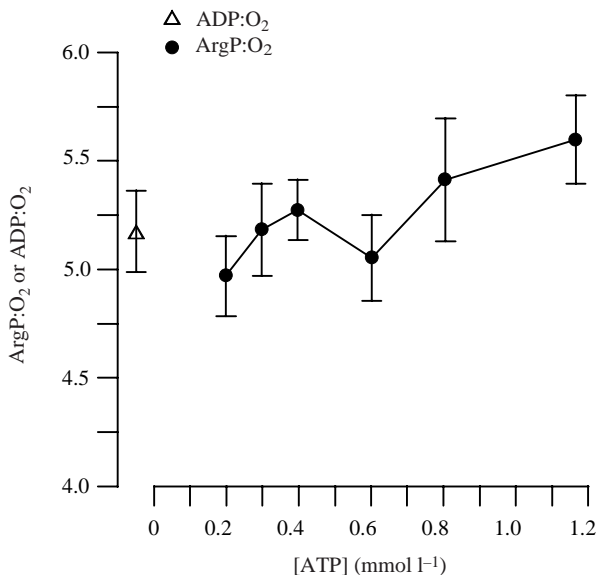


Fig. 3. The arginine phosphate:O<sub>2</sub> ratio in isolated midgut mitochondria at different ATP concentrations. The ADP:O<sub>2</sub> ratio was determined by monitoring oxygen consumed after the addition of 164 nmol of ADP. Values are means  $\pm$  S.E.M. ( $N=6$ ). ArgP, arginine phosphate.

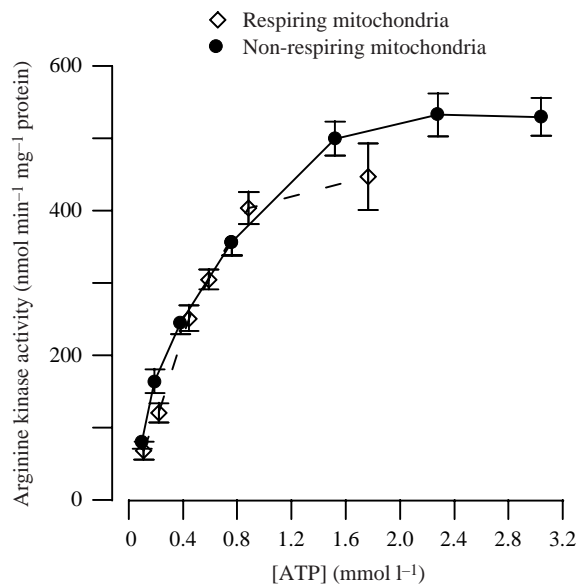


Fig. 4. Arginine kinase activity in respiring and non-respiring mitochondria at different ATP concentrations. The arginine kinase activity in respiring mitochondria was calculated from the arginine-stimulated respiration rate (Fig. 2A) as explained in the text. Values are means  $\pm$  S.E.M.,  $N=6$ .

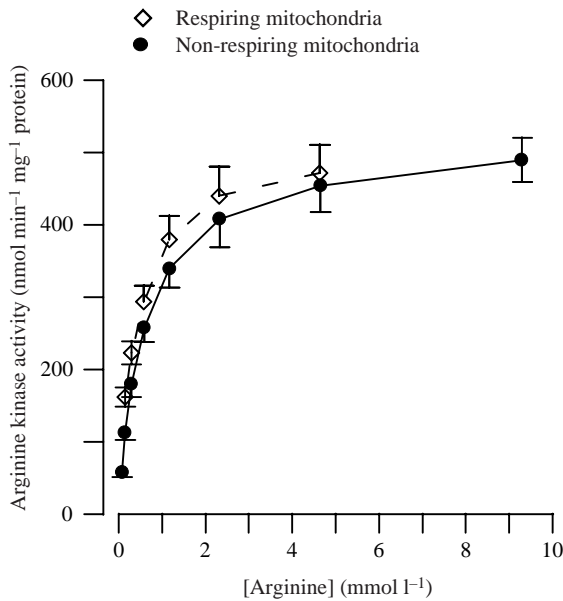


Fig. 5. Arginine kinase activity in respiring and non-respiring mitochondria at different arginine concentrations. Values are means  $\pm$  S.E.M.,  $N=6$ .

### Discussion

Mitochondrial AK has been detected in crustaceans (Chen and Lehninger, 1973; Hird and Robin, 1985; Ellington and Hines, 1991) and Merostomata (Doumen and Ellington, 1990a). A search for a mitochondrial form of the enzyme in insects has been unsuccessful (Ellington and Hines, 1991; Schneider *et al.* 1989; Wyss *et al.* 1995), although Munneke and Collier (1988) claimed to have detected insect mitochondrial AK by assaying whole-body homogenates of *Drosophila melanogaster*. Their study, however, did not check for cytosolic contamination of mitochondrial AK, and Wyss *et al.* (1995) could not replicate Munneke and Collier's (1988) findings. Furthermore, Wyss *et al.* (1995) showed that *Drosophila* muscle contains no mitochondrial AK. The present study clearly demonstrates the presence of a functional AK in insect mitochondria. Although there is a small amount of cytosolic contamination in the midgut mitochondrial preparation, this contamination cannot account for all the AK activity present in the mitochondrial fraction. Although experiments were not specifically designed to determine what fraction of the total AK pool is mitochondrial, an estimate can be made from the study with marker enzymes. Citrate synthase is enriched 4.6-fold by the isolation procedure, and therefore the same enrichment of mitochondrial AK would be expected if it is assumed that citrate synthase and mitochondrial AK are not differentially affected by the isolation procedure. A 4.6-fold enrichment of mitochondrial AK at an activity of  $156 \text{ nmol min}^{-1} \text{ mg}^{-1}$  cellular protein would yield the observed AK activity in isolated mitochondria ( $716.1 \text{ nmol min}^{-1} \text{ mg}^{-1}$  mitochondrial protein).  $156 \text{ nmol min}^{-1} \text{ mg}^{-1}$  cellular protein is 14% of the total AK pool ( $1099.0 \text{ nmol min}^{-1} \text{ mg}^{-1}$  cellular protein). In contrast, mitochondrial AK in *Limulus*

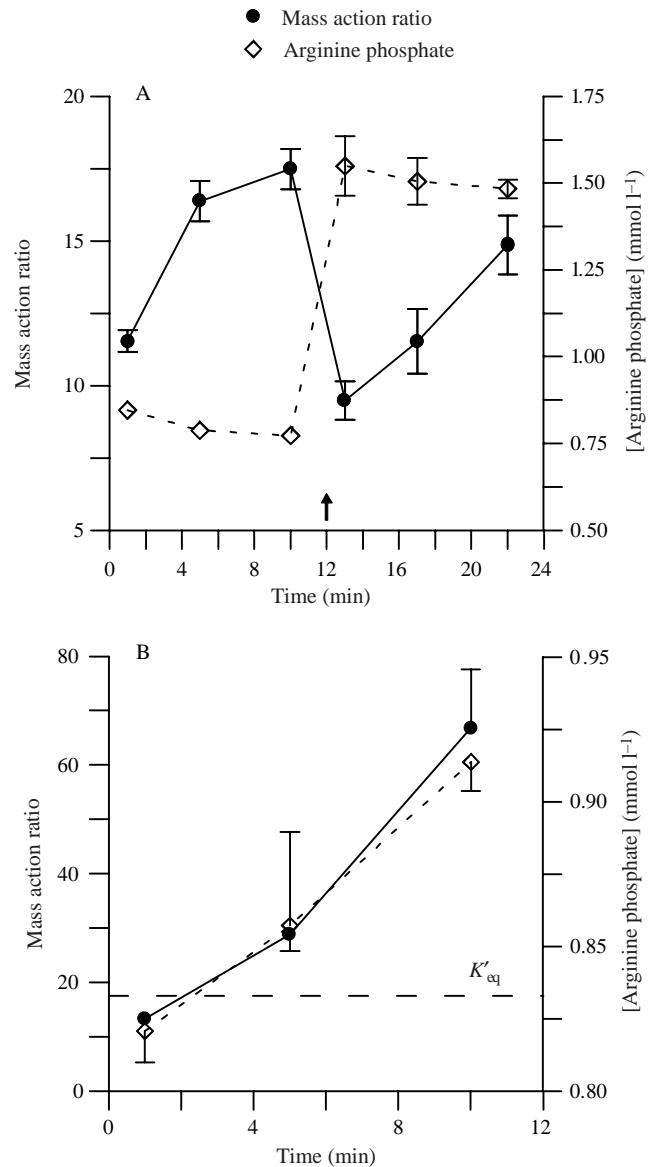


Fig. 6. The mass action ratio and arginine phosphate concentration of mitochondria added at time zero to reaction medium containing arginine, ATP, arginine phosphate and ADP. (A) Mitochondria poisoned with oligomycin and atractyloside.  $1 \text{ mmol l}^{-1}$  arginine phosphate was added at the time indicated by the arrow. (B) Respiring mitochondria. The dashed line represents the apparent equilibrium constant ( $K'_{eq}$ ) determined at 10 min in A. Values are means  $\pm$  S.E.M.,  $N=6$ .

heart is only 1% of the cytoplasmic AK activity (Doumen and Ellington, 1990a).

The location of midgut AK within the mitochondria can be inferred from the respiration experiments. Atractyloside inhibits the arginine-stimulated respiration in isolated mitochondria, indicating that ADP must be transported across the inner mitochondrial membrane to stimulate respiration. Therefore, mitochondrial AK must be located outside the matrix, but these studies do not permit localization to the

intermembrane space, to the outer face of the inner membrane or to either side of the outer membrane.

The mitochondrial AK in the tobacco hornworm midgut appears to be specific to L-arginine since the D-isomer as well as analogs of arginine failed to stimulate mitochondrial respiration in the presence of ATP. This finding is supported by the work of Rosenthal *et al.* (1977) in which it was demonstrated that AK isolated from entire tobacco hornworms could phosphorylate canavanine, but the  $K_m$  for canavanine was  $22 \text{ mmol l}^{-1}$ , 44 times that for arginine. Similarly, Gindling *et al.* (1995) showed that AK purified from *Heliothis virescens* midgut showed only modest activity with canavanine or homoarginine.

The kinetic variables of the midgut AK reaction were unaffected by oxidative phosphorylation. Similarly, Doumen and Ellington (1990b) demonstrated that the  $K_m$  for ATP and arginine of *Limulus polyphemus* AK was the same in the presence or absence of mitochondrial respiration. These findings indicate that, unlike the mitochondrial CK system in mammals, the AK of these arthropod mitochondria does not have preferred access to ATP transported across the inner mitochondrial membrane.

Using a thermodynamic approach that compared the  $K'_{eq}$  with the mass action ratio of the AK reaction, it is clear that the AK reaction is displaced from equilibrium in respiring midgut mitochondria. This probably reflects an AK activity that is low relative to the rate of mitochondrial ATP synthesis. Because arginine phosphate was synthesized when the mass action ratio exceeded the  $K'_{eq}$ , it appears that the midgut AK reaction is governed by the thermodynamic properties of the bulk solution. This provides further evidence that the reaction is not restricted to some microenvironment in the intermembrane space, such as the vicinity of ANT. This is similar to the observation of Doumen and Ellington (1990b), who found no evidence supporting compartmentation of the *Limulus polyphemus* enzyme. Given that *Manduca sexta* and *Limulus polyphemus* are widely divergent arthropods, perhaps a lack of coupling with ANT may be a general characteristic of arthropod mitochondrial AKs.

The role of mitochondrial AK in the midgut is still uncertain. The tobacco hornworm midgut actively transports ions at very high rates (reviewed by Dow, 1986), and energy for this process is provided by aerobic metabolism (Chamberlin, 1987). The small diffusion distances between mitochondrial and plasma membrane ion-motive ATPases in the midgut (Cioffi, 1984) would seem to preclude the need for an energy shuttle system in this tissue. Nevertheless, CK is often associated with  $\text{Na}^+/\text{K}^+$ -ATPase in vertebrate epithelia, and evidence for a phosphocreatine circuit has been provided for vertebrate epithelia such as elasmobranch rectal gland, mammalian nephron (Wallimann and Hemmer, 1994) and teleost gill (Kültz and Somero, 1995). Additional experiments will be required to determine whether arginine phosphate hydrolysis is directly involved in supporting active ion transport in the tobacco hornworm midgut.

Another function of the AK system would be to decrease the free energy required for mitochondrial ATP synthesis by

maintaining a low [ATP]/[ADP] ratio in the vicinity of the adenine nucleotide translocase. Doumen and Ellington (1990b) have argued that functional coupling of AK and the adenine nucleotide translocase is not an absolute prerequisite for increasing the efficiency of oxidative phosphorylation. Simply by having the kinase in the vicinity of the site of ATP production, a low [ATP]/[ADP] ratio near the translocase will be maintained, resulting in a high rate of adenylate exchange even in the face of a high [ATP]/[ADP] ratio in the cytosol. Furthermore, converting the ATP that has just been transported out of the matrix into arginine phosphate would lower the free energy of ATP synthesis and increase the efficiency of oxidative phosphorylation. This role for mitochondrial AK in the tobacco hornworm midgut remains to be tested. Interestingly, adult *Manduca sexta* flight muscle, a tissue with a very high metabolic rate, does not have mitochondrial AK (Ellington and Hines, 1991). It is difficult to speculate why the midgut has mitochondrial AK whereas the flight muscle does not. It is interesting to note, however, that unlike flight muscle, with its intermittent periods of high metabolic demand, tissues in which mitochondrial AK is found are those that work continuously such as pumping hemolymph (*Limulus polyphemus* heart, Doumen and Ellington, 1990a,b; crayfish heart, Ellington and Hines, 1991) or digesting nutrients and transporting solutes (*Callinectes sapidus* hepatopancreas, Chen and Lehninger, 1973; insect midgut; the present study). The mitochondrial AK in these tissues may facilitate the efficient synthesis of ATP over long periods of energy demand.

Midgut AK could be a component of a temporal energy buffering system. The ATP levels in the tobacco hornworm midgut measured in the present study agree with values reported previously (Mandel *et al.* 1980). Although the arginine phosphate levels are lower than those of ATP, arginine phosphate could provide ATP for a brief period should oxidative phosphorylation fail to meet the metabolic demands of the tissue. The rate of oxygen consumption of the midgut tissue is  $2.2 \text{ nmol O}_2 \text{ min}^{-1} \text{ mg}^{-1} \text{ tissue}$  (Chamberlin *et al.* 1997) and, assuming an ADP:O<sub>2</sub> ratio of 5.2 (Fig. 3),  $0.45 \text{ } \mu\text{mol g}^{-1}$  arginine phosphate would provide ATP for 2.4 s. Normally, the caterpillar feeds constantly and the midgut is always filled with food. Under these conditions, the midgut would not experience wide swings in metabolic demand (e.g. post-prandial digestion and absorption). During molting, however, the gut is purged and therefore the first meal after ecdysis may place a brief but severe metabolic demand on the tissue. It may be during this period that the role of AK in temporal energy buffering may come into play.

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