Changes in Intramitochondrial Adenine Nucleotides in Blowfly Flight-Muscle Mitochondria

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1. With freshly isolated blowfly mitochondria 38% of the intramitochondrial adenine nucleotide was present as AMP. 2. On incubation with oxidizable substrates the AMP and ADP concentrations fell and that of ATP rose; with pyruvate together with proline the ATP concentration reached its maximum value at 6 min; with glycerol phosphate the phosphorylation of endogenous nucleotide was more rapid. 3. Addition of the uncoupling agent carbonyl cyanide phenylhydrazone caused a rapid fall of ATP and a parallel rise in ADP, then ADP was converted into AMP. 4. This was in contrast with rat liver mitochondria endogenous AMP concentrations, which were always lower than those of blowfly mitochondria and changed little under different metabolic conditions. 5. Evidence is presented that adenylate kinase (EC 2.7.4.3) has a dual distribution in blowfly mitochondria, a part being located in the matrix space and a part in the space between the outer and inner mitochondrial membranes, as in liver and other mitochondria. 6. The possible regulatory role of changing AMP concentrations in the mitochondrial matrix was investigated. Partially purified pyruvate carboxylase (EC 6.4.1.1) and citrate synthase (EC 4.1.3.7) were inhibited 30% by 2 mM-AMP, whereas pyruvate dehydrogenase (EC 1.2.4.1) was unaffected. 7. AMP activated the NAD⁺-linked isocitrate dehydrogenase (EC 1.1.1.41) activity of blowfly mitochondria in the absence of ADP, but in the presence of ADP, AMP caused inhibition. 8. It is suggested that AMP may exert a controlling effect on the oxidative activity of blowfly mitochondria.

Sacktor & Hurlbut (1966) reported that the concentration of AMP in blowfly (*Phormia regina*) flight muscle *in vivo* at rest was very low (0.15 μ mol/g wet wt.). Within 5 s of the initiation of flight the AMP concentration increased 2.5-fold and after 1 min a steady concentration twice that in resting muscle was reached. Sacktor & Wormser-Shavit (1966) measured the intermediates of carbohydrate metabolism in flight muscle and found that the changes were consistent with phosphofructokinase (EC 2.7.1.11) being activated by the increased AMP and ADP concentrations. It has also been reported that AMP activates phosphorylase (EC 2.4.1.1) in *Phormia regina* flight muscle (Childress & Sacktor, 1970).

AMP is metabolized by the enzyme adenylate kinase (EC 2.7.4.3); this enzyme has been reported in flight muscle (Sacktor, 1952), but its localization has not been studied. In rat liver there is an adenylate kinase in the cytoplasm (Chappell, 1968) and one between the inner and outer mitochondrial membranes (Schnaitman & Greenawalt, 1968).

In the present paper the phosphorylation state of endogenous adenine nucleotides of blowfly flight-muscle mitochondria was investigated and a limited investigation of the adenine nucleotide carrier was made. The phosphorylation state of endogenous

adenine nucleotides and the localization of adenylate kinase in rat liver and flight-muscle mitochondria were compared.

Experimental

Materials

Enzymes, coenzymes and adenine nucleotides were obtained from Boehringer Corp. (London) Ltd., London W.5, U.K. [¹⁴C]Bicarbonate and H₃³²PO₄ were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Atractyloside, bovine serum albumin and firefly lantern extract were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. Triton X-100 was obtained from BDH Chemicals Ltd., Poole, Dorset, U.K.

Methods

Mitochondria. Mitochondria were prepared from the flight muscles of *Calliphora* blowflies by the proteinase method described by Chappell & Hansford (1972). Rat liver mitochondria were also prepared as described by these authors.

Measurement of radioactivity. A toluene-based scintillation solution (Brand *et al.*, 1973) was used for counting ¹⁴C radioactivity. The Čerenkov method

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was used for counting ^{32}P . The samples were assayed in a Packard Tri-Carb liquid-scintillation counter with internal standard.

Labelling of endogenous adenine nucleotides and their separation and assay. The principle employed was that described by Pfaff & Klingenberg (1968). A concentrated suspension of mitochondria is incubated with exogenous ^{14}C -labelled adenine nucleotide for a relatively long period. Owing to the actions of the adenine nucleotide translocase and to enzymic exchange reactions catalysed by, for example, the mitochondrial ATPase* and adenylate kinase, the internal adenine nucleotides are equally labelled (Rafael *et al.*, 1972). Exogenous adenine nucleotides are then removed by washing by centrifugation and resuspension. In these experiments mitochondria were incubated with $2.5\mu\text{Ci}$ of $[8\text{-}^{14}\text{C}]\text{ADP}$ at 0°C for 40 min and then the mitochondria were washed twice with isolation medium {250 mM-sucrose–1 mM-EGTA [ethanedioxybis(ethylamine)tetra-acetic acid]–5 mM-Tris–HCl, pH 7.7} at 0°C and then sedimented by centrifuging for 5 min at 9000 rev./min in an MSE 18 centrifuge. The labelled mitochondria were then incubated in a medium containing 130 mM-KCl, 10 mM-Tris–HCl, 25 mM-potassium phosphate and oxidizable substrates as indicated in the text. The pH was 7.4 and the temperature 25°C . Samples ($400\mu\text{l}$) were taken at intervals and added to a HClO_4 solution so that the final concentration of acid was 2% (w/v). Samples were centrifuged for 2 min in an Eppendorf 3200 centrifuge (approx. 15000g) and the supernatants ($200\mu\text{l}$) chromatographed on Bio-Rad 1 X8 ion-exchange resin (200–400 mesh, Cl^- form) to separate ATP, ADP and AMP as described by Heldt *et al.* (1972). The fractions were evaporated to dryness in glass scintillation bottles at 70°C and the residue was taken up in $150\mu\text{l}$ of 6% (w/v) HClO_4 followed by 15 ml of scintillation fluid. The composition of the adenine nucleotides with respect to ATP, ADP and AMP was calculated for each nucleotide as a percentage of the total radioactivity eluted from the column.

Phosphorylation of endogenous ATP. Mitochondria were incubated in a medium containing 130 mM-KCl, 10 mM-Tris–HCl, pH 7.4, 0.05 mM-potassium $[\text{}^{32}\text{P}]\text{phosphate}$ and an oxidizable substrate at 25°C . Samples ($400\mu\text{l}$) were taken at various time-intervals and added to HClO_4 solution to a final concentration of 2% (w/v) of the acid. The samples were centrifuged in an Eppendorf 3200 centrifuge for 2 min and $200\mu\text{l}$ of supernatant was treated with $10\mu\text{l}$ of 10% (w/v) ammonium molybdate solution and then extracted with 1 ml of 2-methylpropan-1-ol–benzene (1:1, v/v). The distribution of radioactivity in the aqueous (ADP, ATP) and in the organic (phosphate) phases was then determined as described by Heldt *et al.* (1972).

* Abbreviation: ATPase, adenosine triphosphatase.

Exchange of adenine nucleotides. Mitochondria (approx. 8 mg of protein) loaded with ^{14}C -labelled adenine nucleotides, as described above, were incubated with unlabelled adenine nucleotides in a medium containing 130 mM-KCl, 10 mM-Tris–HCl, 5 mM-EDTA and $16\mu\text{g}$ of rotenone at pH 7.2 and at 1.5°C . Samples ($250\mu\text{l}$) were taken at various time-intervals and further transport of adenine nucleotides was stopped by the addition of $50\mu\text{l}$ of an atractylo-side solution to give a final concentration of $170\mu\text{M}$. The pellet and supernatant fractions were individually extracted with HClO_4 and counted for radioactivity. The percentage exchange was calculated by the method of Pfaff & Klingenberg (1968).

Luciferase assays for ATP. The method of Stanley & Williams (1969) was used. For each sample, 2 ml of buffer containing 30 mM-sodium arsenate, 15 mM- MgCl_2 and 7 mM-potassium phosphate at pH 7.4 was used and $30\mu\text{l}$ of dissolved firefly extract was added.

Enzyme assays. Adenylate kinase was assayed in the direction $\text{AMP} + \text{ATP} \rightarrow 2\text{ADP}$ by using pyruvate kinase (Bergmeyer, 1965) and in the presence of oligomycin to inhibit ATPase activity. AMP was also assayed by using this method. NAD^+ -linked isocitrate dehydrogenase (EC 1.1.1.41) was assayed by the method of Hansford (1972).

Pyruvate carboxylase was assayed in mitochondria that had been frozen and thawed three times in liquid N_2 . Rotenone ($2.5\mu\text{g}$), 1 mM-NADH, 5 units of malate dehydrogenase and 0.1 ml of disrupted mitochondria were added to 0.5 ml of a medium containing 50 mM-Tris–HCl, 5 mM- MgCl_2 , 2 mM-ATP, 5 mM-potassium pyruvate, 10 mM- $\text{KH}^{14}\text{CO}_3$ at a final pH of 7.4 at 25°C . The reaction was stopped after various time-intervals with 0.1 ml of 5 M-HCl. The sample ($600\mu\text{l}$) was spun in an Eppendorf 3200 centrifuge and a portion ($500\mu\text{l}$) was dried and counted for radioactivity. Citrate synthase was assayed by the method of Srere *et al.* (1963). Pyruvate dehydrogenase was assayed by the method of Coore *et al.* (1971). Malate dehydrogenase was assayed by the method of Ochoa (1955).

Protein. This was measured by a modified biuret method (Gornall *et al.*, 1949), with bovine serum albumin as the standard.

Results

Phosphorylation state of adenine nucleotides in the matrix

Fig. 1(a) shows the changes in the phosphorylation state of intramitochondrial adenine nucleotides in flight-muscle mitochondria with time, in the presence of 2.2 mM-potassium pyruvate and 2.2 mM-proline, i.e. State 4; where the rate of respiration is limited by lack of phosphate acceptor (Chance &

Fig. 1. Phosphorylation state of intramitochondrial adenine nucleotides in flight-muscle mitochondria

Mitochondrial protein (27mg) was added to a medium containing 130mM-KCl, 10mM-Tris-HCl, pH 7.2, 2.2mM-potassium pyruvate, 2.2mM-proline and 25mM-potassium phosphate. Carbonyl cyanide phenylhydrazone was added at the arrow to a final concentration of 10 μ M. The temperature was 25°C. (a) ●, AMP; ○, ADP; ▲, ATP; (b) ▲, ATP/ADP ratio.

Fig. 2. Phosphorylation state of intramitochondrial adenine nucleotides in rat liver mitochondria

Mitochondrial protein (24mg) was added to a medium containing 130mM-KCl, 10mM-Tris-HCl, pH 7.2, 7.6mM potassium phosphate, 7mM-potassium glutamate, 7mM potassium malate and 2.5mM-EDTA. Carbonyl cyanide phenylhydrazone was added at the arrow to a final concentration of 10 μ M. The temperature was 25°C. ●, AMP; ○, ADP; ▲, ATP.

Williams, 1955). It can be seen that as prepared these mitochondria contain a significant amount of AMP, amounting to 38% of the total intramitochondrial adenine nucleotides. This high proportion of AMP was confirmed by enzymic assay, as described under 'Methods'.

In the presence of substrate the ATP concentration increased with time, but whereas the ATP was formed initially from both ADP and AMP, after 0.5min the ADP concentration remained constant whereas that of AMP decreased until it accounted for 13% of the total adenine nucleotide. The addition of uncoupler (10 μ l of carbonyl cyanide phenylhydrazone) caused the rapid conversion of ATP into ADP, but then ADP was converted into AMP by a 1 mol for 1 mol conversion.

Fig. 1(b) shows the change in the ATP/ADP ratio with time. It can be seen that initially this ratio was low (0.55), but it increased steadily to a maximum value of 2.6 after 6 min.

Fig. 2 shows a similar experiment with rat liver mitochondria, with glutamate (7mM) and malate (7mM) as substrates. This method gave the same changes in intramitochondrial adenine nucleotides found by Heldt *et al.* (1972). After 1min the

ATP/ADP ratio had reached a maximum of 4.0, whereas that in flight-muscle mitochondria was only 1.9 after 1 min.

Phosphorylation of ATP

The gradual formation of ATP in flight-muscle mitochondria in State 4 was confirmed by measuring the synthesis of ATP by using potassium [32 P]phosphate. Fig. 3 shows the rate of formation of ATP by using this method with (1) pyruvate (2.9mM) and proline (2.9mM) and (2) glycerol phosphate (2.5mM) with a Ca^{2+} -EGTA buffer giving $40\mu\text{M}$ of free Ca^{2+} (to activate glycerol phosphate oxidation) (see Chappell & Hansford, 1967) as substrates. This confirms that with pyruvate and proline as substrates the ATP was formed as shown in Fig. 1(a), but with glycerol phosphate as substrate ATP was formed more rapidly.

ATP was also assayed directly by using luciferase. By this method it was shown that the addition of 3.5mM-EDTA had no effect on the rate of ATP formation.

Adenine nucleotide exchange

Table 1 shows the percentage exchange of exogenous ATP ($170\mu\text{M}$), ADP ($170\mu\text{M}$) and AMP

Fig. 3. Formation of ATP by using potassium [32 P]phosphate

Flight-muscle mitochondrial protein (18 mg) was incubated in a medium containing 130mM-KCl, 10mM-Tris-HCl, pH 7.2, and 0.4mM-potassium [32 P]phosphate at 25°C . Δ , 2.9mM-potassium pyruvate and 2.9mM-proline were added; $\blacksquare$, 2.5mM-glycerol phosphate with a Ca^{2+} -EGTA buffer (as described in the Results section) was added.

($170\mu\text{M}$) with labelled endogenous adenine nucleotides in flight-muscle mitochondria at 1.5°C . ADP and ATP could exchange with approx. 50% of the intramitochondrial adenine nucleotides, whereas AMP could only exchange with approx. 6%.

Localization of adenylate kinase

Malate dehydrogenase was used as a 'marker' enzyme for the mitochondrial matrix. The malate dehydrogenase activity measured by using whole mitochondria gives the percentage of broken mitochondria when compared with the activity measured in mitochondria lysed with Triton X-100 (final concn. 0.02%), which gives the total enzyme activity. Similarly the adenylate kinase activity measured in unlysed mitochondria was the sum of that outside the inner mitochondrial membrane and the activity derived from the matrix of the broken mitochondria.

Table 2 shows the malate dehydrogenase and adenylate kinase activities in rat liver and flight-muscle mitochondria in the presence and absence of Triton X-100. This demonstrated that adenylate kinase in rat liver mitochondria was localized outside the inner mitochondrial membrane as shown by Schnaitman & Greenawalt (1968). In flight-muscle mitochondria, accounting for the broken mitochondria, 48% of the mitochondrial adenylate kinase activity was in the matrix, the other 52% was outside the inner mitochondrial membrane. The same results were obtained in the presence of $20\mu\text{M}$ -atractyloside.

Effect of AMP on enzymic activity

Since there was a large percentage of AMP in flight-muscle mitochondria, AMP might control the activities of some enzymes in the mitochondrial matrix. Table 3 shows the effect of AMP on four enzymes. Pyruvate dehydrogenase, which does not

Table 1. Exchange of adenine nucleotides

Mitochondria (8.5mg of protein), loaded as explained under 'Methods', were incubated in a medium containing 130mM-KCl, 10mM-Tris-HCl, pH 7.2, 25mM-potassium phosphate, 0.1mg of rotenone and 6.2mM-EDTA at 1.5°C .

Time (s)	Percentage exchange with		
	AMP	ADP	ATP
0	0	0	0
5	0	37	29
10	0.3	39	20
15	2.6	48	42
20	6.4	49	23
25	6.2	48	33
30	6.2	44	23

Table 2. *Adenylate kinase activities*

Malate dehydrogenase and adenylate kinase were assayed in 2.5 ml of a medium containing 130mM-KCl and 10mM-Tris-HCl, pH 7.2, in the presence and absence of 0.5mg of Triton X-100 at 25°C. The values are quoted $\pm$ S.E.M. Numbers of mitochondrial preparations measured are given in parentheses.

	Malate dehydrogenase (μ mol/min per mg of protein)		Percentage of broken mitochondria
	Intact mitochondria	Mitochondria+Triton X-100	
Rat liver mitochondria	0.26 $\pm$ 0.05 (3)	3.27 $\pm$ 0.17 (3)	8
Flight-muscle mitochondria	0.9 $\pm$ 0.27 (4)	4.1 $\pm$ 1.4 (4)	22
	Adenylate kinase (μ mol/min per mg of protein)		Percentage of enzyme in the matrix
	Intact mitochondria	Mitochondria+Triton X-100	
Rat liver mitochondria	0.31 $\pm$ 0.03 (3)	0.33 $\pm$ 0.02 (2)	0
Flight-muscle mitochondria	0.22 $\pm$ 0.01 (4)	0.33 $\pm$ 0.02 (3)	48

Table 3. *Effect of AMP on some matrix enzyme activities*

The enzymes were assayed as described under 'Methods' at 25°C.

Enzyme	% inhibition with 2mM-AMP
Citrate synthase	32
Pyruvate dehydrogenase	0
Pyruvate carboxylase	30
Isocitrate dehydrogenase	0

(AMP inhibits ADP stimulation)

have a phosphorylated form in flight-muscle mitochondria (S. M. Danks & R. M. Denton, unpublished work), was unaffected by 2mM-AMP.

Although pyruvate carboxylase activity increased twofold in the presence of 200 μ M-acetyl-CoA, 2mM-AMP inhibited pyruvate carboxylase by 30% in the presence and absence of acetyl-CoA.

The activity of citrate synthase in flight-muscle mitochondria was high, 3.31 $\pm$ 0.20 μ mol/min per mg of mitochondrial protein at 30°C (mean of four determinations $\pm$ S.E.M.), and 2mM-AMP inhibited this enzyme by 32%. ADP and ATP also inhibited citrate synthase, the inhibition by adenine nucleotides being the same as that for the enzyme from adipose tissue (Halestrap, 1973).

Hansford (1972) reported that the activity of the flight-muscle mitochondrial NAD⁺-linked isocitrate dehydrogenase was dependent on the percentage of ADP in a mixture of ADP and ATP. A similar experiment was carried out with an ADP-AMP mixture to the same final total adenine nucleotide concentration of 5mM. Fig. 4 compares the activity of isocitrate dehydrogenase with a mixture of ADP and AMP to that with ADP alone. At high concentrations of AMP (5mM) isocitrate dehydrogenase was stimulated, but in a mixture of ADP and AMP, AMP inhibited the ADP stimulation of isocitrate dehydrogenase.

Fig. 4. *Effect of ADP or AMP plus ADP on NAD⁺-linked isocitrate dehydrogenase*

Sonicated mitochondria were assayed in 2ml of a medium containing 130mM-KCl, 10mM-Tris-HCl, pH 7.2, 25mM-potassium phosphate, 10mM-MgCl₂, 3.2mM-DL-isocitrate, 0.75mM-NAD⁺, 0.1mg of rotenone and 20 μ g of oligomycin. □, ADP plus AMP to a final concentration of 5mM were added; ○, ADP only was added. The temperature was 25°C.

Discussion

The intramitochondrial adenine nucleotides of blowfly flight-muscle mitochondria, as prepared, were 38% AMP (see Fig. 1a). This AMP cannot be transported into or out of the mitochondria by the adenine nucleotide carrier; this would indicate that the AMP was metabolized by an enzyme in the mitochondrial matrix. The large percentage of untransportable adenine nucleotide in the matrix would account for the fact that ADP and ATP do not exchange with 100% of the intramitochondrial adenine nucleotides.

The changes of the intramitochondrial adenine nucleotides in the presence of substrate (Fig. 1a) were consistent with the action of adenylate

kinase and ATPase. Since EDTA, which chelates Mg^{2+} [an activator of adenylate kinase (Rose, 1968)], had no effect on these changes, it was deduced that the adenylate kinase was within the matrix. This was confirmed by enzymic assay, which also demonstrated that there was some adenylate kinase activity between the inner and outer mitochondrial membranes.

An adenylate kinase in the mitochondrial matrix has not been previously reported, but the results obtained by Rafael *et al.* (1972) for brown-adipose-tissue mitochondria from newborn guinea pigs could be explained by an adenylate kinase in the matrix. These mitochondria also contained a large amount of AMP on preparation.

From the results presented here AMP apparently had no significant inhibitory effects on the enzymes examined except with NAD^+ -linked isocitrate dehydrogenase. The possibility that isocitrate dehydrogenase and glycerophosphate dehydrogenase were limited by lack of activators in the resting state of the fly was discussed by Hansford (1972). Under such conditions the AMP, and therefore the adenylate kinase in the matrix could be important since AMP has an inhibitory effect on isocitrate dehydrogenase.

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