Evidence for a Calcium-Ion-Transport System in Mitochondria Isolated from Flight Muscle of the Developing Sheep Blowfly *Lucilia cuprina*

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The EGTA [ethanedioxybis(ethylamine)tetra-acetic acid]–Ruthenium Red-quench technique (Reed & Bygrave, 1974a) was used to measure initial rates of Ca^{2+} transport in mitochondria from flight muscle of the blowfly *Lucilia cuprina*. Evidence is provided for the existence in these mitochondria of a Ca^{2+} -transport system that has many features in common with that known to exist in rat liver mitochondria. These include requirement for energy, saturation at high concentrations of Ca^{2+} , a sigmoidal relation between initial rates of Ca^{2+} transport and Ca^{2+} concentration, a high affinity for free Ca^{2+} (K_m approx. $5\mu\text{M}$) and high affinity for the Ca^{2+} -transport inhibitor, Ruthenium Red (approx. 0.03nmol of carrier-specific binding-sites/mg of protein; K_i approx. $1.6 \times 10^{-8}\text{M}$). Controlled respiration can be stimulated by Ca^{2+} after a short lag-period provided the incubation medium contains KCl and not sucrose. The ability of *Lucilia* mitochondria to transport Ca^{2+} critically depends on the stage of mitochondrial development; Ca^{2+} transport is minimal in mitochondria from pharate adults, is maximal between 0 and 2 h post-emergence and thereafter rapidly declines to reach less than 20% of the maximum value by about 2–3 days post-emergence. Respiration in mitochondria from newly emerged flies does not respond to added Ca^{2+} ; that from 3- to 5-day-old flies is stimulated approx. 50%. Whereas very low concentrations of Ca^{2+} inhibit ADP-stimulated respiration and oxidative phosphorylation in mitochondria from newly emerged flies (K_i approx. 60ng -ions of Ca^{2+} /mg of protein); much higher concentrations (approx. 200ng -ion/mg of protein) are needed to inhibit these processes in those from older flies. The potential of this system for studying the function and development of metabolite transport systems in mitochondria is discussed.

It has been argued that mitochondria from flight muscle of the blowfly *Phormia* do not possess the Ca^{2+} -transport system known to exist in mitochondria obtained from a range of tissues and species (Carafoli *et al.*, 1971). Failure to observe high-affinity Ca^{2+} -binding sites, failure of Ca^{2+} to saturate when initial rates of Ca^{2+} transport were measured as a function of Ca^{2+} concentration (Carafoli & Lehninger, 1971; Carafoli *et al.*, 1971) and failure of Ca^{2+} to both stimulate respiration and induce a reversible shift in the oxidation–reduction state of cytochrome *b* in these mitochondria (Carafoli *et al.*, 1971) together formed the basis of this argument.

We have determined the extent to which Ca^{2+} transport takes place in mitochondria from *Lucilia* flight muscle by using techniques that allow one to accurately measure initial rates of Ca^{2+} transport without interference from any external specific or non-specific Ca^{2+} binding (Reed & Bygrave, 1974a). We show here that mitochondria from flight muscle of *Lucilia* possess a transport system with high affinity for Ca^{2+} . This system is saturated at high concentra-

tions of Ca^{2+} and inhibited by concentrations of Ruthenium Red that inhibit Ca^{2+} transport in rat liver mitochondria. Our experiments also demonstrate that the ability of these mitochondria to transport Ca^{2+} is critically dependent on the stage of insect metamorphosis and thus of mitochondrial development.

Experimental

Maintenance of flies

Flies were obtained from the Division of Entomology, CSIRO, Canberra, at pupariation or shortly before, where they had been reared on sheep liver at 28°C with a 12 h photoperiod. The pupae and pharate adults were kept at 30°C with a 24 h photoperiod until just before emergence. At this stage the pharate adults were sieved away from the vermiculite medium in which pupariation had occurred, and kept at 30°C until approx. 25% of the flies had emerged. The whole culture was then cooled to 4°C for periods

of 3–8 h. The emerged flies were removed and discarded and the remaining culture was warmed to room temperature, at which several hundred flies emerged virtually simultaneously. The above method of cooling was necessary to obtain sufficient flies of the same age for the experiments.

As the flies emerged they were placed on ice. When sufficient were obtained they were either processed immediately (newly emerged) or caged in 900 ml bottles covered with nylon net and fed sugar cubes and water. They were kept at 30°C for a 24 h photo-period and cooled to 4°C before being processed.

Isolation of mitochondria

All procedures were carried out at 0–4°C. Generally 80 flies were immobilized on ice. The thoraces were dissected out and placed in 10 ml of cold isolation medium (154 mM-KCl–2 mM-Hepes*–Tris–1 mM-EDTA–0.3% bovine serum albumin, pH 7.4) contained in a Thomas homogenizer (size C). The thoraces were disrupted by a gentle squeezing action of the pestle; any grinding action was avoided. The homogenate was filtered by suction through a small glass-wool pad on a sintered glass funnel (porosity 3DO) into a 15 ml glass Sorvall tube and the pad washed with 5 ml of isolation medium. Nagarse (0.3 mg) dissolved in a few drops of isolation medium was added and the tube stood on ice for 15 min. It was then centrifuged for 3 min at 120g in a Sorvall RC2B refrigerated centrifuge. The supernatant was transferred with a Pasteur pipette to a clean tube and centrifuged for 5 min at 4300g. The pellet was gently resuspended in 15 ml of wash medium (154 mM-KCl–2 mM-Hepes–Tris–0.3% bovine serum albumin, pH 7.4) by using a small Teflon pestle and then centrifuged for 5 min at 4300g. The pellet was finally resuspended in 0.5 ml of wash medium.

The method described above was applied to flies of all ages examined except pharate adults and newly emerged flies where the treatment with Nagarse was omitted. Examination of the mitochondrial preparations by electron microscopy revealed intact structures with very few contaminating myofibrils.

Mitochondrial protein concentration was assayed by the biuret method after solubilization with deoxycholate; cyanide was added subsequently to allow for corrections to be made for non-biuret colour and turbidity (Szarkowska & Klingenberg, 1963).

Ca²⁺ transport

Initial rates of Ca²⁺ transport were measured by the EGTA–Ruthenium Red-quench technique described by Reed & Bygrave (1974a). The reaction

* Abbreviations: EGTA, ethanedioxybis(ethylamine)-tetra-acetic acid; Hepes 2-(N-2-hydroxyethylpiperazin-N'-yl)ethanesulphonic acid.

system initially contained, in a final volume of 1.0 ml: 100 mM-KCl; 25 mM-K₂HPO₄; 25 mM- α -glycerophosphate. The final pH and temperature were 7.4 and 25°C respectively. Mitochondria (0.5 mg of protein) were added in a volume of approx. 30 μ l and after a 1 min preincubation Ca²⁺, containing approximately 0.2 μ Ci of ⁴⁵Ca²⁺, was added to initiate the reaction. Further details are in the legends to the Figures.

Measurement of mitochondrial respiration and oxidative phosphorylation

Respiration was measured with a Clark-type oxygen electrode designed and built in this laboratory (Reed, 1972). The reaction mixture consisted of 100 mM-KCl–25 mM-KH₂PO₄–10 mM-Tris and approx. 300 μ g of mitochondrial protein contained in a total volume of 2.4 ml. When oxidation of pyruvate (plus malate) was measured, ATP (1 mM) and K₂CO₃ (2 mM) were also added to the reaction system. Several additions (each 150 nmol) of ADP could be made each inducing a stimulation of respiration. The controlled or resting rate of respiration (i.e. State 4) generally did not increase after each respiratory 'jump', indicating a high degree of coupling of respiration to phosphorylation. This was seen also with mitochondria isolated from pharate adults and newly emerged flies.

Oxidative phosphorylation of ADP was measured as described elsewhere (Bygrave & Reed, 1970) by the method of Nielson & Lehninger (1955) to determine the esterification of ³²P_i (obtained from The Australian Atomic Energy Commission, Lucas Heights, Sydney, Australia).

Materials

The pure Ruthenium Red used was recrystallized from a commercial sample (Sigma Chemical Co., St. Louis, Mo., U.S.A.) as described by Fletcher *et al.* (1961). The product's extinction coefficient was identical with that found by Luft (1971). ⁴⁵Ca²⁺ (specific radioactivity 25 mCi/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. All reagents were of analytical grade.

Results

Ca²⁺ transport

Fig. 1 shows several basic features of Ca²⁺ transport in mitochondria isolated from flight muscle of *Lucilia*. When 200 μ M-Ca²⁺ (Fig. 1a) is added to the mitochondria (i.e. 200 nmol/0.5 mg of mitochondrial protein) it is rapidly removed from the incubation medium into a pool in the mitochondria which is inaccessible to EGTA. We have argued that only Ca²⁺

Fig. 1. Time-course of Ca²⁺ transport by mitochondria isolated from flight muscle of *Lucilia*

The reaction medium contained 100 mM-KCl, 25 mM-K₂HPO₄ (pH 7.4), 25 mM- α -glycerophosphate and 0.5 mg of mitochondrial protein. The reaction was started by the addition of (a) or 200 μ M-Ca²⁺ (b) 100 μ M-Ca²⁺. At the times indicated 100 μ l of the reaction mixture was removed and rapidly added to the Ruthenium Red (5 μ M)-EGTA (10 mM) quench medium and the extent of transport determined (see Reed & Bygrave, 1974a). Mitochondria were obtained from 2-h-old flies. Total vol. 1.0 ml; temp. 25°C. Insets show the initial rates of Ca²⁺ transport for the mitochondrial concentrations of Ca²⁺ indicated.

that is contained within this EGTA-inaccessible pool represents that Ca²⁺ that has been transported into mitochondria (Reed & Bygrave, 1974a). This period of rapid Ca²⁺ transport continues for about 4 min; after 6–8 min the mitochondria have transported as much Ca²⁺ as they will under the conditions of the assay system. A similar curve relating Ca²⁺ transport to incubation time is obtained when only one-half the amount of Ca²⁺ is added (Fig. 1b). Again, not all of the Ca²⁺ is transported into the mitochondria but rather only a proportion of the total amount added to the system. The techniques adopted in this work thus allow one to accurately measure initial rates of Ca²⁺ transport (see insets to Fig. 1).

The transported Ca²⁺ remains in the mitochondria for up to 20 min and is not released as would be the case with rat liver mitochondria (Drahota *et al.*, 1965; McIntyre & Bygrave, 1974). As reported by Carafoli *et al.* (1971), addition of the uncoupler of oxidative phosphorylation carbonyl cyanide *m*-chlorophenylhydrazine to the mitochondria leads to the release of transported Ca²⁺ (results not shown).

In other experiments the following points were also established (results not presented). Ca²⁺ transport supported by the oxidation of α -glycerophosphate

does not occur either in the presence of antimycin A or when P_i is omitted from the incubation system (see also Carafoli *et al.*, 1971) and confirms the requirement of energy for Ca²⁺ transport. It should be noted that the concentration of α -glycerophosphate used in the present work was 25 mM. At this concentration Ca²⁺ is unable to stimulate glycerophosphate oxidation (Hansford & Chappell, 1967).

Influence of Ca²⁺ concentration on initial rate of Ca²⁺ transport

Fig. 2 shows how the initial rate of Ca²⁺ transport in *Lucilia* flight-muscle mitochondria varies with the concentration of Ca²⁺ added to the system. Between 0 and 150 μ M added Ca²⁺ there is an exponential-like rise in the initial rate of Ca²⁺ transport, but further addition of Ca²⁺ fails to increase this, indicating saturation by Ca²⁺ of the transporting mechanism. The experiment in Fig. 2 was one of about 15 carried out with *Lucilia* flight-muscle mitochondria obtained from different stages of the life-cycle of the insect; all gave data of a similar nature. The other important feature of the experiment shown in Fig. 2 is that when the concentration of 'free' Ca²⁺ is computed by using

Fig. 2. Influence of Ca^{2+} concentration on the initial rate of Ca^{2+} transport by *Lucilia* mitochondria

The reaction system was as described in Fig. 1 except that the concentration of Ca^{2+} was varied as indicated. Mitochondria (0.5 mg of protein) were obtained from 1-day-old flies. Total vol. 1.0 ml; temp. 25°C. Initial rates were determined from measurements made as shown in the insets to Fig. 1.

known stability constants for the interacting species, it is seen that the Ca^{2+} -transport system has a high affinity for 'free' Ca^{2+} ; the K_m is approx. 5 μM .

Influence of Ruthenium Red on Ca^{2+} transport

Ruthenium Red is a potent inhibitor of Ca^{2+} transport in mammalian mitochondria (Moore, 1971; Reed & Bygrave, 1974a; Vasington *et al.*, 1972). Fig. 3(a) shows how the initial rate of Ca^{2+} transport in *Lucilia* mitochondria changes in the presence of increasing concentrations of Ruthenium Red. Having such high potency in this system, Ruthenium Red must be considered as a 'tightly bound' inhibitor (see Reed & Bygrave, 1974a). The kinetic data obtained in Fig. 3(a) were therefore analysed by treatments designed for this class of inhibitor (Henderson, 1972; Morrison, 1969). The derived plot (Fig. 3b) has a positive slope and when extrapolated, intersects the ordinate above the origin. The concentration of Ruthenium Red-binding sites is approx. 0.03 nmol/mg of protein and the K_i about $1.6 \times 10^{-8} \text{ M}$.

Influence of Ca^{2+} on respiration

Ca^{2+} will stimulate controlled (State 4) respiration in mitochondria from *Lucilia* flight muscle provided the incubation medium contains KCl and not sucrose (as used by Carafoli *et al.*, 1971) and provided the concentration of mitochondria present is not excessive (Fig. 4). A short (approx. 1 min) lag-period follows the addition of Ca^{2+} before the onset of a

Fig. 3. Inhibition by Ruthenium Red of initial rate of Ca^{2+} transport by *Lucilia* mitochondria

(a) Reaction system as described in Fig. 1 except that the concentration of Ca^{2+} was 200 μM . Incubations were carried out in the presence of the indicated concentrations of Ruthenium Red with 0.5 mg of mitochondrial protein isolated from 1-day-old flies. Total vol. 1.0 ml; temp. 25°C. Data are from two separate experiments: (1) ●; (2) ○. (b) Data from (a) replotted according to Henderson (1972). The abscissa is $1/\alpha$, where $\alpha = v_i/v_0$ (the ratio of the inhibited rate to the uninhibited rate at the same substrate concentration), and the ordinate is $i_i/(1-\alpha)$, where i_i is the total concentration of Ruthenium Red (nmol/mg of protein). The intercept on the ordinate gives the concentration of inhibitor binding sites (E_T) and the intercept on the abscissa is $-E_T K_i$.

Fig. 4. Ability of Ca²⁺ to stimulate respiration in *Lucilia* mitochondria

The reaction mixture contained 100 mM-KCl, 25 mM-KH₂PO₄ and 25 mM- α -glycerophosphate. The pH was 7.4; the final vol. 2.4 ml and the reaction temp. 25°C. Mitochondria (300 μ g of protein) from flight-muscle of 3-day-old flies were used. (a), (b) and (c) are three different experiments with additions as indicated on respiratory curves, for details see the text.

Fig. 5. Respiratory and Ca²⁺-transport activities in tightly coupled mitochondria isolated from *Lucilia* flight muscle at different stages of development

(a) Respiration of mitochondria obtained at the indicated stages of development was measured polarographically in the presence of either 25 mM- α -glycerophosphate in the absence ($\circ$) and the presence ($\bullet$) of ADP or 10 mM-pyruvate (plus malate) in the absence (Δ) and presence ($\blacktriangle$) of ADP. For further details see the Experimental section. (b) Transport of Ca²⁺ was measured at the indicated stages of development by the procedures outlined in Fig. 1. $\circ$, Values calculated from the maximum rate of Ca²⁺ transport from initial-rate measurements (see Fig. 1); $\bullet$, values determined from the extent to which 200 μ M-Ca²⁺ could be transported into mitochondria (0.5 mg of protein) after 10 min of incubation. Total vol. 1.0 ml; temp. 25°C. (c) Relation of ability of Ca²⁺ to stimulate resting respiration. The reaction mixture was as described in Fig. 4 except that mitochondria (250–300 μ g of protein) were obtained from *Lucilia* at the age indicated. Vol. 2.4 ml; temp. 25°C.

stimulated respiratory rate; the stimulated rate fails to return to the initial controlled rate of respiration. Addition of low amounts of Ruthenium Red before that of Ca²⁺ prevents the increase in respiratory rate.

The degree of stimulation of respiration is dependent on the concentration of added Ca²⁺ (K_m approx. 100 ng-ion of Ca²⁺/mg of protein, 25 μ M-total Ca²⁺ or 2 μ M-'free' Ca²⁺).

*Relation of Ca^{2+} transport to mitochondrial development in *Lucilia* mitochondria*

In the present work mitochondrial development in the functional sense was assessed by measuring the ability of mitochondria obtained at different stages of the insect life-cycle to oxidize α -glycerophosphate and pyruvate (plus malate) in the presence (State 3 respiration) or absence (State 4 respiration) of ADP.

Data from a number of individual experiments are shown in Fig. 5(a). In the period of pre-emergence (–1 day to 0) respiration is observed with α -glycerophosphate but not with pyruvate (plus malate). Tightly coupled mitochondria could be prepared as a routine which, with α -glycerophosphate as substrate, exhibited acceptor control ratios of up to 2. Over the period of emergence, particularly 0 to 2–3 h, the mitochondria rapidly develop their ability to further oxidize α -glycerophosphate and to oxidize pyruvate (plus malate). As State 4 respiration does not change significantly in this period, mitochondria with acceptor control ratios of 4–5 and up to 10 can be obtained with α -glycerophosphate and with pyruvate (plus malate) respectively. In the period 4–48 h, the maximum rates of oxidation of both substrates increase still further but more gradually to reach a peak at or near 48 h.

Fig. 5(b) shows how the mitochondria obtained at different stages of development, alter in their ability to transport Ca^{2+} . Two sets of experimental points are combined in the presentation; the first, obtained from initial-rate measurements (cf. insets to Fig. 1), indicate the rates of Ca^{2+} transport at saturating concentrations of Ca^{2+} . The second indicate the extent to which $200\text{ }\mu\text{M}$ - Ca^{2+} could be transported into the mitochondria. The ability of mitochondria from flight muscle of *Lucilia* to accumulate Ca^{2+} is greatest in the period quite soon (0–2 h) after emergence. Data obtained with mitochondria from pharate adults is difficult to assess because of the difficulties inherent in determining the precise time at which the flies would have emerged. Within several hours of emergence the mitochondria quickly lose their ability to transport Ca^{2+} so that by day 2 or 3 it is less than 20% of the maximum value obtained. Mitochondria isolated from 9-day-old flies similarly retained less than 20% of the maximum ability to transport Ca^{2+} . In this respect it is noteworthy that the mitochondria used by Carafoli *et al.* (1971) from *Phormia* were obtained from adults 7–10 days old. The maximum rates of Ca^{2+} transport obtained from initial-rate studies in the present work were approx. $120\text{ nmol of Ca}^{2+}/\text{min per mg of mitochondrial protein measured at } 25^{\circ}\text{C}$.

Finally, Fig. 5(c) shows that the degree to which Ca^{2+} is able to stimulate controlled respiration also bears a direct relationship to the age of the flies from

which the mitochondria are isolated. At or near emergence Ca^{2+} has little effect. As the mitochondria develop, however, so does the ability of Ca^{2+} to stimulate respiration. The attainment of this ability occurs rapidly after emergence, and reaches a maximum at about the second or third day. The maximum rate of Ca^{2+} -stimulated respiration, however, is still only about one-half of that obtained with ADP.

Influence of Ca^{2+} on ADP-stimulated respiration and oxidative phosphorylation

The experiments shown in Fig. 6 were carried out in an identical manner to those previously carried out with mitochondria from rat liver (Bygrave & Reed, 1970) and ascites tumour cells (Thorne & Bygrave, 1974). The experimental procedure differs from that undertaken in the experiments shown in Fig. 4 in that, here, Ca^{2+} at the indicated concentrations was added to the incubation medium before the mitochondria. Approximately 0.5 min after the addition of the mitochondria, sufficient ADP (1 mM) was added to allow respiration to proceed maximally until anaerobiosis was reached. Results from an experiment carried out with mitochondria from newly emerged flies are shown in Fig. 6(a). Very low concentrations of added Ca^{2+} inhibit ADP-stimulated respiration and, as well, the ability of these mitochondria to phosphorylate ADP. At about $100\text{ ng-ion of Ca}^{2+}/\text{mg of protein}$, respiration is inhibited by about 80% and the P/O ratio has decreased by about 50%.

When the experiment is carried out with mitochondria from 4 h-old flies (Fig. 6b) a greater resistance of ADP-stimulated respiration to added Ca^{2+} is seen. Little inhibition of ADP-stimulated respiration and little diminution of the P/O ratio is observed at $100\text{ ng-ions of Ca}^{2+}/\text{mg of protein}$. Not until the concentration of Ca^{2+} is about $200\text{ ng-ions/mg of protein}$ does Ca^{2+} begin to have any noticeable effect on respiration and on the P/O ratio.

Discussion

This work has shown that many features of the *Lucilia* Ca^{2+} -transport system are identical with those previously found in mammalian mitochondria. The sigmoidal relation between initial rates of Ca^{2+} transport and Ca^{2+} concentration, the very high affinity for 'free' Ca^{2+} and saturation of the transport system by high Ca^{2+} concentrations are seen both with the *Lucilia* mitochondria and those from rat liver (cf. Bygrave *et al.*, 1971a,b; Spencer & Bygrave, 1973; K. C. Reed & F. L. Bygrave, unpublished work; Vinogradov & Scarpa, 1973). Equally revealing is the finding that the concentrations of Ruthenium Red required for inhibition of Ca^{2+} transport are almost identical for both *Lucilia* and rat liver mitochondria (cf. Reed & Bygrave, 1974a). Wohlrab (1974) pre-

Fig. 6. Effect of Ca²⁺ on ADP-stimulated respiration and on the P/O ratio (▲) in *Lucilia* mitochondria isolated at different stages of mitochondrial development

The reaction mixture was as described in Fig. 1, with the following additions: 100mM-glucose, 10 μ g of hexokinase, 1×10^{-6} c.p.m. of ³²P_i and various concentrations of Ca²⁺. Mitochondria (about 300 μ g of protein) were added, immediately followed by 1 mM-ADP. The rate of respiration was then measured for approx. 4 min (to about 80% anaerobiosis), when samples (500 μ l) were removed into 10% (w/v) trichloroacetic acid. The extent of ATP formation was determined as indicated in the Experimental section. Mitochondria were isolated from flight muscle of (a) newly emerged, (b) 4h-old flies. Vol. 2.4ml; temp. 25°C. ●, ADP present; ○, ADP absent.

sented evidence for the existence of a Ca²⁺-transport system in flight-muscle mitochondria of the blowfly *Sarcophaga bullata*. It is now clear that interpretation of a failure to observe high-affinity Ca²⁺ binding in any type of mitochondria should be made with caution, especially in the light of criticism regarding the measurement of such binding (Akerman *et al.*, 1974; Reed & Bygrave, 1974b; Southard & Green, 1974).

The most noteworthy finding in our study is that during insect development, mitochondrial change in their capacity to transport Ca²⁺. The changes seen are remarkable for several reasons. First, the degree of change is very substantial with much of it taking place within a few hours of the period of emergence of the adult fly. Secondly, the change (i.e. decrease) in the ability to transport Ca²⁺ is opposite to the change (i.e. an increase, in the bioenergetic sense) in the functional development and maturity of the mitochondrial population.

These observations can be correlated with the knowledge that in *Lucilia*, wing movement occurs at about 2h, short flight at about 4h and prolonged

flight at about 24h after emergence. It seems possible that the changes in the nature of the interaction of Ca²⁺ with energy-linked reactions of flight-muscle mitochondria that are seen early in the development of the adult fly (the present work; Walker & Birt, 1969) are intimately related: first, to the development of a musculature that will adequately support flight; secondly, to the development of a mitochondrial population whose primary function is to provide energy for the rapid contraction-relaxation cycles this musculature eventually undergoes; and thirdly, to the development of a metabolic network involving Ca²⁺ that will allow these mitochondria to respire and to synthesize ATP with maximum efficiency. The detailed interrelationship of these events can be elucidated by further examination of the developmental system described in this study. Moreover this system (see also Williams *et al.*, 1974) should provide further insight into fundamental questions related to the function, development and molecular biology of the Ca²⁺ and other metabolite-transport systems located in the mitochondrial membrane.

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