ENRICHMENT OF RUTHENIUM RED-SENSITIVE Ca^{2+} TRANSPORT IN A POPULATION OF HEAVY MITOCHONDRIA ISOLATED FROM FLIGHT-MUSCLE OF *LUCILIA CUPRINA*

Further evidence for its heterogeneous distribution in the inner mitochondrial membrane

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1. Introduction

Evidence has been provided [1] that Ruthenium Red-sensitive calcium-ion (Ca^{2+}) transport is enriched in specific populations of mitochondria isolated from rat liver. In these populations, evidence was also provided that the Ca^{2+} transport system is concentrated in the inner peripheral membrane [1].

The implications of these findings to aspects of the biogenesis of mitochondria and the role of mitochondrial Ca^{2+} transport in cell physiology and metabolism (see e.g. [1] and reviews on Ca^{2+} transport in [2–6]) is such that we felt it important to determine whether evidence for heterogeneity of Ca^{2+} transport in mitochondria also exists in other tissues and species. We chose, therefore, to re-examine Ca^{2+} transport in flight-muscle mitochondria of the sheep blowfly *Lucilia cuprina*. Firstly, *Lucilia* represents a species phylogenetically far removed from liver, and secondly, detailed analyses of the Ca^{2+} transport system in the flight-muscle of this species has been undertaken [7–9]. In particular we have established that the ability of flight-muscle mitochondria to transport Ca^{2+}, changes markedly during the development of the adult fly. The data presented here show that a considerable degree of heterogeneity of mitochondrial Ca^{2+} transport does exist in *Lucilia* flight-muscle, similar to that found in rat liver.

2. Experimental

Lucilia were reared and maintained as in [7,8].

2.1. *Isolation of mitochondrial fractions*

An homogenate of the thoraces of 200 flies was prepared as in [7,8]. In the present work however, the centrifugation procedure was modified, adopting a centrifugation time of 5 min for each step. Using the Sorvall RC2B centrifuge with rotor SS-34, as follows: A very low speed pellet containing cell debri, nuclei and some mitochondria was obtained and discarded after an initial centrifugation at 75 × *g*. The supernatant from this step was centrifuged at 480 × *g* and the pellet obtained designated fraction 1. Similarly, pellets were obtained after further centrifugation of the supernatant at 1480 × *g*, 4350 × *g* and 17 300 × *g*; these are designated fractions 2–4, respectively. Finally, pellets 1–3 were washed with buffered KCl [7] by resuspension and centrifugation at 4350 × *g*. Fraction 4 was washed by resuspension and centrifugation at 17 300 × *g*. The pellets were resuspended in wash medium in ~1 ml to give ~10 mg protein/ml. The concentration of mitochondrial protein was determined using the Folin reagent [10].

Ca^{2+} transport was measured using the EGTA–Ruthenium Red 'quench' technique [11]. The incubation medium, identical to that in [8], contained 100 mM KCl, 25 mM α-glycerophosphate, 2 mM K_2HPO_4 and 1 mg mitochondrial protein in final vol. 1.0 ml, at pH 7.4 and 25°C. 50 μM $CaCl_2$ (containing ~0.3 μCi $^{45}Ca^{2+}$) was added to initiate the reaction. Measurements were made over 5 min. Cytochrome oxidase was measured by the method in [12] and α-glycerophosphate dehydrogenase as in [13] but

using 20–50 μg mitochondrial protein in the latter assay.

Materials were obtained from the sources in [7].

2.2. *Reproducibility*

The data presented were obtained from individual experiments each of which was carried out at least twice.

3. Results

In the present work with *Lucilia* flight-muscle, a similar approach to that adopted for rat liver [1] was used to determine the extent to which Ca^{2+} transport is present in the various mitochondrial preparations.

At 6 h and 72 h following the emergence of the adult fly, 4 mitochondrial fractions were isolated from the flight-muscle on the basis of their sedimentation properties in buffered iso-osmotic KCl as detailed in section 2. Ca^{2+} transport over this period, as measured in the presence of 2 mM P_i, retains near-maximal activity (cf. [8]).

Immediately following isolation of the fractions, initial rates of Ca^{2+} transport were measured and, as time allowed, so were α-glycerophosphate dehydrogenase, and cytochrome *c* oxidase activities (cf. [1]).

Data in fig.1a reveal that initial rates of Ca^{2+} transport were maximal by far in fraction 1. The activity diminished to reach quite low values in fraction 4. This pattern was seen in mitochondria prepared from each of the stages of adult development examined (i.e. 6–72 h). By 5 days after emergence, Ca^{2+} transport activity in all 4 fractions was minimal (data not presented, see [8]).

Data in fig.1b reveal that initial rates of α-glycerophosphate dehydrogenase exhibited a similar activity profile to Ca^{2+} transport at all the developmental stages examined; it was greatest in fraction 1 and least in fraction 4. A gradual increase in activity in all fractions occurred however, between 6 h and 72 h after emergence, consistent with knowledge about the development of this enzyme activity during adult life (see, e.g. [14]).

By contrast with the activity profiles of each of Ruthenium Red-sensitive Ca^{2+} transport and α-glycerophosphate dehydrogenase, that of cytochrome *c* oxidase was clearly different. In all mitochondrial

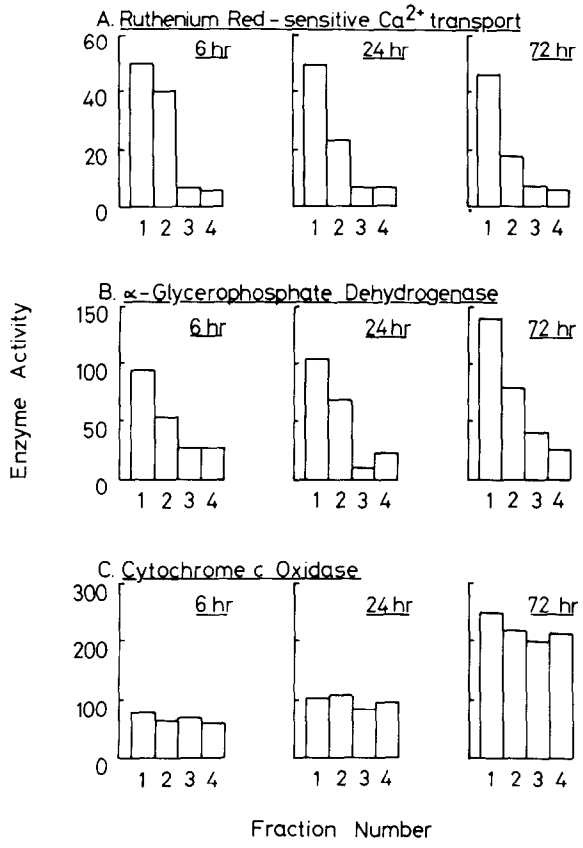

Fig.1. Distribution of Ruthenium Red-sensitive Ca^{2+} transport, α-glycerophosphate dehydrogenase and cytochrome *c* oxidase activities in fractions of *Lucilia* flight-muscle mitochondria obtained at different times following emergence of the adult. All assays were carried out by the procedures in section 2. Fractions 1–4 refer to the pellets obtained after centrifuging the homogenate for 5 min at 480 × *g*, 1480 × *g*, 4350 × *g* and 17 300 × *g*, respectively (see section 2). Enzyme activities are expressed as nmol/min.mg protein (Ca^{2+} transport and cytochrome *c* oxidase) or μmol/min.mg protein (α-glycerophosphate dehydrogenase).

fractions at all times of development, the activity of this latter enzyme was similar (fig.1c). As with α-glycerophosphate dehydrogenase, an increase in activity occurred in each mitochondrial fraction over the developmental period examined again consistent with knowledge about the increase in activity of this enzyme during development of *Lucilia* [14].

Control experiments revealed that the rates of respiration in the absence or presence of ADP in each

of the fractions at a given stage of development, were not significantly different from each other indicating that the different rates of Ca^{2+} transport in the mitochondrial fractions were not the result of a difference in the ability of the fractions to generate a membrane potential that drives Ca^{2+} transport in these organelles.

4. Discussion

The following evidence underlies the conclusion [1] that Ruthenium Red-sensitive Ca^{2+} transport is enriched in specific populations of rat liver mitochondria:

(i) Initial rates of Ca^{2+} transport were maximal in the 'heavier' fractions of a range prepared from rat liver homogenates by differential centrifugation in buffered iso-osmotic medium; this occurred despite the fact that the potential across the inner membrane (i.e., the driving force for Ca^{2+} transport) was similar in the fractions.

(ii) Of the 3 enzyme activity profiles measured in these fractions namely, cytochrome *c* oxidase, uncoupler-stimulated ATPase and α-glycerophosphate dehydrogenase, only that of the latter was similar to Ruthenium Red-sensitive Ca^{2+} transport.

The present work has shown that Ca^{2+} transport in *Lucilia* flight-muscle mitochondria, like that in rat liver mitochondria, exhibits greatest rates in the population sedimenting at the lowest centrifugal forces (i.e., 480 × *g* for 5 min). This compares with the centrifugal force of 4300 × *g* for 5 min used in [7–9] to sediment the mitochondrial fraction from *Lucilia* flight-muscle (see [15]).

That the activity profiles for Ca^{2+} transport and α-glycerophosphate dehydrogenase were similar and together different from that of cytochrome *c* oxidase, also is of significance since it strongly indicates that the distribution of the former two enzymes in the inner mitochondrial membrane is different from that of cytochrome *c* oxidase, a commonly-employed 'marker' for this membrane in cell fractionation studies (see [16]). Because of the assignment of α-glycerophosphate dehydrogenase to the peripheral region of the inner membrane [17,18], we suggest that, as in rat liver mitochondria [1], the Ca^{2+} transport system in *Lucilia* flight-muscle mitochondria is located in that region of the inner membrane.

Because Ca^{2+} transport, during the 72 h period post-emergence, declined slightly (fig.1a, [8]) while the activities of α-glycerophosphate dehydrogenase (fig.1b) and especially of cytochrome *c* oxidase (fig.1c) increased, it is clear that the ratios of the activities Ca^{2+} transport to each of the latter enzymes, declines quite markedly over the developmental period studied (see [7]). Thus in flight-muscle mitochondria of *Lucilia*, Ca^{2+} transport does not retain a 'constant proportionality' [14,19,20] with electron transport chain components during development of the organelle post-emergence, a conclusion that can be gleaned also from data in [7,8,21].

Finally it is of considerable interest that mitochondria from such widely diverse species as rat liver and blowfly flight-muscle exhibit a heterogeneous distribution of Ca^{2+} transport in different mitochondrial populations. While the biological significance of this phenomenon remains to be assessed, it is pertinent that mitochondrial populations with different Ca^{2+} transporting properties recently have been isolated also from cardiac muscle [22,23].

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References

[1] Bygrave, F. L., Heaney, T. P. and Ramachandran, C. (1978) Biochem. J. 174, 1011–1019.
[2] Lehninger, A. L. (1970) Biochem. J. 119, 129–138.
[3] Carafoli, E. and Crompton, M. (1976) Soc. Exp. Biol. Symp. 30, 89–115.
[4] Mela, L. (1977) Current Topics in Membranes and Transport 9, 322–366.
[5] Bygrave, F. L. (1978) Biol. Revs. (Camb. Phil. Soc.) 53, 43–79.
[6] Bygrave, F. L. (1978) Trends Biochem. Sci. 3, 175–178.
[7] Bygrave, F. L., Daday, A. A. and Doy, F. A. (1975) Biochem. J. 146, 601–608.
[8] Smith, R. L. and Bygrave, F. L. (1978) Biochem. J. 170, 81–85.
[9] Bygrave, F. L. and Smith, R. L. (1978) Biochem. J. in press.
[10] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.

[11] Reed, K. C. and Bygrave, F. L. (1974) Biochem. J. 140, 143–155.
[12] Tolbert, N. E. (1974) Methods Enzymol. 31, 734–746.
[13] Lee, Y. P. and Lardy, H. A. (1965) J. Biol. Chem. 240, 1427–1436.
[14] Lennie, R. W. and Birt, L. M. (1967) Biochem. J. 102, 338–350.
[15] Harada, N. and Sato, T. (1975) Agr. Biol. Chem. 39, 899–900.
[16] De Duve, C., Pressman, B. C., Gianetto, R., Wattiaux, R. and Appelmans, F. (1955) Biochem. J. 60, 604–617.
[17] Werner, S. and Neupert, W. (1972) Eur. J. Biochem. 25, 379–396.
[18] Brdiczka, D., Dolken, G., Krebs, W. and Hofmann, D. (1974) Hoppe-Seyler's Z. Physiol. Chem. 355, 731–743.
[19] Pette, D., Klingenberg, M. and Bücher, Th. (1962) Biochem. Biophys. Res. Commun. 7, 425–429.
[20] Bücher, Th. (1965) Symp. Biochem. Soc. 25, 15–28.
[21] Doy, F. A., Daday, A. A. and Bygrave, F. L. (1975) FEBS Lett. 54, 245–248.
[22] McMillin Wood, J. and Wolkowicz, P. E. (1978) Fed. Proc. Fed. Am. Soc. Exp. Biol. 37, 1615.
[23] Palmer, J. (1978) Fed. Proc. Fed. Am. Soc. Exp. Biol. 37, 1616.