RECONSTRUCTION OF THE α-GLYCEROLPHOSPHATE SHUTTLE USING RAT KIDNEY MITOCHONDRIA

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1. Introduction

In mammalian cells NADH produced in the cytosol does not have direct access to the mitochondrial respiratory chain. Instead, excess cytosolic reducing equivalents are made available to the respiratory chain indirectly by way of various 'shuttle' systems (reviewed [1]). Two of these, the malate–aspartate shuttle [2] and the fatty acid shuttle [3] have been reconstructed experimentally by using isolated mitochondria from certain mammalian tissues together with appropriate shuttle components [4–9]. However, reconstruction of the α-glycerolphosphate shuttle [10], depicted in fig.1, has proved difficult [6,11]. A purportedly successful reconstruction of this shuttle with mitochondria from brown adipose tissue was marred by a long, and not easily explained delay between the addition of shuttle components and the establishment of a sustained increase in the rate of oxidation of extramitochondrial NADH [12].

In this communication we describe a system in which L-glycerol-3-phosphate together with L-glycerol-3-phosphate: NAD^+ oxidoreductase (EC 1.1.1.8) facilitated the rotenoneinsensitive oxidation of extramitochondrial NADH by rat kidney mitochondria. The system represents, to the best of our knowledge, the first unequivocal reconstruction of the α-glycerolphosphate shuttle with mammalian mitochondria.

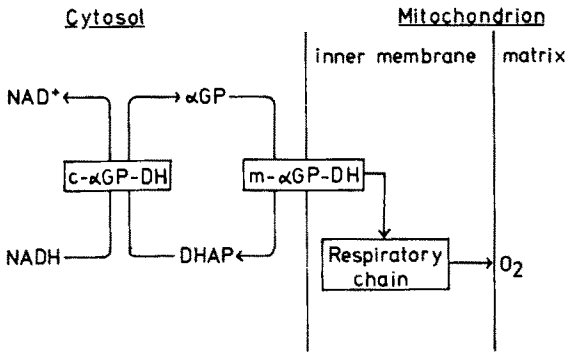

Fig.1. The α-glycerolphosphate shuttle.
Abbreviations: αGP, L-glycerol-3-phosphate; DHAP, dihydroxyacetonephosphate; c-αGP-DH, cytoplasmic L-glycerol-3-phosphate:NAD^+ oxidoreductase; m-αGP-DH, mitochondrial L-glycerol-3-phosphate: flavin oxidoreductase

2. Materials and methods

L-glycerol-3-phosphate, L-glycerol-3-phosphate: NAD^+ oxidoreductase (crystalline type X), rotenone and bovine serum albumin (fraction V) were purchased from Sigma Chemical Co., St Louis, MO. Carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone (FCCP) and dihydroxyacetone phosphate dimethyl ketal were obtained from Boehringer Mannheim GmbH, Mannheim, FRG.

Mitochondria were isolated from kidneys of adult albino rats (Wistar strain) by the method in [13] except that the final 20 000 × *g* 20 min spin was replaced by a 7000 × *g* 10 min spin in an attempt to minimise contamination of the final mitochondrial pellet with broken mitochondria. Protein was determined by a biuret method [14].

The α-glycerolphosphate shuttle was reconstructed at 35°C as follows: into each of two cell cuvettes were placed mitochondria (1–2 mg protein)

suspended in 2.5 ml buffered medium comprising 220 mM mannitol, 70 mM sucrose, 2.4 mM potassium phosphate buffer, 2 mM Hepes buffer, 1.8 mM $MgCl_2$ and 0.5 mM sodium EDTA, at pH 7.2. The cuvettes were inserted into the sample and reference cuvette holders (thermostatted at 35°C) of a Varian Superscan 3 ultraviolet-visible spectrophotometer set to operate in double-beam mode and to measure ΔA_{340} (sample minus reference). NADH (0.12 mM) was added to the sample cuvette only. Then to both cuvettes were added, in varying order for different experiments, 5 μM rotenone, 0.4–2 units L-glycerol-3-phosphate:NAD^+ oxidoreductase (c-αGP-DH), 0.2–3.2 mM L-glycerol-3-phosphate (αGP) and, lastly, 1 mM KCN. The rate of oxidation of NADH was followed at 340 nm after each successive addition.

The activities of the flavin-linked and NAD^+-linked glycerol-3-phosphate dehydrogenases in a kidney homogenate and in isolated mitochondria were measured at 35°C in the medium described above. A Rank oxygen electrode (Rank Bros., Bottisham, Cambs) was used for the assay of the mitochondrial flavin-linked enzyme (m-αGP-DH; EC 1.1.99.5), activity being measured in the presence of 5 μM rotenone and 0.4 mM αGP. The cytoplasmic NAD^+-linked enzyme (c-αGP-DH) was assayed spectrophotometrically by following the oxidation of NADH (0.12 mM) at 340 nm in the presence of 0.06 mM dihydroxyacetone phosphate.

3. Results

The activity of c-αGP-DH in an homogenate of kidney was 126 nmol NADH oxidised/min.mg $protein^{-1}$, and the activity of m-αGP-DH was 2.6 nmol oxygen atoms used/min.mg $protein^{-1}$. The cytoplasmic enzyme was, therefore, ~50 times more active than the mitochondrial enzyme. In the shuttle reconstruction experiments, a purified form of the cytoplasmic enzyme was added to mitochondrial suspensions and an attempt was made to keep the activity ratio fairly close to that measured in the whole homogenate.

In the first reconstruction experiment, shown in fig.2a, it was observed that the mitochondrial preparation oxidised added NADH even in the absence

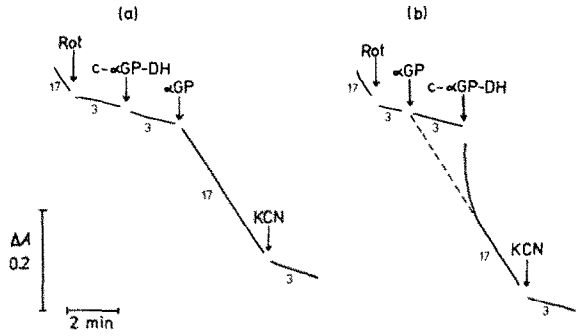

Fig.2. Oxidation of extramitochondrial NADH by rat kidney mitochondria. Mitochondria (1.7 mg protein) were incubated at 35°C in the buffered reaction mixture containing 0.12 mM NADH as in section 2. The additions were 5 μM rotenone (Rot), 2 units L-glycerol-3-phosphate:NAD^+ oxidoreductase (c-αGP-DH), 0.4 mM L-glycerol-3-phosphate (αGP) and 1 mM KCN. The trace shows the decrease in A_{340} and the figures alongside give the rate of NADH oxidation in nmol/min.mg mitochondrial $protein^{-1}$.

of further additions. However, this oxidation was strongly inhibited by rotenone, suggesting that damaged mitochondria were responsible for it. With NADH oxidation thus lowered, first c-αGP-DH and then αGP were added. It can be seen that addition of the enzyme did not affect the NADH oxidation rate but the introduction of αGP brought about an immediate and marked increase in the oxidation rate. This increase was abolished by cyanide, indicating its dependence on the mitochondrial respiratory chain.

Other experiments revealed that NADH oxidation was totally dependent on the presence of mitochondria as well as c-αGP-DH and αGP. When rotenone was omitted the basal rate of NADH oxidation was greater but the responses elicited by c-αGP-DH and αGP were unaltered (data not shown). Neither uncoupler (2 μM FCCP) nor ADP (0.1 mM) affected the rate of rotenone-insensitive NADH oxidation; nor did the lowering of added c-αGP-DH from 2–0.4 units. Moreover, it was found that the increase in the rate of NADH oxidation brought about by αGP together with c-αGP-DH (13.6 nmol/min.mg $protein^{-1}$) was identical to the increase in the rate of mitochondrial oxygen consumption upon addition of αGP (13.8 nmol oxygen atoms/min.mg $protein^{-1}$). The observations

all suggested that the overall rate of NADH oxidation was determined by the activity of m-αGP-DH.

The above results implicate the α-glycerolphosphate shuttle as the mediator of extramitochondrial NADH oxidation by rat kidney mitochondria, and this is supported by data presented in fig.2b. In this experiment αGP alone did not stimulate NADH oxidation but subsequent addition of c-αGP-DH led to a very rapid oxidation which soon gave way to a lower sustained rate identical to that seen in the first experiment (cf. fig.2a). The conclusion was that the rapid phase of NADH oxidation occurred because dihydroxyacetone phosphate (DHAP) had accumulated in the system prior to the addition of c-αGP-DH, and it is suggested that DHAP could have been produced only through the oxidation of αGP by m-αGP-DH. This interpretation is supported by the fact that extrapolation of the later sustained rate intercepts the earlier line at the point at which αGP was added (see broken line in fig.2b).

The only reasonable explanation we can offer for the events shown in fig.2 is that added αGP was oxidised by the mitochondrial m-α GP-DH to DHAP which, in turn, was reduced to αGP by the added c-αGP-DH with added NADH as the reductant. The αGP so formed would then be available once more to the mitochondrial oxidation system, thus closing the operation into a cycle identical to the proposed α-glycerolphosphate shuttle (fig.1).

In other experiments it was found that the rate of rotenone-insensitive NADH oxidation depended on the concentration of added αGP (fig.3), and a maximum rate of 23 nmol NADH oxidised/min.mg mitochondrial protein^{-1} was achieved. However, in contrast with [12], the rate of NADH oxidation was unaffected by the omission of EDTA or the inclusion of 0.4% bovine serum albumin. Furthermore, Ca^{2+}, which activates m-αGP-DH of insect flight muscle [15,16], did not stimulate shuttle activity when present at concentrations up to 0.5 mM.

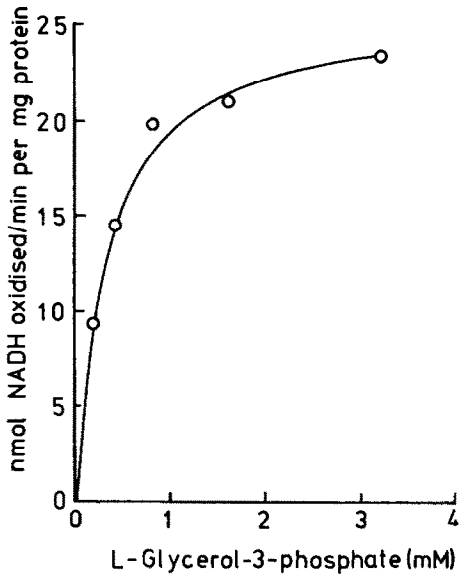

Fig.3. Effect of glycerol phosphate concentration on the rate of rotenone-insensitive NADH oxidation. Conditions as in fig.2 but with L-glycerol-3-phosphate concentrations from 0.2–3.2 mM.

4. Discussion

The α-glycerolphosphate shuttle is theoretically the simplest of the reducing equivalent shuttle systems in that it involves only two enzymes and does not require components of the shuttle to cross the mitochondrial inner membrane [16]. Nonetheless its reconstruction with mammalian mitochondria has proved difficult despite suggestions that it is active in several tissues of mammalian origin [12,17–20]. Some reasons for the failure to reconstruct the shuttle convincingly have been given by other authors. For instance it was pointed out [1] that the measurement of extramitochondrial ethanol oxidation as an index of shuttle activity [6] did not foresee the effect that rotenone, by inhibiting the mitochondrial oxidation of acetaldehyde, would have on the oxidation of ethanol by alcohol dehydrogenase and NAD^+. The work in [12] was also less conclusive than it might have been, possibly because, in a well-justified attempt to keep experimental conditions as close to physiological as possible, cell supernatant was used as the source of cytoplasmic glycerol phosphate dehydrogenase even though it might have contained fatty acids or other inhibitors of the mitochondrial glycerol phosphate dehydrogenase. In our work we attempted to avoid such pitfalls by adding only those components directly involved in the cytoplasmic arm of the shuttle and in this way achieved a successful reconstruction.

On the question of whether the observed activity is sufficient to contribute significantly to the oxidation of extramitochondrial reducing equivalents produced during glucose oxidation in kidney cells the following figures may give some guide. According to [21] kidney cells can convert glucose to CO_2 at rates up to 0.15 μmol/h. mg cell protein^{-1}. Thus, if mitochondrial protein constitutes ~20% total cell protein [22] extramitochondrial NADH could be produced at < 1.5 μmol/h. mg mitochondrial protein^{-1}. In our experiments the α-glycerolphosphate shuttle oxidised, at maximum, ~1.4 μmol NADH/h. mg mitochondrial protein^{-1} and thus appears capable of playing a significant role in intact cells. Support for this view may be found in figures showing the concentration of α-glycerol phosphate in the in vivo kidney [23] and in unpublished results from our laboratory which indicate that the anti-inflammatory drug indomethacin, which strongly inhibits the oxidation of glucose to CO_2 and promotes its conversion to lactate in kidney cells [24], is a potent inhibitor of the reconstructed α-glycerolphosphate shuttle.

In conclusion it should be pointed out that to suggest a significant role for the α-glycerolphosphate shuttle in kidney cells is not to imply a lack of involvement of other shuttles in kidney metabolism. It might be expected that in the kidney, as in the liver, various shuttles are employed in regulating the metabolic activity of the cell.

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