"MALIC ENZYME" AND ALKALINE PHOSPHATASE IN MITOCHONDRIA FROM PIG AND RAT OVARIES

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1. Introduction

The utilisation of malate by two tissues involved with steroidogenesis, the ovary and the adrenal cortex, has been of interest since 1954 when Brownie and Grant [1] showed that in the adrenal cortex malate could supply electrons for steroid hydroxylation reactions. Simpson and Estabrook have since postulated that an intra mitochondria malic enzyme (malate dehydrogenase (decarboxylating) (NADP) (EC 1.1.1.40) is in fact the direct source of NADPH for SCC* in this tissue [2]. During an investigation of porcine corpora lutea we showed that although mitochondrial malic enzyme was of relatively low specific activity it could supply enough NADPH to support all the SCC which occurred in that tissue. However, malic enzyme and SCC appeared to reside in different mitochondrial compartments [3].

The aim of this work was to find the relative positions of cytochrome P450, which is part of the mixed function oxidase responsible for SCC, and malic enzyme in mitochondria from rat and pig corpora lutea. On fractionating mitochondria from each of these sources "malic enzyme" activity was lost. It was demonstrated that "malic enzyme" from these mitochondria is an artefact and probably consists of a combination of alkaline phosphatase which converts NADP to NAD in the outer membrane, and of malic dehydrogenase (EC 1.1.1.37), present in the inner membrane and the mitosol, which reduces the NAD.

* SCC = cholesterol side chain cleavage.

2. Methods and materials

L-malic acid, L-lactate (lithium salt), p-nitrophenylphosphate, nitrophenol, NAD, NADP and lactic dehydrogenase (beef heart) (EC 1.1.1.27) were obtained from Sigma Chemical Co.

Mitochondria, prepared from pig corpora lutea and superovulated rat ovaries [4] by the method described previously [3], were separated into 3 fractions using the technique which Sottocasa et al. [5] employed for separating outer from inner mitochondrial membranes. After swelling the mitochondria in hypotonic Tris-phosphate buffer, shrinking the inner membranes with Mg^{2+}-ATP in hypertonic sucrose, and sonicating, the membranes were separated by layering 7 ml of sonicate over 10 ml of 1.18 M sucrose and centrifuging for 3 hr at 30.6×10^3 rpm (60,000 *g* at centre of the tube) in an MSE 8 × 25 ml fixed angle head. The pellet, "inner mitochondrial membranes" was resuspended in 0.25 M sucrose, and the clear layer immediately above the pellet which had no enzymic activity, was discarded. The upper 2 layers (outer membranes and mitosol) were not clearly separated and were therefore recentrifuged at 104,000 *g* for 1 hr. The "outer membranes" which then formed a pellet were resuspended in 0.25 M sucrose.

Malic enzyme, and malate dehydrogenase were assayed at 30° in 3 ml of 0.1 M methylaminopropanol buffer, pH 9.5, containing 1.5 μmoles $MnCl_2$, 3 μmoles NAD(P) and an appropriate amount of enzyme. The reaction was started by adding 50 μmoles Na^+ malate and was followed spectrophotometrically at 340 nm. Lactic dehydrogenase was assayed in the same system using lactate at a concentration of 20 mM in place of malate.

Cytochrome *P*450 was measured from its CO difference spectrum, obtained with a Shimadzu split beam recording spectrophotometre after mitochondrial fractions, suspended in sucrose, had been reduced by dithionite. The mmolar extinction coefficient $\epsilon(450-490) = 91$ was used to calculate its concentration [6].

Alkaline phosphatase was assayed in 3 ml of 0.1 M methylamino–propanol buffer pH 9.5 containing 1.5 μmoles $MnCl_2$ and 6 μmoles p-nitrophenylphosphate. The assay was initiated by the addition of an appropriate amount of enzyme. The reaction was followed spectrophotometrically at 402 nm. The calculated mmolar extinction coefficient of nitrophenol under the conditions of the assay was 23.4: activity was expressed as the number of μmole of p-nitrophenylphosphate converted to nitrophenol/min/mg protein. Protein concentrations were estimated by the biuret reaction using bovine serum albumin as standard [7].

Chromatographic separation of NAD and NADP was achieved by the method recommended on the Sigma data sheets which accompany their products; viz. on Whatman No. 1 paper in ethanol – 1 M ammonium acetate (7:3 by vol) by ascending chromatography. Spots were visible under UV illumination.

3. Results and discussion

No claim is made that pure mitochondrial membranes were prepared, but the results illustrated in table 1 demonstrate that some separation of enzymic activities occurred as a result of the fractionation. The results reported throughout refer to mitochondria from porcine corpora lutea, but mitochondria from luteinized rat ovaries behaved in the same way. Malic enzyme activity was apparently lost during the fractionation of mitochondria from both sources.

Fig. 1 illustrates that the "malic enzyme" activity was regained by mixing the outer membrane fraction with the inner membranes, or the outer membranes with the mitosol. It further shows that lactic dehydrogenase which was NAD specific could apparently reduce NADP when outer mitochondrial membranes were present.

These assays are all based on spectrophotometric measurements at 340 nm, which do not differentiate between the reduction of NAD and NADP. It was proposed that the "malic enzyme" activity measurable in unfractionated luteal mitochondria is an artefact which in fact consists of two entities: (1) a phosphatase or phosphoro-transferase (present in the outer membrane) which converts NADP to NAD, and (2) malic dehydrogenase, present in the inner membranes and the mitosol, which reduces NAD with concomitant increase in extinction at 340 nm.

The following experimental results support this hypothesis. Alkaline phosphatase was very much more active in outer membrane fractions than in inner membranes (table 1). When NADP and malate were preincubated with outer mitochondrial membranes there was an initial burst of activity on addition of inner membranes, equivalent to the rate of the NAD-dependent malic dehydrogenase. The rate then fell, presumably when all the preformed NAD was exhausted, and proceeded at a rate of the same order as alkaline phosphatase, which must be rate limiting. There was no initial burst of activity when the membranes were added in the reverse order.

Table 1
Analysis of mitochondrial fractions prepared from porcine corpora lutea.

	Malic enzyme	Malid dehydrogenase	Alkaline phosphatase	Cyt. *P*450 (mM/mg protein)
Outer membrane	0.007	0.045	0.047	9×10^{-5}
Inner membrane	0.0014	0.91	0.004	37×10^{-5}
Mitosol	none	0.57	none	none
Whole mitochondria	0.25	none	–	–

Mitochondria were fractionated, and assays performed as described in the text. Activities are expressed as μmole product formed/min/mg protein. The cytochrome *P*450 results and those of the enzyme assays were obtained from two different preparations. *P*450 was measured in three preparations only, but in each case its ratio in outer and inner membranes was the same as for malic dehydrogenase.

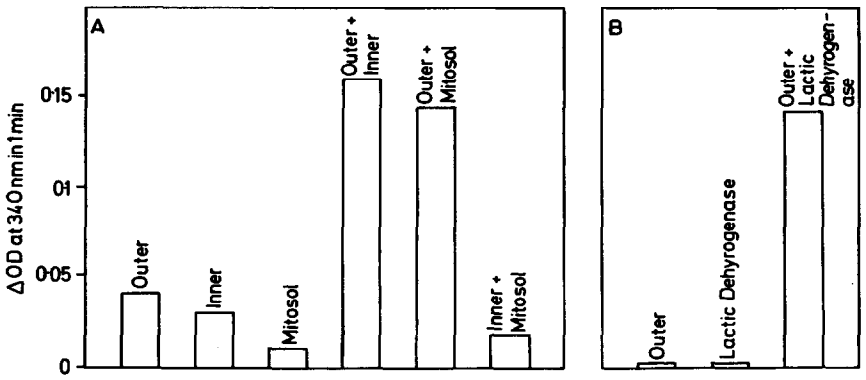

Fig. 1. Mitochondrial membranes, or commercial lactic dehydrogenase, were incubated separately and together as designated on the histogram, in 3 ml of 0.1 M methylaminopropanol buffer pH 9.5 containing 1.5 μmoles, $MnCl_2$ and 3 μmoles NADP. In A the substrate was malate (50 μmoles) and in B substrate was lactate (60 μmoles). Change in extinction at 340 nm was measured on a Shimadzu recording spectrophotometer.

Both alkaline phosphatase and "malic enzyme" were inhibited by 0.13 M phosphate and by 0.01 M EDTA. The Lineweaver-Burk plots illustrated in fig. 2 demonstrate that NADP is a competitive inhibitor of alkaline phosphatase when nitrophenylphosphate is substrate: K_m for substrate was 1.3×10^{-4} M and K_i for NADP under the conditions of the assay was 4.4×10^{-4} M. Added NAD has no effect on the enzymic cleavage of p-nitrophenylphosphate.

Chromatography of the products of reaction when 10 μmoles NADP was incubated for 1 hr at room temp. with outer membranes (0.1 mg protein) at pH 9.5, indicated that NAD, or a substance with the same

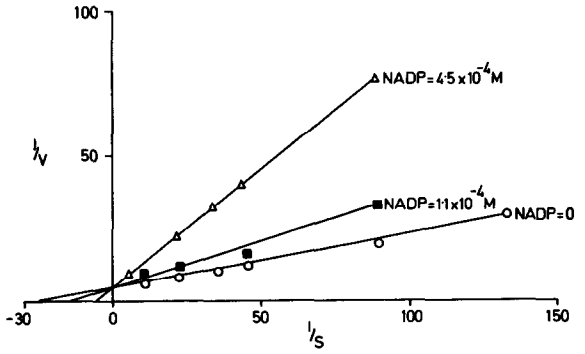

Fig. 2. Alkaline phosphatase assays were carried out as described in the text, using substrate (p-nitrophenylphosphate) concentrations ranging from 1×10^{-5} to 6×10^{-4} M. The results of the assays performed in the presence or absence of NADP as shown are presented as Lineweaver-Burk plots.

chromatographic characteristics as NAD, was formed. One preparation of rat mitochondria which had no malic enzyme activity had no phosphatase activity, although malic dehydrogenase was present with a very high specific activity.

The four following observations suggest, tenuously, that the outer membrane phosphatase might be part of an enzyme complex which removes phosphate from NADP and transfers the NAD into the inner mitochondria. (a) When "malic enzyme" activity is measured in intact mitochondria by following the reduction of NADP at 340 nm there is an initial lag period after the mitochondria have been added to NADP + malate; this lag is followed by steady reduction of coenzyme. (b) Neither the course of reduction of coenzyme nor the activity of "malic enzyme" are changed by sonication of the whole mitochondria. (c) Exogenous NAD is not available to malic dehydrogenase unless the mitochondria are sonicated. (d) "Malic enzyme" activity is lost on freezing and thawing although neither the phosphatase nor malic dehydrogenase are affected by this procedure.

It is clear that in luteinized rat and pig ovary malic enzyme is not involved in the supply of NADP *per se* for mitochondrial steroid hydroxylation reactions, as it is in the adrenal cortex [2]. We have shown that in pig corpora lutea free fatty acids derived from cholesterol esters can supply the reducing power necessary for cholesterol side chain cleavage [8]. Both malate and NAD are necessary for potentiating fatty acid

oxidation. Malate and fatty acid oxidation are mutually dependent in that their end products, oxaloacetate and acetyl CoA, are removed by condensing with each other, (acetyl CoA shifts the equilibrium of malic dehydrogenase in favour of oxaloacetate formation in sonicated mitochondria from rat ovaries [9]) and both are dependent on NAD. If an NAD transferase is being measured by the "malic enzyme" assay, it is possible that its rate may contribute to the control of fatty acid oxidation and hence of steroidogenesis.

Acknowledgement

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