THE ROLE OF ADRENOCORTICAL MITOCHONDRIAL MALIC ENZYME IN NADPH GENERATION*

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1. Introduction

It was first suggested by Grant [1] that adrenal steroid 11β-hydroxylation reaction might be associated with C_4-dicarboxylic acids oxidation. Simpson and Estabrook [2, 3] later demonstrated that bovine adrenal cortex contains not only a cytosol malic enzyme, similar to pigeon liver enzyme characterized by Hsu and Lardy [4], but also an apparently intramitochondrial malic enzyme which is only seen after sonication. The activity of the mitochondrial enzyme was nearly sufficient to account for observed rates of steroid 11β-hydroxylation with intact mitochondria using malate as reductant [3, 5]. The method used to assay the malic enzyme activity did not, however, distinguish which reduced pyridine nucleotide was being formed upon malate oxidation. The increases in absorbance at 340 nm obtained by oxidation of malate were interpreted as direct reduction of NADP through malic enzyme, implying that adrenocortical mitochondria contain malic enzyme unlike heart, liver and kidney mitochondria.

In 1971, Stevenson and Taylor [6] demonstrated that in mitochondria obtained from corpora lutea of superovulated pig and rat ovaries, the apparent mitochondrial "malic enzyme activity" is due to the combined action of an inner membrane or mitosol malic dehydrogenase and an outer membrane NADP-phosphatase.

This raises a question on the reality of the "malic enzyme" activity of Simpson and Estabrook [3]. In this report, we shall demonstrate that at physiological pH, sonicated mitochondria contain a true malic enzyme, converting malate to pyruvate and generating NADPH. Only at very high pH, is the phenomenon of Stevenson and Taylor observed.

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2. Materials and methods

Adrenal cortex mitochondria were prepared from fresh slaughter house material. Cortex tissue was carefully dissected from the medulla. 20 g of tissue was ground in 8 vol of homogenizing media (0.32 M sucrose, 10 mM Tris-HCl, pH 7.8) by the use of a very loose teflon homogenizer (approx. 1/32 inch clearance), then homogenized by two full passes of a tight homogenizer (0.009 inch clearance). All centrifugations were carried out at 0°in a Sorvall SS-34 rotor. The homogenate was centrifuged at 750 *g* for 10 min. The supernatant was decanted from the pellet and the pH readjusted to 7.8 by NaOH. The mitochondrial fraction was collected by initial centrifugation at 2,000 *g*, 3 min followed by 9,500 *g* for 10 min. To decant the supernatant, the tubes were rapidly inverted allowing the "fluffy layer" to slough off the pellet. The heavy mitochondria were mechanically separated from any residual material at the bottom of the tube and gently resuspended in 80 ml of homogenizing media. This procedure was repeated 3 additional times using 40 ml of media for resuspension. The final pellet (typically 30–40 mg) was suspended to a protein concentration of approx. 10 mg/ml. These mitochondrial preparations were found to be nearly homogeneous as examined by electron microscopy.

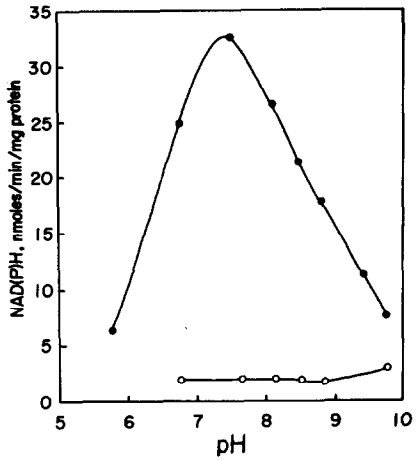

Fig. 1. Malic enzyme activity as a function of pH. The reaction mixture contained in a final volume of 3.25 ml, 0.32 M sucrose, 5 mM $MgCl_2$, 20 mM KCl, 10 mM Tris, 10 mM Glycine, 1.04 mM $NADP^+$ and appropriate amounts of mitochondrial preparations at a pH as indicated. Reaction was carried out at 25° and initiated by the addition of $NADP^+$. Rates were calculated for the initial linear period by comparing the relative fluorescence increase with time to that of a NADPH solution of known concentration. Sonication was conducted in homogenizing media (see Methods) using a Biosonik III at power setting 70. Sonication time was 2 min at 0° at a protein concentration of about 2 mg/ml. (○–○–○): intact mitochondria; (●–●–●): sonicated mitochondria.

Reduced pyridine nucleotide formation was determined by the fluorescent method using the excitation wavelength as 350 nm and the emission wavelength as 450 nm. Fluorometry was carried out by the use of a Hitachi spectrofluorometer, model 204.

Protein was determined by the biuret method. All chemicals used in this investigation were obtained from Sigma Chemical Company.

3. Results and discussion

Fig. 1 shows that sonicated mitochondria contain an enzyme(s) which produces reduced pyridine nucleotide when incubated with malate and NADP. At optimal pH (7.6)*, 32 nmoles of reduced pyridine nucleotide are produced per min per mg protein. No detectable reduction was observed if NAD was substituted for NADP. These results confirm the report of Simpson and Estabrook [3], and also indicate that the reduced pyridine nucleotide produced is probably NADPH and not NADH. This conclusion was more firmly established by the use of specific dehydrogenases to reoxidize the reduced pyridine nucleotide: muscle lactic dehydrogenase and pyruvate for NADH, and glutathione reductase and oxidized glutathione for NADPH. The results (fig. 2) show that the reduced pyridine nucleotide produced by sonicated mitochondria with malate and NADP at pH 7.6 is completely oxidized by glutathione reductase and glutathione (curve 1) but not by lactic dehydrogenase and pyruvate (curve 2), indicating that the reduced pyridine nucleotide is NADPH. The formation of NADPH is accompanied by the formation of pyruvate as evidenced by the fact that added NADH is reduced by lactic dehydrogenase without addition of pyruvate (curve 3).

In contrast to this, at pH 9.9 (fig. 2, curve 4), lactic dehydrogenase and pyruvate can reoxidize about 65% of the reduced pyridine nucleotide formed. The remaining part of the reduced nucleotide can be reoxidized by glutathione reductase and oxidized glutathione (curve 5). These results confirm the report of Stevenson and Taylor [6] that at elevated pH, incubation of the mitochondria with malate and NADP can produce NADH by the combined action of NADP-phosphatase and malic dehydrogenase. The presence of phosphatase is further shown in curve 6. Pre-incubation of NADP with sonicated mitochondria in the absence of malate at pH 9.9, yielded, upon subsequent addition of malate, a burst of pyridine nucleotide reduction, which was identified as NADH.

The percentage of NADPH and NADH formed is a function of pH (fig. 3). In the physiological pH region, the malic enzyme reaction produces exclusively NADPH. As the pH increases, the percentage of NADH increases until, at pH 9.9, 65% of the product is NADH. Since the endogeneous content of NAD or that produced by non-enzymatic hydrolysis of NADP was insignificant, and since malic enzyme is nearly inactive at high pH, the NADH formed must be the result of the combination of a phosphatase and malic dehydrogenase. This phosphatase activity was 4.0 nmoles per min per mg sonicated mitochondrial protein at pH 9.9, which is only 2% of the apparent value reported by Stevenson

* The pH profile resembles that of partially purified malic enzyme from adrenal glands (M.K. Mostafapour and T. Kimura, unpublished data).

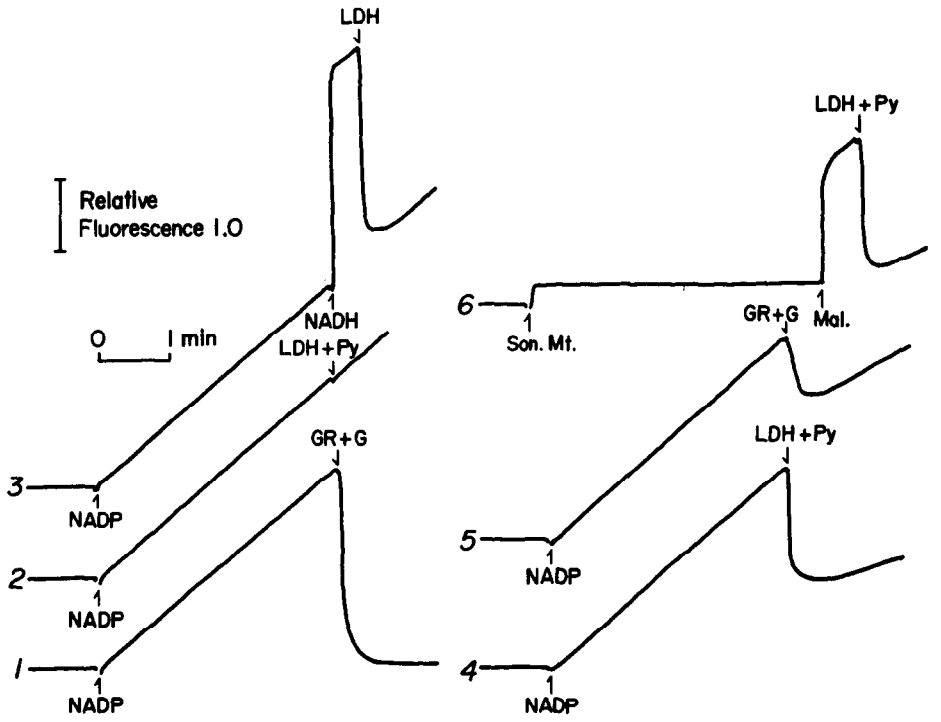

Fig. 2. Reoxidation of reduced pyridine nucleotides by specific enzymes. The reaction mixture was as described in fig. 1 using sonicated mitochondria (Son. MT.) as enzyme source. The pH for curves 1–3 was 7.6 and 9.9 for curves 4–6. Additions were as shown. Glutathione reductase (GR) from yeast (Sigma Type III) and beef heart lactic dehydrogenase (LDH) (Sigma Type III) when used were added to a final activity of 1.0 and 10.0 μmoles NADPH (NADH) oxidation/min, respectively. Pyruvate (Py) and oxidized glutathione (G) were used in excess of the total reduced nucleotide pool.

and Taylor for intact mitochondria [6]. Under these conditions, the rate of reduction of NAD by malate and malic dehydrogenase was in far excess of the phosphatase activity, as illustrated in curve 6 of fig. 2. It is not known whether this is due to tissue differences or due to different purity of the mitochondrial preparations.

Although malic enzyme is present and functional in adrenocortical mitochondria, the question whether or not energy-linked transhydrogenase (NADH → NADPH) has an important physiological role for steroid hydroxylation reaction cannot be answered. The fact that α-ketoglutarate-supported steroid 11β-hydroxylase activity in the presence of malonate is about as active as the malate-supported activity, is evidence in support of the involvement of transhydrogenase. Perhaps malic enzyme and transhydrogenase both contribute to intramitochondrial NADPH-production depending on energy levels in the mitochondria.

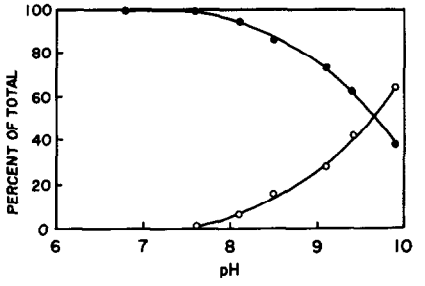

Fig. 3. The percentage of reduced pyridine nucleotides formed by malate oxidation as a function of pH. Incubations were conducted as described in fig. 1 at pH as indicated. After approx. 0.10 μmole reduced pyridine nucleotide had accumulated glutathione reductase plus oxidized glutathione or lactic dehydrogenase plus pyruvate was added. (Similar to experiments in fig. 2). The percentage of either nucleotide was then calculated from the immediate decrease in fluorescence representing reoxidation. (●–●–●): NADPH; (○–○–○): NADH.

Acknowledgements

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