THE ROLE OF Ca^{2+} IN CONTROL OF MALIC ENZYME ACTIVITY IN BOVINE ADRENAL CORTEX MITOCHONDRIA

by

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Summary: Low physiological levels of Ca^{2+}, in the presence of Mg^{2+} allow the reduction of extramitochondrial NADP+ via intramitochondrial malic enzyme. The rate of reduction is dependent on the concentration of Ca^{2+} and Mg^{2+}. The Ca^{2+} levels producing the appearance of malic enzyme activity also cause an ultrastructural transformation from the aggregated to the orthodox form. The phenomenon requires electron transport and is blocked by agents which interfere with active Ca^{2+} accumulation.

INTRODUCTION

The adrenal cortex mitochondria are known to have an unusual ultrastructure which may be associated with their function of performing steroid hydroxylations, involving cytochrome P450 and an electron transport chain. The source of intramitochondrial NADPH for these reactions remains uncertain but is generally believed to be derived from a mitochondrial malic enzyme and/or pyridine nucleotide transhdrogenase(s). Exogenous NADPH can be utilized in the presence of Ca^{2+}, reaching an optimum at high Ca^{2+} (11 mM) where mitochondrial swelling is maximal (1). As these authors pointed out, these high Ca^{2+} levels are not likely to be of physiological significance. Malic enzyme has been found in adrenal cortex (2, 3, 4) bovine heart (5) and rat brain (6) mitochondria but only after sonication. The evidence supporting malic enzyme

involvement in steroid biosynthesis is largely based on the stoichiometric appearance of pyruvate during 11β hydroxylation (7).

Low levels of Ca^{2+}, 100-500 nmoles/mg protein, are known to have definite effects on adrenal mitochondria. These include an increased rate of oxidation of Krebs cycle intermediates (8) and a marked change in ultrastructure which is accompanied by a general decrease in the efficiency of coupled processes including oxidative phosphorylation (9). The data to be presented in this communication show that low physiological concentrations of Ca^{2+} can unveil the malic enzyme activity of "intact" mitochondria and generate free NADPH. These results suggest that extramitochondrial steroid hydroxylation, such as 21 and 17 α hydroxylases, may also be dependent, in part, on the mitochondria for their supply of NADPH in a system where cytoplasmic $NADP^{+}$ reduction via intramitochondrial malic enzyme is controlled by the level of free Ca^{2+} in the cells.

MATERIALS AND METHODS

Bovine adrenal cortex mitochondria were prepared as previously described (4) with the exception that the isolation media contained 0.10 mM sodium EDTA and 0.4% w/v bovine serum albumin, low fatty acid type. This solution was used throughout the isolation except during the final wash when EDTA and albumin were omitted. In addition to this modification, readjustment of pH to 7.8 after the low speed centrifugation was omitted. All sucrose solutions used in these studies were deionized prior to use according to the method of Green (10). Malic enzyme was assayed fluorometrically, or alternatively, spectrophotometrically at 340 nm using an Aminco-Chance dual wavelength/split-beam spectrophotometer. Protein concentration was determined by the biuret method in the presence of deoxycholate. All reagents utilized in this work were obtained from Sigma.

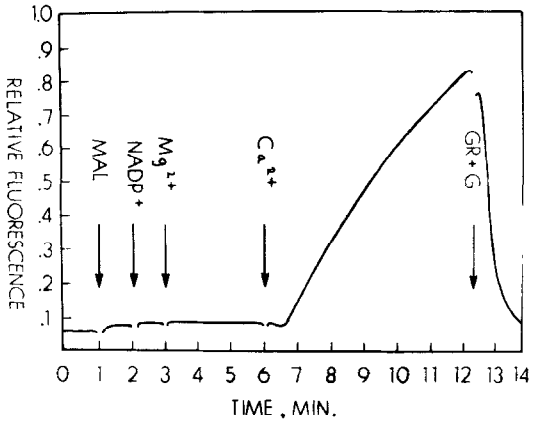

Fig. 1.

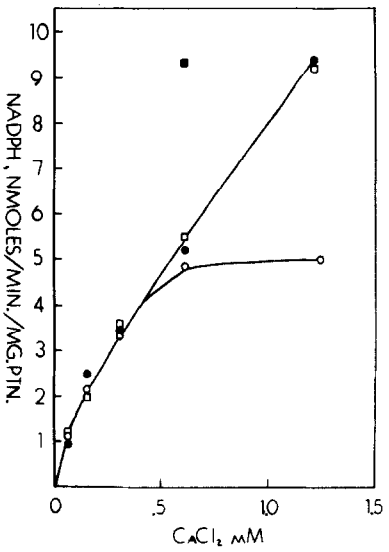

Fig. 2.

Figure 1

Ca^{2+} dependent reduction of extramitochondrial $NADP^{+}$ by malic enzyme in "intact" mitochondria.

NADPH formation was monitored fluorometrically using the Hitachi Perkin Elmer model 203 spectrofluorometer, excitation-350 nm, emission-450 nm. At t = 0, the reaction mixture contained in a volume of 3.0 ml, 0.32 M sucrose 10 mM Tris-Cl, pH 7.4 and 1.0 mg mitochondrial protein. Additions made as indicated in the figure were 7 mM sodium malate, 2 mg NADP+, 5mM $MgCl_2$ 0.5 mM $CaCl_2$ and excess glutathione reductase plus oxidized glutathione (GR+G). A fluorescence intensity of 1.0 is equivalent to 20 μM NADPH.

Figure 2

The rate of NADPH formation by intramitochondrial malic enzyme as a function of Ca^{2+} and Mg^{2+} concentrations.

NADPH formation was monitored at 340 nm. The reaction media contained sucrose, Tris-Cl, mitochondria, sodium malate and $NADP^{+}$ as described in the legend to Figure 1. $MgCl_2$ was present at concentrations of 1 mM (O), 5 mM (□) and 20 mM (●) respectively. Reduction was initiated by addition of $CaCl_2$ to a final concentration as shown. The single point designated as ■ contained all assay components at 5 mM $MgCl_2$ plus 7 mM sodium succinate and 5 mM sodium phosphate.

RESULTS AND DISCUSSION

Figure 1 shows that in agreement with our earlier work (4) and that of

Estabrook (3, 7) intact adrenal mitochondria incubated in the presence of malate and Mg^{2+} do not reduce exogenous $NADP^{+}$. Addition of Ca^{2+} resulted in rapid formation of NADPH which could be rapidly reoxidized by excess glutathione reductase and oxidized glutathione indicating that the NADPH was extramitochondrial. Altering the order of addition of Ca^{2+}, Mg^{2+}, $NADP^{+}$ and malate gave similar results, indicating that all components are required. These results are reproducible only with mitochondria prepared in the presence of bovine serum albumin which is believed to remove free fatty acids from the mitochondria.

The relationship between malic enzyme activity and the concentration of Ca^{2+} and Mg^{2+} is shown in Figure 2. In the presence of 1 mM Ca^{2+} and 5 mM Mg^{2+} (chosen for approximation with in vivo concentrations) the rate of NADPH formation is 8 nmoles/min/mg protein. This rate is variable from one preparation of mitochondria to another and can be as high as 14 nmoles/min/mg protein at 0.66 mM Ca^{2+}. The figure also shows that more malic enzyme activity can be obtained with "intact" mitochondria by increasing cation concentration or the addition of phosphate and succinate. The possible existence of other activators and the optimal conditions for observing malic enzyme are currently under investigation.

The observed reduction of $NADP^{+}$ appears to require active Ca^{2+} uptake coupled to the standard electron transport chain. NADPH formation is inhibited (over 80%) by either antimycin A (2 μg/mg protein) or ruthenium red (10 nmoles/mg protein). Both agents were found to have no effect on solubilized malic enzyme. Ruthenium red inhibition was overcome by the addition of ADP which is known to restore rapid oxidation rates in liver mitochondria when the Ca^{2+} stimulated rate has been previously blocked by addition of ruthenium red (12).

Also worthy of mention is the observation that α-ketoglutarate plus 5 mM malonate cannot replace malate for the reduction of extramitochondrial $NADP^+$, indicating that free NADPH is not formed by transhydrogenation between NADH and $NADP^+$. The reason for a Mg^{2+} requirement is not clear at present. Hsu and Lardy found that the soluble pigeon liver malic enzyme requires either Mn^{2+} or Mg^{2+} as cofactor (13). The adrenal cortex mitochondrial enzyme shows a similar requirement (T. Kimura and M. Mostafapour, unpublished observations) suggesting that Mg^{2+} is required for malic enzyme per se. However, Green found that the intramitochondrial Mg^{2+} level is not depleted by isolation in 0.1 mM EDTA (14). The latter worker suggested that external Mg^{2+} is required for Ca^{2+} transport. In the present study both Mg^{2+} and Ca^{2+} are required. It is not possible at the present to say what are the mechanisms involved.

The mitochondria used in these studies are in the aggregate form when isolated. Addition of Ca^{2+}(0.5 mM) in the presence of Mg^{2+} (5 mM) caused a change to the orthodox form (unpublished results of Dr. T. Kuo), somewhat at variance with the results of Green and coworkers who observed that adrenal cortex mitochondria, prepared in the absence of bovine serum albumin, remained in the aggregate form when exposed to 1 mM Ca^{2+} and 10 mM Mg^{2+} (9). Whether this ultrastructural change is required for the appearance of malic enzyme activity remains to be determined.

The concentration of Ca^{2+} required to invoke malic enzyme dependent reduction of extramitochondrial $NADP^+$ is within the physiological concentration of Ca^{2+} of the adrenal cortex (1 mM) (14). It is not known how much of this Ca^{2+} is free, chelated by ATP or bound to membranes. As shown by Sutherland et al., hormonal activation of adenyl cyclase can raise c-AMP levels as high as 400 nmoles/g adrenal in rat (15). As has been suggested by Rasmussen and Tennenhause (16) this could release significant amounts of Ca^{2+} from Ca^{2+}-ATP

complex or otherwise result in elevated levels of free intracellular Ca^{2+}. This Ca^{2+} could activate the mitochondrial malic enzyme. Reduction of cytoplasmic $NADP^{+}$ would be expected which should be available to microsomal hydroxylases. Whether this NADPH is also utilized intramitochondrially is not known at present. The possibility that cytoplasmic NADPH could be generated in a similar manner in other tissues which contain a mitochondrial malic enzyme should also be considered.

The results reported here, together with the implication of mitochondrial malic enzyme in 11β-hydroxylation and the enhanced 11β-hydroxylation accompanying Ca^{2+}-induced orthodox mitochondrial structure formation (17), suggest a close relationship between Ca^{2+}, mitochondrial structure and steroid hydroxylation. A more detailed investigation of this phenomenon is in progress.

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