ANION TRANSPORT IN BEEF ADRENAL CORTEX MITOCHONDRIA

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1. Introduction

Adrenal cortex mitochondria are able to catalyze at least two reactions in the conversion of cholesterol to corticosterone, namely the shortening of the iso-octyl chain of cholesterol and the hydroxylation at C-11. These reactions are located in the inner membrane matrix space of mitochondria [1–3] and require a mixed function oxidation system for which NADPH is the donor of reducing equivalents. Since the inner mitochondrial membrane is impermeable to pyridine nucleotides [4] the enzymatic reduction of NADP must occur within the matrix space. This could be brought about by NADP linked dehydrogenases present in adrenal cortex mitochondria (NADP isocitrate dehydrogenase for rat mitochondria and NADP malate dehydrogenase for beef mitochondria [5]), if an adequate supply of substrate is maintained. One may ask therefore whether adrenal cortex mitochondria, similarly to liver mitochondria possess transport systems for citric acid cycle intermediates to ensure the entry of specific substrates for NADP dehydrogenases. The present paper provides experimental evidence for the presence of malate, succinate and oxoglutarate carriers in beef adrenal cortex mitochondria. A preliminary account of this work has been communicated [6].

2. Materials and methods

Beef adrenal cortex mitochondria were prepared as previously described [2] in a medium containing 0.27 M sucrose, 10 mM Tris-HCl pH 7.4 and either 0.2% bovine serum albumin (BSA mitochondria) or 0.5 mM EGTA (EGTA mitochondria). The twice-washed mitochondria were stored at 0° in a concentrated suspension (50–70 mg protein/ml).

The entry of various anions of the citric acid cycle was followed either by reduction of endogenous pyridine nucleotides or by swelling in the ammonium salts of the corresponding anions or by uptake of labelled anions.

The reduction of intramitochondrial pyridine nucleotides was assayed with a double beam spectrophotometer at 340–374 nm [7]. In this assay, mitochondria were depleted of endogenous pyridine nucleotides by a preincubation with FCCP (carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone) and then supplemented with rotenone to block the respiratory chain.

Mitochondrial swelling was followed by light scattering changes as described by Chappell and Crofts [8]. The variation of extramitochondrial pH corresponding to the uptake of malate was monitored potentiometrically using a fast responding low resistance glass electrode (Ingold) and a radiometer pH-meter connected to a Servotrace recorder.

The technique used for measuring the kinetics of anion uptake was that described by Quagliariello et al. [9]. The uptake was started by addition of ^{14}C-labelled anion and terminated by addition of the specific inhibitor [10] and rapid centrifugation in a 3200 Eppendorf centrifuge. The sediment was dissolved in 4% sodium cholate and the radioactivity was determined by liquid scintillation. The ^{14}C-radioactivity external to the matrix space was calculated in a parallel assay where the inhibitor was added before the ^{14}C-anion.

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3. Results

Two types of mitochondrial preparation were used in these experiments: EGTA mitochondria and BSA mitochondria. EGTA mitochondria differed from BSA mitochondria in particular in that they are loosely coupled and that their respiratory carriers are more oxidized, probably because they are partially depleted of endogenous substrates.

Fig. 1 shows assays where the reduction of intramitochondrial pyridine nucleotides by malate was followed spectrophotometrically. The rate of reduction of pyridine nucleotides was greater and the extent of the reduction was larger in EGTA mitochondria than in BSA mitochondria. Furthermore, whereas phosphate stimulated both the rate and extent of NAD(P) reduction in EGTA mitochondria, it had no effect in BSA mitochondria. Provided phosphate and a trace of malate (30 μM) were present, the addition of oxoglutarate resulted in NAD(P) reduction. By contrast to oxoglutarate, citrate did not bring about any reduction of NAD(P). The pH dependence of the rate

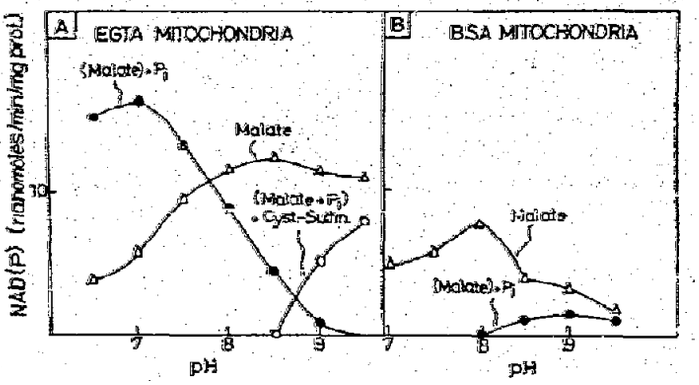

Fig. 2. pH dependency of the rate of NAD(P) reduction by L-malate and of the stimulation by phosphate and cysteine sulfinate. The same conditions as in fig. 1.

of NAD(P) reduction by malate is shown in fig. 2. An optimum pH around 8 was found for the reduction of NAD(P) by malate alone. The optimum pH for phosphate stimulation was 7 in the case of EGTA mitochondria and higher than 8 for BSA mitochondria. Finally cysteine sulfinate which transaminates oxaloacetate

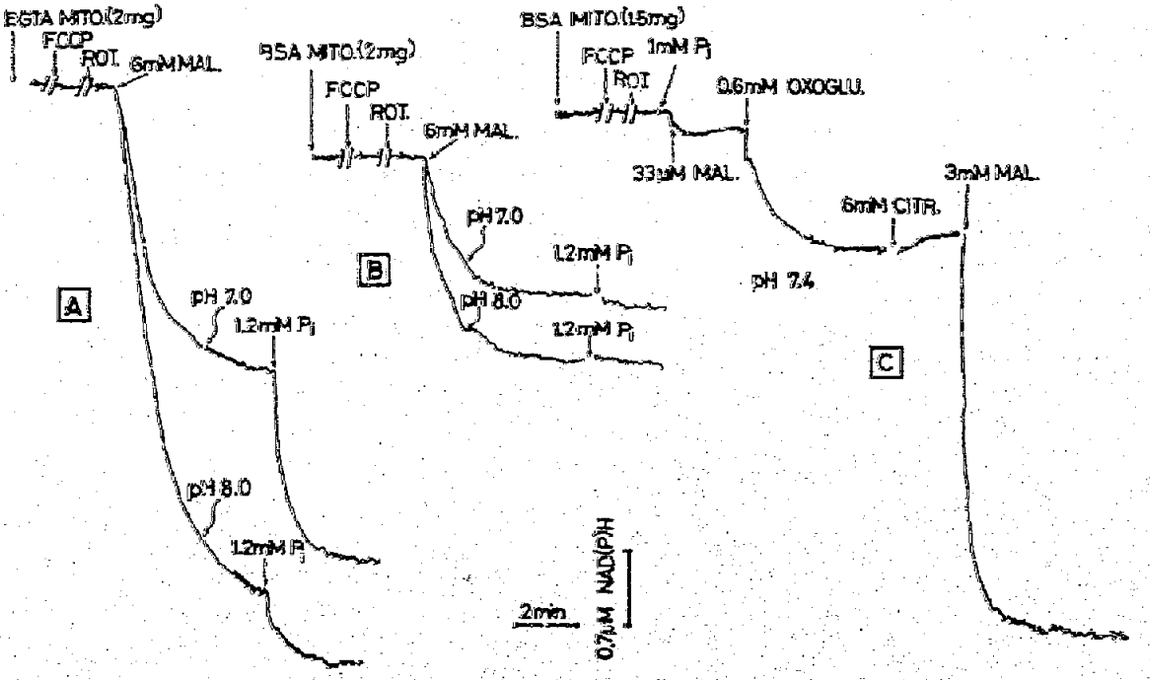

Fig. 1. Reduction of NAD(P) in adrenal cortex mitochondria by L-malate and α-ketoglutarate. Absence of reduction by citrate. Measurements were made at 340–374 nm with a double beam spectrophotometer. Beef adrenal cortex mitochondria (EGTA and BSA mitochondria) were incubated for 3 min at 25° in 3.3 ml of a medium containing 0.27 M sucrose, 10 mM Tris-Hepes, 1.2 μM FCCP was added to allow an extensive oxidation of endogenous substrates followed after 3 min by 3 μM rotenone to block the respiratory chain. Other additions were as indicated.

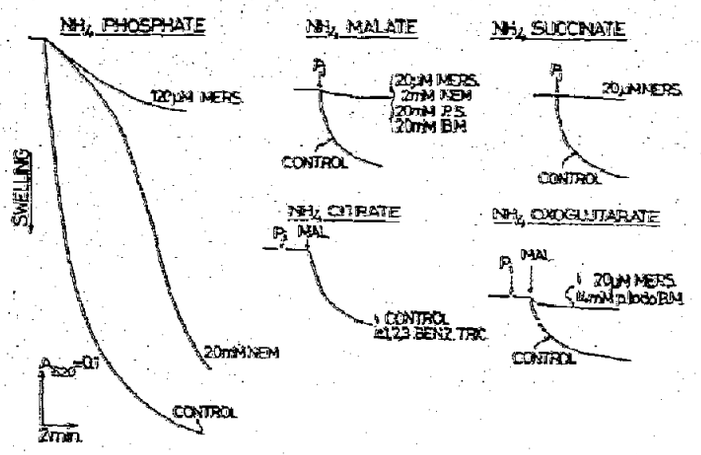

Fig. 3. Swelling of beef adrenal cortex mitochondria suspended in isoosmotic solutions of NH_4 phosphate, malate, succinate, citrate and oxoglutarate. 2.2 mg of mitochondria in 0.1 ml sucrose were added to 2.8 ml of a medium made of 20 mM Tris, 1 mM EDTA and 100 mM of the various ammonium salts. The pH was 7.4 and the temperature 25°. Where indicated, 2 mM phosphate and 2 mM malate (final conc.) were added 1 and 2 min, respectively, after mitochondria. The light scattering change was followed at 520 nm.

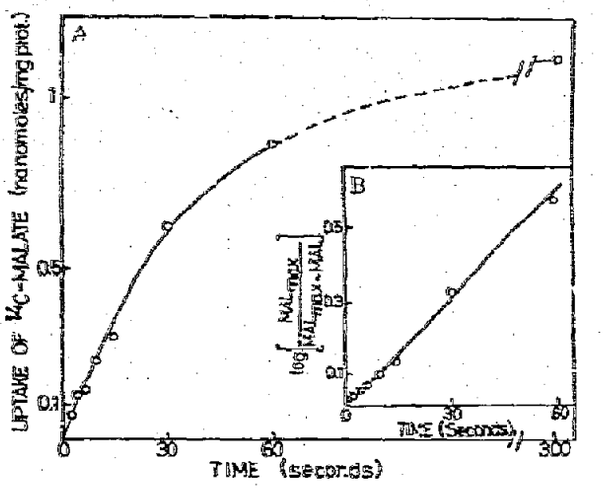

Fig. 4. A) [^{14}C]Malate uptake by adrenal cortex mitochondria. Mitochondria (6.3 mg protein) were preincubated for 30 sec in a reaction medium containing: 100 mM KCl, 25 mM Tris-MES, 1 mM EGTA, 5 μM rotenone, 2 μg/ml antimycin, 2.5 μg/ml oligomycin. Final volume 0.9 ml, final pH 6.5, temperature 0°. Then 23 μM [^{14}C]malate were added. The malate uptake was stopped after various periods of incubation by addition of 10 mM mersalyl followed by rapid centrifugation of the mitochondria in a 3200 Eppendorf centrifuge. B) Logarithmic plot of [^{14}C]malate uptake. MAL_{max} refers to malate accumulated after the equilibrium was reached (after 5 min).

further enhanced NAD(P) reduction after addition of malate and phosphate above pH 8.5. These spectrophotometric assays deserve a few comments: i) although reduction of NAD(P) is indicative of the penetration of added malate into mitochondria, the overall process leading to NAD(P) reduction depends on other factors like the dehydrogenation reaction itself which may account for the pH effect; ii) The stimulatory effect of cysteine sulfinate above pH 8.5 suggests accumulation of oxaloacetate at pH higher than 8.5.

The entry of phosphate and various anions of the citric acid cycle into beef adrenal cortex mitochondria has also been followed by light scattering changes when mitochondria are suspended in isoosmotic solutions of ammonium salts of the respective anions (fig. 3). As in liver mitochondria, swelling was induced by addition of NH_4 phosphate, NH_4 malate or succinate in the presence of phosphate, NH_4 oxoglutarate in the presence of phosphate and malate. In all assays, swelling was sensitive to the sulfhydryl-blocking reagent, mersalyl which, at low concentrations, is a specific inhibitor of phosphate transport.

Inhibitors of dicarboxylate transport in liver mitochondria, namely phenylsuccinate, butylmalonate, *p*-iodobenzylmalonate blocked also the entry of malate, succinate or oxoglutarate in beef adrenal cortex mitochondria. A special case is that of citrate. A swelling in isoosmotic NH_4 citrate was obtained after addition of small amounts of phosphate and malate. However, 1,2,3-benzene tricarboxylate, which is an inhibitor of citrate entry in rat liver mitochondria [11] had no inhibitory effect on the citrate-induced swelling of beef adrenal cortex mitochondria (fig. 3).

Results from spectrophotometric and light scattering experiments were corroborated by isotopic assays. Adrenal cortex mitochondria incubated with [^{32}P]phosphate, [^{14}C]malate, [^{14}C]succinate, [^{14}C]malonate, [^{14}C]oxoglutarate rapidly incorporated, even at 0°, radioactivity into the matrix space, correction being made for the extramatrix space in a parallel experiment carried out with [^{14}C]sucrose. By contrast the entry of [^{14}C]citrate was not significant even in the presence of phosphate and malate. As shown in fig. 4, the [^{14}C]malate uptake follows a first order kinetics with a rate constant of 1.4 min^{-1} at 0°. From the maximum amount of malate accumulated at equilibrium (1.2 nmole/mg protein), a rate of transport of 1.7 nmole/min/mg protein can be calculated. The K_m for malate entry calculated for experiments was in the

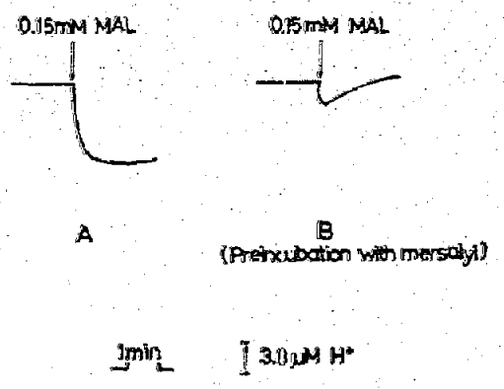

Fig. 5. Proton movements accompanying the penetration of malate into mitochondria. BSA mitochondria (20 mg) were preincubated in 6.5 ml of a medium made of 0.125 M KCl, 0.5 mM EGTA and 2 μg/ml antimycin. The pH was adjusted to 6.35. Temperature was 0°. In experiment (A) 0.150 mM malate (pH 6.35) was added after 2 min incubation. In experiment (B) 1.5 mM mersalyl was added to the incubation mixture 2 min before malate.

range of 0.06 to 0.1 mM. In other experiments (not shown), it was found that malate or malonate added to [^{14}C]malate-loaded adrenal cortex mitochondria initiates a rapid efflux of [^{14}C]malate.

The entry of malate was accompanied by a rapid disappearance of protons from the medium as shown by the increase of external pH (fig. 5). This increase of pH was paralleled by an efflux of internal phosphate (of the order of 2.5 nmoles/mg protein) and was inhibited by mersalyl.

4. Discussion

Transport of citric cycle anions into beef adrenal cortex mitochondria has been investigated with three different approaches. i) Reduction of intramitochondrial NAD(P) upon addition of anions, ii) light scattering changes in isoosmotic solutions of NH_4 salts of corresponding anions, iii) direct uptake of ^{14}C-labelled anions. Mitochondria prepared in the presence of EGTA (EGTA mitochondria), which are largely depleted of endogenous substrates, were found more suitable than mitochondria prepared in the presence of BSA for studying the anion carriers. The data obtained with the three above methods concur to indicate that beef adrenal cortex mitochondria are able to transport malate, succinate and oxoglutarate, but apparently not citrate, in disagreement with a recent report [12]. A lack or poor efficiency of citrate carrier in beef heart mitochondria has also been reported [13].

Malate entry into beef adrenal cortex mitochondria is accompanied by a proton uptake and a phosphate efflux. This finding together with the sensitivity of the proton uptake to mersalyl (cf. fig. 5) affords suggestive evidence for the occurrence of a malate-phosphate carrier in beef adrenal cortex mitochondria. The demonstration of a malate and oxoglutarate transport allows us to conclude that beef adrenal cortex mitochondria are equipped, like rat liver mitochondria, with a dicarboxylate-oxoglutarate carrier. Finally a malate–malate exchange in beef adrenal cortex mitochondria is evidenced by the observation that malate efflux from [^{14}C]malate-loaded mitochondria is initiated by addition of malate. A rapid reduction of intramitochondrial NADP is required for some steps in steroidogenesis occurring in the mitochondrial compartment of the adrenal cortex, for instance the cleavage of the side chain of cholesterol and the 11β-hydroxylation of deoxycorticosterone into corticosterone. This ability of adrenal cortex mitochondria to efficiently transport malate makes it likely that the cytosol is a major source of reducing equivalents for intra-mitochondrial steroid metabolism.

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