SHORT COMMUNICATIONS

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The kinetics of tricarboxylate anion oxidation by rat liver mitochondria in relation to the availability of L-malate

The requirement for L-malate for the transport of tricarboxylate anions across the mitochondrial membrane has been demonstrated by the use of several techniques[1-8]. The present investigation attempts to elucidate the kinetics of the tricarboxylate anion transporting system, taking into account the activities of the enzymes of mitochondrial citrate metabolism.

When rat liver mitochondrial NAD(P)$^+$ redox changes are followed by 340–373-nm double-beam spectrophotometry, a rapid oxidation of pyridine nucleotides occurs on addition of uncoupling agent. After the addition of respiratory inhibitors such as rotenone or antimycin, addition of *threo*-D_s-isocitrate causes little reduction until L-malate is added, when intramitochondrial NAD(P)$^+$ is rapidly reduced (Fig. 1). Experiments were performed in a similar manner varying the concentration of L-malate at constant isocitrate concentrations and Lineweaver–Burk plots constructed for the reciprocal of the rate of mitochondrial NAD(P)$^+$ reduction *versus* the reciprocal

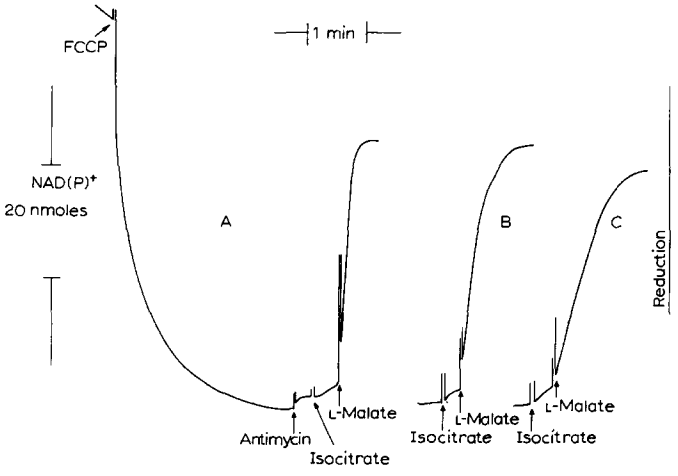

Fig. 1. Effect of different concentrations of L-malate on the rate of reduction of intramitochondrial NAD(P)$^+$ by isocitrate. Rat liver mitochondria (3 mg protein) were suspended in 2.5 ml of a medium containing 125 mM KCl, 20 mM Tris–HCl and 2 mM P_i, pH 7.4, at 30°. The redox changes of intramitochondrial NAD(P)$^+$ were monitored by 340–373-nm double-beam spectrophotometry. Additions were as indicated using the following final concentrations of reagents. A. Carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), 1 μM; antimycin, 0.16 μg/ml; *threo*-D_s-isocitrate, 250 μM; L-malate, 800 μM. B. FCCP, 1 μM; antimycin, 0.16 μg/ml (not shown); *threo*-D_s-isocitrate, 250 μM; L-malate, 200 μM. C. FCCP, 1 μM; antimycin, 0.16 μg/ml (not shown); *threo*-D_s-isocitrate, 250 μM; L-malate, 100 μM.

Abbrevation: FCCP, *p*-trifluoromethoxyphenylhydrazone.

of the L-malate concentration (Fig. 2). The K_a for L-malate was found to be independent of isocitrate concentration at 220 μM. Similar plots of *cis*-aconitate and citrate oxidation confirmed the K_a for L-malate to be independent of tricarboxylate anion concentration and values of 160 μM and 70 μM, respectively, were obtained. v_{max} was dependent on tricarboxylate anion concentration.

Fig. 3 shows reciprocal plots for the reduction of intramitochondrial NAD(P)$^+$ in the presence of two different concentrations of L-malate with respect to isocitrate concentration. As can be seen, the K_m for isocitrate was independent of L-malate concentration while v_{max} was dependent. The K_m values obtained for citrate, *cis*-aconitate and isocitrate were 90 μM, 77 μM and 67 μM, respectively.

In order to assess the restriction imposed by the permeability barrier on tricarboxylate anion oxidation, the mitochondrial enzymes were investigated as regards to their kinetic parameters but assayed in the same proportions as they existed together in the rat liver mitochondria. Sonication of the liver mitochondria allowed the activities of the enzymes to be assessed, but in some cases the enzymes needed to be protected during sonication. For the estimation of aconitate hydratase, liver mitochondria (60–80 mg protein per ml) were diluted to twice their volume in a buffer containing 125 mM KCl, 20 mM Tris–HCl, pH 7.4, and sonicated while cooling with a freezing mixture at $-5°$. The estimation of NADP-linked isocitrate dehydrogenase could be performed on a sonicate of this type, but for the NAD-linked isocitrate dehydrogenase the mitochondria were diluted as before but sonicated in the presence of 2 mM ADP, 2 mM $MgCl_2$ and 10 mM reduced glutathione. In the latter case the sonicate was kept at room temperature as the enzyme is cold labile[9].

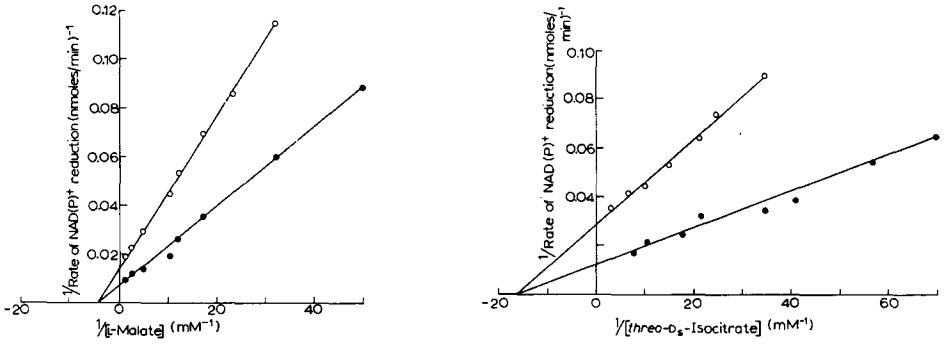

Fig. 2. Lineweaver–Burk plots to show the dependence on L-malate concentration of *threo*-D_s-isocitrate oxidation by mitochondria. The reciprocal of the rate of intramitochondrial NAD(P)$^+$ reduction is plotted against the reciprocal of L-malate concentration. The initial conditions were as for Fig. 1 using 3 mg mitochondrial protein. After antimycin addition, isocitrate (60 or 250 μM) was added and the reduction of intramitochondrial NAD(P)$^+$ on the addition of L-malate was recorded by 340–373-nm double-beam spectrophotometry. The experiment was repeated at a range of malate concentrations, at 60 μM isocitrate (○—○) and 250 μM isocitrate (●—●).

Fig. 3. Lineweaver–Burk plots at two L-malate concentrations to show the dependence of isocitrate oxidation on the concentration of *threo*-D_s-isocitrate. The reciprocal of the rate of intramitochondrial NAD(P)$^+$ reduction is plotted against the reciprocal of *threo*-D_s-isocitrate concentration. The initial conditions of incubation were as for Fig. 1 using 3 mg mitochondrial protein per incubation. After antimycin addition isocitrate (varying concentrations) was added and the reduction of intramitochondrial NAD(P)$^+$ on the addition of L-malate was recorded by 340–373-nm double-beam spectrophotometry. Malate concentrations were 1 mM (●—●), and 0.12 mM (○—○).

TABLE I

THE ACTIVITY OF ENZYMES FROM RAT LIVER MITOCHONDRIA CONCERNED IN CITRATE METABOLISM

For No. 1, 2 and 3 below, 100 μl sonicated rat liver mitochondria (2 mg protein) were suspended in a medium containing 125 mM KCl–20 mM Tris–HCl, pH 7.4. 0.2 mM $NADP^+$ and 2 mM $MgCl_2$ were added and the reaction started by the addition of substrate. $NADP^+$ reduction was followed at 340 nm spectrophotometrically. For No. 4, 100 μl rat liver mitochondria sonicated in the ADP–Mg^{2+}–reduced glutathione medium described below were suspended in a medium containing 95 mM KCl–20 mM Tris–HCl–20 mM potassium phosphate, pH 7.4, in the presence of 2 mM NAD^+, 2 mM $MgCl_2$ and 2 mM ADP; the reaction was started by the addition of isocitrate and NAD^+ reduction followed at 340 nm spectrophotometrically. The v_{max} values for whole rat liver mitochondria were obtained from experiments performed as described for Fig. 1 using 2 mM citrate, *cis*-aconitate and *threo*-D_s-isocitrate in the presence of 2 mM L-malate. The temperature was 30°.

Enzyme	*Substrate*	K_m (μM)	*Activity at* v_{max} (*nmoles/min per mg*)	
			Broken mitochondria	*Whole mitochondria*
1. Aconitate hydratase	Citrate	90	9	9
2. Aconitate hydratase	*cis*-Aconitate	13	48	38
3. NADP–isocitrate dehydrogenase	*threo*-D_s-Isocitrate	2.5	66	52
4. NAD–isocitrate dehydrogenase	*threo*-D_s-Isocitrate	280	11	

Table I sets out the parameters found for the two dehydrogenases and aconitate hydratase. The K_m values for aconitate hydratase and NADP–isocitrate dehydrogenase were obtained from Lineweaver–Burk plots but for NAD–isocitrate dehydrogenase the K_m was obtained from a velocity *versus* substrate concentration plot, as the latter enzyme showed allosteric kinetics. The enzymic activity of NAD–isocitrate dehydrogenase obtained from sonicated rat liver mitochondria agrees with that found by STEIN *et al.*[10] and PLAUT AND AOGAICHI[9], but is somewhat lower than that found by GARLAND *et al.*[11] and is much lower than that reported by GARLAND[12]. NADP–isocitrate dehydrogenase activity was corrected for contaminant extramitochondrial enzyme, by performing a parallel assay for activity with whole mitochondria using a double-beam spectrophotometer. This may be the reason for the lower activities obtained compared with other values reported[10,11]. It was found that the kinetic constants obtained for *cis*-aconitate and NADP-linked isocitrate oxidation were much lower than those obtained with whole mitochondria. The K_m for citrate oxidation through aconitate hydratase by NADP–isocitrate dehydrogenase was virtually the same in whole and broken preparations and in fact the rates of reduction of $NAD(P)^+$ obtained with whole mitochondria with citrate at saturating L-malate concentrations were the same as those obtained in a broken preparation. Thus for citrate, the limitation imposed by aconitate hydratase is greater than that imposed by the membrane permeability so long as L-malate is saturating. The situations with *cis*-aconitate and isocitrate are more complex due to the presence of both isocitrate dehydrogenases, but under these conditions of measurement when both the NAD and NADP are almost fully oxidised before the addition of substrate, it seems reasonable to suppose that the enzyme possessing the greater activity and lower K_m for the substrate will pre-

dominate in the ensuing oxidation. Taking this into consideration it does appear that the membrane imposes a limitation on the rates of oxidation of *cis*-aconitate and isocitrate by rat liver mitochondria under these conditions, even at saturating L-malate concentrations. This limitation, it must be emphasised would probably have little bearing on the situation *in vivo* where mitochondrial $NADP^+$ is substantially reduced and the NAD–isocitrate dehydrogenase would predominate in the oxidation of isocitrate derived from citrate.

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1 J. B. CHAPPELL, *Biochem. J.*, 90 (1964) 225.
2 J. B. CHAPPELL, *Abstr. 6th Intern. Congr. Biochem., New York, 1964*, 8 (1964) 625.
3 J. B. CHAPPELL AND K. N. HAARHOFF, in E. C. SLATER, Z. KANIUGA AND WOJTCZAK, *Biochemistry of Mitochondria*, Academic Press and Polish Scientific Publishers, London and Warsaw, 1966, p. 75.
4 S. M. P. FERGUSON AND G. R. WILLIAMS, *J. Biol. Chem.*, 241 (1966) 2696.
5 B. H. ROBINSON AND J. B. CHAPPELL, *Biochem. Biophys. Res. Commun.*, 28 (1967) 249.
6 J. B. CHAPPELL AND B. H. ROBINSON, *Biochem. Soc. Symp.*, 27 (1968) 123.
7 S. PAPA, R. D'ALOYA, A. J. MEIJER, J. M. TAGER AND E. QUAGLIARIELLO, in S. PAPA, J. M. TAGER, E. QUAGLIARIELLO AND E. C. SLATER, *The Energy Level and Metabolic Control in Mitochondria*, Adriatica Editrice, Bari, 1969, p. 159.
8 A. J. MEIJER, N. E. LOFRUMENTO, S. PAPA, J. M. TAGER AND E. QUAGLIARIELLO, *Biochem. J.*, in the press.
9 G. W. E. PLAUT AND T. AOGAICHI, *Biochem. Biophys. Res. Commun.*, 28 (1967) 628.
10 A. M. STEIN, J. H. STEIN AND K. KIRKMAN, *J. Biol. Chem.*, 246 (1967) 1371.
11 P. B. GARLAND, D. SHEPHERD, D. G. NICHOLLS AND J. ONTKO, in G. WEBER, *Advan. Enzyme Regulation*, 6 (1968) 3.
12 P. B. GARLAND, *Biochem. Soc. Symp.*, 27 (1968) 41.

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The active chlorophyll a_{II} in suspensions of lyophilized and Tris-washed chloroplasts

In photosynthesis, light absorbed in two different pigment systems is used for two different light reactions[1,2]. The reaction center for System I (the P700) was discovered by KOK[3]. Suggestions for the existence of a trap in System II came first from EMERSON AND RABINOWITCH[4]. Further hints came from fluorescence studies at low temperatures[5–7]. Recently DÖRING *et al.*[8] have discovered absorbance changes due to chlorophyll *a* (Chl *a*) in System II with peaks at 435 nm and 682–690 nm[9] and a half-life a 100 times shorter than that of P700.

Abbreviations: Chl, chlorophyll; DCMU, 3(3,4-dichlorophenyl)-1,1-dimethylurea.