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The choline transporter is the major site of control of choline oxidation in isolated rat liver mitochondria

Claude P. Kaplan, Richard K. Porter and Martin D. Brand

Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW, UK

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The degree of control exerted by the mitochondrial choline transporter over the choline oxidation pathway was measured in isolated rat liver mitochondria. Choline transporter activity was titrated with hemicholinium-3, a known competitive inhibitor of the transporter. It was shown that the rate of betaine efflux from mitochondria was an accurate measure of choline oxidation. The relative rate of choline oxidation was measured as a function of the relative degree of inhibition of the transporter. The resulting data gave a flux control coefficient over choline oxidation of 0.9 for the choline transporter. It is concluded that the choline transporter is the major site for control of choline oxidation in isolated rat liver mitochondria

Metabolic control analysis; Mitochondrion; Choline transporter, Choline oxidation; Betaine

1. INTRODUCTION

In mammals, betaine acts as a major source of methyl groups in the liver [1] and plays an essential role as an organic osmolyte in the kidney [2]. The main intracellular source of betaine is from the oxidation of choline. Choline oxidation occurs in mitochondria mainly in the liver [3] and kidney [4], using an FAD-linked choline dehydrogenase (EC 1.1.99.1) which forms betaine aldehyde, and an NAD-linked betaine aldehyde dehydrogenase (EC 1.2.1.8) which forms betaine. Choline dehydrogenase is situated on the inner side of the mitochondrial inner membrane [5], whereas betaine aldehyde dehydrogenase is in the mitochondrial matrix [6]. At physiological concentrations of choline (60–340 μM) [3,7], the main means of access for choline to the mitochondrial matrix is via a specific transporter in the inner membrane of rat liver mitochondria [8]. The transporter has Michaelis–Menten kinetics with a K_m of 220 μM. Hemicholinium-3 is a high affinity competitive inhibitor of the choline transporter, with a K_i of 17 μM [8]. Betaine, the major product of choline oxidation, appears to diffuse out of the matrix across the mitochondrial inner membrane [9]. In the liver, betaine can act to re-methylate homocysteine to methionine via the cytosolic enzyme betaine-homocysteine methyltransferase [10]. To quantify the degree of control exerted by the choline transporter over choline oxidation and, by extrapolation, over the supply of betaine to betaine-homocysteine methyltransferase, we measured the flux control coefficient of choline transporter activity over the rate of choline oxidation in isolated rat liver mitochondria.

Correspondence address: R.K. Porter, Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW, UK.

2. MATERIALS AND METHODS

Rat liver mitochondria were isolated from Wistar rats (200–250 g) according to the procedure of [11]. Mitochondrial protein was determined by the biuret method [12]. Mitochondria (3 mg of mitochondrial protein/ml) were stirred at 37°C in a medium containing 120 μM KCl, 5 μM HEPES, 1 μM EGTA (free acid), 5 μM KH_2PO_4, 0.5 μM $MgCl_2$, 5 μM L-glutamate (monosodium salt) and 1.2 μM L-malate (disodium salt), adjusted to pH 7.2 with KOH. Incubations were initiated by addition of 500 μM [*methyl*-^{14}C]choline chloride at a specific activity of 0.8 mCi/mmol. Hemicholinium-3 (0, 10, 25, 50, 100 or 500 μM) was added before choline. Oxidation rates were determined from the difference in the sum of the radiolabelled oxidation products of choline between 30 and 40 min. Choline was distinguished from its oxidized products betaine aldehyde and betaine using thin layer chromatography as described previously [9]. Choline transporter activity was represented as the percent inhibition of the transporter in the presence, relative to the absence, of hemicholinium-3, using equation 6 for a competitive inhibitor given in Groen et al. [13]. The average choline concentration in the medium between 30 and 40 min was used for calculations of transporter inhibition. 500 μM hemicholinium-3 had no effect on the integrity of the mitochondria over the period of the time course as measured by membrane potential. Mitochondrial matrix volumes and membrane potential were determined as described previously [8].

L-Glutamate (monosodium salt) and L-malate (disodium salt) were from Sigma Chemical Co (Poole, Dorset, UK). All other chemicals were from sources described in [8].

3. RESULTS AND DISCUSSION

Kacser and Burns [14] and Heinrich and Rapoport [15] introduced metabolic control analysis; a method to

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quantify the amount of control exerted by a step in a pathway over flux through that pathway. The flux control coefficient (C_{vi}^{J}) is the fractional change in the flux through the pathway induced by an infinitesimal fractional change in the activity of the enzyme under consideration, i.e.

$$C_{vi}^{J} = \left(\frac{dJ}{dvi} \cdot \frac{vi}{J}\right)_{ss}$$

where J is the steady-state flux through the pathway and vi is the activity of the enzyme. The subscript ss refers to steady state. If the concentration of the initial substrate and end product of the pathway are kept constant, the sum of all the control coefficients in the pathway is equal to one [14,15].

One way to measure the flux control coefficient of an enzyme is to measure the effect of an infinitesimally small change in the activity of the enzyme on the pathway flux. Changes in enzyme or transporter activity can be achieved by the use of specific inhibitors. In the present paper, hemicholinium-3, a competitive inhibitor of the choline transporter [8], was used to titrate the activity of the choline uniporter. The flux control coefficient was calculated from the relationship between flux through the pathway and transporter activity extrapolated to zero inhibitor concentration.

Hemicholinium-3 does not affect betaine efflux from mitochondria [9]. Hemicholinium-3 is also known not to inhibit the dicarboxylate carrier or the adenine nucleotide carrier [8], however, it is not known whether it affects other mitochondrial transporters. It was assumed that hemicholinium-3, a large cationic quaternary amine, does not cross the mitochondrial inner membrane.

Fig. 1 shows that under the incubation conditions described, the majority of the betaine produced by oxidation of choline appeared in the medium. The production of radiolabelled betaine in the medium was reasonably linear (approx. 0.8 nmol betaine/min/mg mitochondrial protein). Furthermore, very little betaine or choline were measured in the matrix. Therefore, it was concluded that the appearance of radiolabelled betaine in the medium was an accurate measure of choline oxidation. A similar overall pattern for the time course of choline oxidation was obtained by Porter et al. [8] in the presence succinate. However, the rate of choline transport and oxidation appears to be greater under the conditions in the present investigation. This most probably reflects an increase in the activity of the transporter, diffusion processes and the choline oxidising enzymes as a consequence of the incubation temperature difference between the two sets of experiments (25°C and 37°C).

Fig. 2 shows the relative rate of choline oxidation as a function of relative inhibition of the choline transporter. The slope in Fig. 2 is 0.89 ± 0.04 (mean ± S.E.M., $n = 3$) indicating that the choline

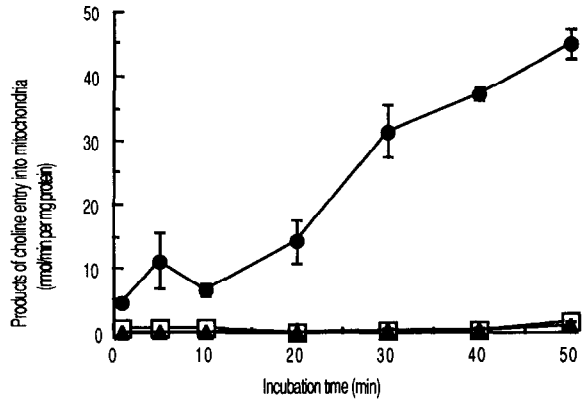

Fig. 1. Products of choline entry into rat liver mitochondria as a function of incubation time. ●, total choline oxidized i.e. the radiolabelled choline, betaine aldehyde and betaine present in the matrix plus the radiolabelled betaine produced and exported into the medium; □, choline in the matrix; ▲, betaine in the matrix. Betaine aldehyde in the matrix was below the limits of detection. Matrix concentrations are corrected for contamination of the pellet by the extramatrix compartment (measured as the sucrose impermeable space). Data are from 2 independent experiments, each determination was made in triplicate (mean ± range, $n = 2$).

transporter has a flux control coefficient of 0.9, i.e. 90% of the control over choline oxidation under these conditions. The data shows some scatter, but different ways of fitting would not change the conclusion that most of the control over choline oxidation is exerted by the choline carrier.

A high degree of control by the transporter is predicted when one compares the kinetic parameters of the choline transporter with those for the enzymes that oxidize choline. The choline transporter has a K_m of 220 μM and a V_{max} of 0.4 nmol/min/mg mitochondrial protein at 25°C. K_m values for liver choline dehydrogenase have been reported at between 140–700 μM [16,17] with a V_{max} value of 6.3 nmol/min/mg mitochondrial protein at pH 7.6 at 37°C [17]. A K_m for betaine aldehyde dehydrogenase has been estimated at 600 μM [5]. The V_{max} value of this enzyme would appear to be greater than that for choline dehydrogenase under the conditions described in this paper and under those described by Porter et al. [8] and others [18]. This is apparent from the observation that no betaine aldehyde was detectable over the period of the time course, i.e. betaine is the main product of choline oxidation. Betaine would also appear not to cause product inhibition. It has also been shown that betaine is a very weak inhibitor of choline dehydrogenase ($K_i > 50$ μM) [3]. Furthermore, extramitochondrial betaine has no effect on choline uptake rate into mitochondria [8] nor does extramitochondrial choline have any effect on betaine efflux rate from betaine-loaded mitochondria [9]. Hepatic concentrations of betaine in the rat have been estimated to be 20–30 times those of choline [20], equivalent to 1.2–6.8 μM [9]. Betaine concentrations in the kidney medulla cells at the

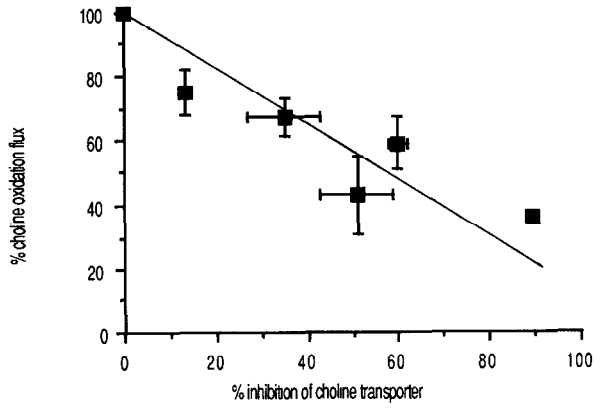

Fig. 2. Percent choline oxidation rate as a function of percent inhibition of choline transport activity. The steady-state choline oxidation rate was measured from the difference in radiolabelled betaine in the mitochondrial supernatant at 30 and 40 min after addition of radiolabelled choline. The choline transporter was inhibited by addition of different concentrations of hemicholinium-3 and the percent inhibition of its activity was calculated as described in section 2. Data are from 3 independent experiments (2 for the 500 μM hemicholinium-3 point) and each determination was made in triplicate The line is unweighted and forced through 100% Its slope (and hence the flux control coefficient of the transporter over choline oxidation) is 0.89 ± 0.04 (mean ± S.E.M., $n = 3$).

tip of the papilla are even higher, approximately 20 μM [2]. To our knowledge, the effect of such high concentrations of cellular betaine on the choline oxidation process in mammals is not known.

So what of the remaining 10% of the control? Previously, workers have implicated the enzymes choline dehydrogenase and betaine aldehyde dehydrogenase as having control in state 3 respiration and uncoupled conditions [16]. Betaine efflux has also been suggested to have some control over the flux through the choline oxidation pathway [5,19]. Certainly, some control may lie with all these pathway steps. However, there may also be control in the diffusion of choline into the mitochondria. At 25°C and a concentration of 220 μM, choline diffuses across the mitochondrial inner membrane at approximately 10% the rate it is transported [8]. If we assume approximately the same Q_{10} for transport and for diffusion then this value will be the same at 37°C. The branching theorem [21] states that $J_1/J_2 = C_1/C_2$ where J_1 and J_2 are the fluxes through the two branches (choline uptake fluxes by diffusion and by transport with $J_1/J_2 = 0.1$) and C_1 and C_2 are the flux control coefficients of the two branches over the common flux (choline oxidation). Thus, the flux control coefficient of choline diffusion over choline oxidation is $0.89 \times 0.1 = 0.09$ and the sum of the flux control coefficients of choline transport and choline diffusion is 0.98, leaving little scope for control by other reactions. We conclude that choline transport (0.9) and most probably choline diffusion (0.1) account for virtually all of the control over the rate of choline oxidation by isolated rat liver mitochondria, and that control by choline dehydrogenase, betaine aldehyde dehydrogenase and betaine efflux is therefore minimal.

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