EVIDENCE FOR THE ROLE OF A SPECIFIC MONOCARBOXYLATE TRANSPORTER IN THE CONTROL OF PYRUVATE OXIDATION BY RAT LIVER MITOCHONDRIA

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1. Introduction

The most convincing evidence for the existence of mitochondrial transporters is the ability shown by certain respiratory poisons to prevent specific exchange 'diffusion' reactions across the inner mitochondrial membrane [1,2]. The absence of such a transport inhibitor for monocarboxylates has cast doubt that the ready penetration and exchange of pyruvate across the mitochondrial inner membrane [3–6] is mediated by a carrier; indeed it has been argued [2] that sufficient undissociated monocarboxylic acid exists in the cell to render redundant such a transporter. Very recently some data on the influence of α-cyano-4-hydroxycinnimate on red cell and mitochondrial pyruvate content have been interpreted to mean that this compound is a specific inhbitor of pyruvate transport in these vesicles [7].

This report presents some studies of the effect of certain 2-oxo-acids on pyruvate content, pyruvate exchange reactions and pyruvate oxidation in rat liver mitochondria. The results are interpreted as evidence for the existence of a monocarboxylate transporter. Palmityl carnitine has been found to be a very powerful counter-transporter of pyruvate. The consequences of such an exchange in the control of the pyruvate dehydrogenase complex are discussed.

2. Materials and methods

Mitochondria from livers of male white rats weighing about 230–250 g were prepared and twice washed in 0.25 M sucrose; 25 mM triethanolamine–HCl; 1 mM ethylene diamine tetraacetic acid buffer, pH 7.2. Finally they were resuspended at about 50 mg per ml in incubation buffer minus rotenone and arsenite. In exchange experiments the medium contained 0.25 M sucrose; 10 mM KCl; 20 mM Tris–HCl; 1.15 mM succinate; 21.5 mM oxamate; 0.75 mM arsenite, pH 7.4. This buffer also invariably contained 2.5 μg rotenone and about 10 μCi [^{3}H]H_2O per ml. The procedure used in exchange experiments was that concentrated mitochondrial suspension was added to about 2 ml of incubation buffer at 25°C containing 1 mM [U-^{14}C]pyruvate so that the final mitochondrial concentration was about 5 mg per ml. After 3 min incubation, 200 μl samples of the suspension were centrifuged as described below. A small volume (5 or 10 μl) of competing unlabelled substrate (100 or 300 mM) was now added to the incubation and the mixture sampled after a further 2 min. Further additions of competing substrate were followed by incubation and sampling. Oxygen electrode experiments were performed in 0.13 M KCl; 2 mM $MgCl_2$; 2 mM EGTA; 5 mM Tris–HCl; 2% defatted albumin solution, pH 7.2. The electrode chamber (Rank Bros., Bottisham, Cambridgeshire) contained in addition to about 3 mg mitochondrial protein in 1.5 ml buffer, 2 μmoles D-glucose and inorganic phosphate, 10 nmoles ADP and 50 μmoles D-glucose and inorganic phosphate, 10 nmoles ADP and 50 μg yeast hexokinase (Boehringer).

[U-^{14}C]pyruvate was obtained from the Radiochemical Centre, Amersham, and used at a specific activity of 135 mCi per mole. α-Ketovaleric acid (KV) and α-ketoisocaproic acid (KIC) were obtained from Fluka AG, Buchs SG, Switzerland, L-carnitine

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and phenylpyruvic acid from Kochlight, Colnbrook, Bucks, and DL-palmityl carnitine from Sigma London Chemical Company Ltd. The substrate content of the mitochondria was measured after sedimenting about 1 mg mitochondria through DC550 silicone oil (Hopkins & Williams Ltd., Chadwell Heath, Essex) into 1.5 M $HClO_4$ in a Zentrifuge 3200 (Eppendorf, Marburg/Lahn) as described by Harris and Van Dam [8]. The extramitochondrial space was determined in parallel experiments using [^{14}C]sucrose and protein was estimated by the method of Lowry et al. [9].

3. Experimental

3.1. *Pyruvate exchange*

Fig. 1 presents results from experiments in which following incubation in 1 mM [^{14}C]pyruvate, the effect of addition of unlabelled pyruvate on the intramitochondrial radioactivity has been measured. At concentrations of pyruvate in suspending medium above 1 mM, the mitochondrial pyruvate content does not substantially increase. Although certain anions are capable of co-transporting pyruvate in rat liver and heart mitochondria and thus, on addition, of increasing the level of intramitochondrial pyruvate they appear

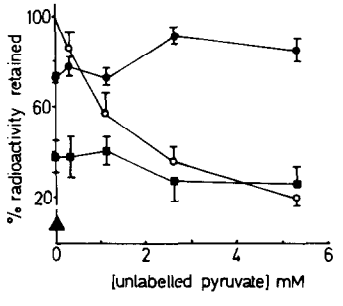

Fig. 1. [^{14}C]pyruvate loaded mitochondria were incubated at 25°C after addition of no inhibitor (○), 50 mM α-ketovalerate (■) or α-ketoisocaproate (●) followed by further addition to give the concentration shown of unlabelled pyruvate to the original 1 mM [^{14}C]pyruvate medium. The radioactivity in the sucrose inaccessible space was determined after centrifugation of about 1 mg of mitochondria through silicone oil. The initial [^{14}C]pyruvate content was 1.10 ± 0.07 nmoles per mg protein and the data show the mean and S.E.M. of 4 different preparations. ▲ represents the content when either KV or KIC were added prior to the [^{14}C]pyruvate.

to influence only the maximum pyruvate content and not the affinity of the mitochondria for pyruvate (J. Mowbray, unpublished results). This being so, further addition of unlabelled pyruvate to the [^{14}C]-pyruvate incubation will be expected to reduce the intramitochondrial radioactivity if free exchange between medium and mitochondrial pyruvate exists. In the absence of inhibitor this is indeed what happens (fig. 1). The values found are very close to those predicted from the known reduction in pyruvate specific activity. When, following the initial incubation in [^{14}C]pyruvate, 100 μl of a 1 M solution in buffer of either 2-ketovalerate or 2-ketoisocaproate is added (50 mM final concentration) and incubation continued for a further 2 min before sampling, the response to unlabelled pyruvate addition is quite different. In neither case does the addition of unlabelled pyruvate substantially alter the initial radioactivity in the sucrose impermeable space. This is behaviour consistent with that expected of compounds which inhibit pyruvate transport. However, the addition of these inhibitors, by themselves, caused an appreciable reduction in the [^{14}C]pyruvate content, especially in the case of KV where the initial content drops by 60%. In these experiments the sucrose-inaccessible space had values between 0.65 and 0.88 μl per mg protein and was not observed to be altered by additions of substrate or inhibitors. The displacement of pyruvate from mitochondria is presumably the result of competition by the inhibitors for pyruvate binding sites, including the transporter. The different amounts of displacement shown by different inhibitors may reflect their ability as competitors at several distinct substrate sites. Interestingly KV has been reported to have a substantially smaller K_m for pig heart pyruvate dehydrogenase complex than KIC: also that KIC has no effect on brain pyruvate dehydrogenase [11,12] though it inhibits brain citrate synthase [12]. For the obvious reason that it reduces the pyruvate content less, KIC seems preferable as a possible transport inhibitor. When either KIC or KV were added prior to [^{14}C]pyruvate, the initial pyruvate content was less than 10% of the control value. Very similar results to those shown in fig. 1 were obtained if the unlabelled pyruvate were replaced by DL-3-hydroxybutyrate. Phenylpyruvate at 23 mM behaves much like KIC: it reduces the mitochondrial pyruvate content by about 45%.

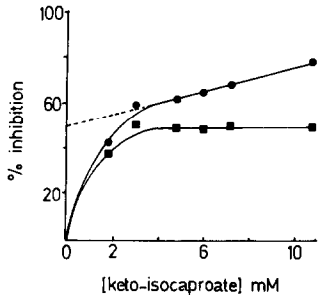

Fig. 2. The inhbition of extra oxygen uptake (23 natoms $0.mg^{-1} \cdot min^{-1}$.) shown by state 3 rat liver mitochondria after 0.6 mM pyruvate addition (•). At this concentration extra oxygen uptake due to pyruvate was proportional to pyruvate concentration. No exogenous malate was provided since malate addition has been found to alter pyruvate penetration to the sucrose-impermeable space. The hyperbolic curve obtained by correcting the original data for the linear component which is observable above 3 mM inhibitor concentration has been plotted (■).

3.2. *Oxygen electrode studies*

Not surprisingly, KIC was found to be a powerful inhibitor of pyruvate oxidation in whole coupled mitochondria, being capable of inhibiting the extra oxygen uptake due to pyruvate by 80% at 11 mM (fig. 2). The initial inhibition is severe reaching 60% by 3 mM inhibitor. Increases in KIC concentration between 3 and 11 mM appear to exert a linear increase in the inhibition. Assuming this linear component to have been present from the beginning, the effect of the inhibitor has been divided into a saturable component inhibiting 50% of the extra oxygen uptake by 3 mM KIC and a less powerful inhibitory function which does not show saturation within the concentration range used. It is worth noting that because of the 'catalytic' effect of added substrates on oxygen uptake the degree of inhibition in this experiment cannot be directly compared with the exchange experiments. However these data would suggest that KIC may be having at least two distinct effects in mitochondria; which effect might be assigned to transporter inhibition is uncertain. It is notable how similar these results are to KIC inhibition of pyruvate oxidation in whole brain mitochondria [13] where KIC inhibits citrate synthase [12] but not extracted pyruvate dehydrogenase. Thus it is possible that the two inhibitory effects seen represent transporter and citrate synthase inhibition.

3.3. *The effect of palmityl carnitine on pyruvate content*

Of the ions observed to undergo exchange reactions with pyruvate across the liver mitochondrial membrane, DL-palmityl carnitine has been found to be by far the most effective in expelling pyruvate. At 0.2 mM Dl-palmityl carnitine reduces the liver mitochondrial pyruvate content by 80% in the presence of 2.2 mM pyruvate (fig. 3). L-carnitine itself, at 5 mM, reduces the pyruvate in the sucrose-inaccessible space by 60%.

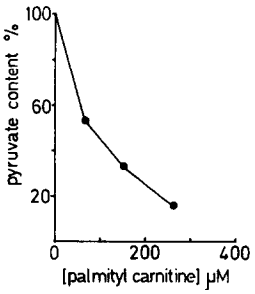

Fig. 3. The effect of DL-palmityl carnitine in the presence of 2.2 mM pyruvate on the content of pyruvate in the sucrose inaccessible space (1.06 μl per mg) of rat liver mitochondria. The initial pyruvate content was 1.19 nmoles per mg protein and the data are means from two experiments.

4. Discussion

The addition of high concentrations of KV, KIC and phenylpyruvate appear capable of sequestering within intact mitochondria a pool of pyruvate such that it will not exchange with pyruvate in the suspending medium. This appears strong evidence for the specific inhibition of pyruvate transport in mitochondria. Attempts to inhibit pyruvate exchange with hemimellitate or 2-*n*-butyl malonate were unsuccessful (J. Mowbray, unpublished results) implying that the operation of the dicarboxylate or tricarboxylate carriers are not required for pyruvate transport. Butyl malonate (15 mM), however, did reduce the pyruvate content by 50%. In the light of this and in view of the fact that any ion which counter-exchanges pyruvate may reduce the pyruvate content, it is necessary to interpret with caution experiments

where a proposed transport inhibitor is shown merely to have reduced the quantity of anion sequestered [cf. 7]. Under conditions where pyruvate efflux appears small and where pyruvate oxidation was probably low, α-cyano-4-hydroxycinnimate addition (0.5 mM) allowed recovery of more intramitochondrial pyruvate after centrifugation of mitochondria than in the absence of inhibitors [7]. These results are consistent with the suggestion that this compound too may be a transport inhibitor, and that the transporter recognises the enol form of α-oxo-acids [7].

A striking similarity has been found between pyruvate and 3-hydroxybutyrate in exchange experiments and it seems possible that these compounds share a carrier. In support of this it has been found that the K_i for pyruvate inhibition of hydroxybutyrate transport has the same value as the absorption coefficient for pyruvate (J. Mowbray, unpublished results). If this is so, counter-exchange of pyruvate and 3-hydroxybutyrate across the mitochondrial membrane may explain the sparing effect of ketone bodies on glucose utilisation in peripheral tissues, since expulsion of pyruvate would lead to inhibition of the pyruvate dehydrogenase complex. The proportion of this complex in the active (dephospho) form appears to depend on the supply of pyruvate [14], possibly because pyruvate protects the complex from kinase attack [15,16]. Incubation of whole rat liver mitochondria with pyruvate has been shown to lead to activation of pyruvate dehydrogenase: also palmityl carnitine and 3-hydroxybutyrate can reverse this effect [17]. Significantly, pyruvate was maximally effective in activating the complex at 2 mM, and half-maximal activation was achieved around 0.4 mM [17]. The Langmuir absorption constant (equivalent to K_m) for pyruvate sequestration in liver mitochondria has been found to be 0.45 mM (J. Mowbray, unpublished results) and the pyruvate content to be maximal at a medium concentration of 2 mM. Further, Portenhaus and Wieland [17] found 100 μM L-palmityl carnitine to be maximally effective in reversing pyruvate activation of the complex; this concentration compares well with the 200 μM DL-palmityl carnitine found to expel virtually all the mitochondrial pyruvate (fig. 3). Since pyruvate dehydrogenase appears only accessible from the matrix space [18], it seems likely that the mechanism by which ketone bodies and fatty acid carnityl derivatives achieve their effect on pyruvate oxidation in through the specific expulsion of mitochondrial pyruvate via the transporter and the subsequent effect of this on the covalent modification of the complex.

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