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The concentration of the mitochondrial pyruvate carrier in rat liver and heart mitochondria determined with α-cyano-β-(1-phenylindol-3-yl)acrylate

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1. α-Cyano-β-(1-phenylindol-3-yl)acrylate inhibited pyruvate transport into both liver and heart mitochondria approximately linearly with respect to its concentration until 65% inhibition was achieved. 2. The extent of inhibition was dependent on the mitochondrial protein concentration. 3. By extrapolation of plots of inhibition versus inhibitor concentration to total inhibition, or by mathematical analysis of the plots, the concentration of pyruvate transporter molecules per mg of protein was calculated to be approximately 100 pmol/mg for both heart and liver mitochondria, and the K_i about 7 nM. 4. The data also suggest that pyruvate transport is rate-limiting for pyruvate oxidation by heart mitochondria in State 3, but not by liver mitochondria.

Pyruvate is transported into the mitochondria of liver and heart using a transporter that is specifically inhibited by α-cyanocinnamate derivatives (Halestrap *et al.*, 1980). These inhibitors bind extremely tightly but reversibly to an essential thiol group on the intramitochondrial surface of the mitochondrial inner membrane (Halestrap, 1975, 1976*a*, 1978*a*; Halestrap *et al.*, 1980). The inhibition is essentially non-competitive, because, once the inhibitor binds, further pyruvate entry is inhibited, so preventing competition between substrate and inhibitor (Halestrap *et al.*, 1980). This feature allows the use of the inhibitor to study the role of pyruvate transport in intact tissue preparations and isolated mitochondria (Halestrap & Denton, 1975; Halestrap, 1978*b*; Halestrap *et al.*, 1980; Thomas & Halestrap, 1981*b*). In the present paper we utilize an exceptionally potent inhibitor of the transport of pyruvate into isolated mitochondria, α-cyano-β-(1-phenylindol-3-yl)-acrylate, which binds so tightly that it titrates out the carrier stoichiometrically until about 65% inhibition is reached. This has allowed determination of the number of carrier molecules per mg of mitochondrial protein and so complements previous work from this laboratory (Thomas & Halestrap, 1981*a*) involving specific labelling of the transporter with [^{3}H]*N*-phenylmaleimide.

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Experimental

Materials

Heart and liver mitochondria were prepared from normal fed female Wistar rats (200–250 g body wt.) as described previously (Halestrap, 1975). Unless otherwise stated, all chemicals and biochemicals were obtained from the sources cited in Halestrap (1975) and Armston *et al.* (1982).

Methods

Oxygen-electrode studies. Oxygen uptake by mitochondria was measured polarographically at 37°C by using a Clark-type oxygen electrode as described previously (Halestrap, 1975). The medium was 125 mM-KCl, 10 mM-Mops (4-morpholinepropanesulphonic acid), 7 mM-Tris base, 2.5 mM-potassium phosphate, 2.5 mM-$MgCl_2$, 1 mM-EGTA, at pH 7.2. For the hypo-osmotic conditions used for studying pyruvate oxidation by liver mitochondria, the KCl concentration was lowered to 25 mM. Pyruvate and L-malate were both added at 1 mM, and CaEGTA was at 0.6 mM. These conditions activated pyruvate dehydrogenase (Denton *et al.*, 1980) and gave maximal rates of oxygen uptake on subsequent addition of ADP (2 mM). In liver, maximal rates of pyruvate oxidation were only achieved if hypo-osmotic medium was used in the presence of uncoupler (0.1 μM-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone) and ADP (2 mM). Heart mitochondria (0.2–1 mg of protein/ml) were preincubated for 1 min with the buffer containing inhibitor at the required concen-

tration and all additions except ADP. The oxygen uptake was then stimulated with ADP and the rate measured. For liver mitochondria, 1–2.5mg of protein was used and the procedure was similar to that outlined above. However, both uncoupler (0.1 μM) and ADP (2mM) were used to stimulate respiration, and the pyruvate-transport inhibitor was added during stimulated respiration before measuring the new steady-state rate of oxygen uptake.

Analysis of α-cyano-β-(1-phenylindol-3-yl)acrylate inhibition of pyruvate oxidation. Detailed analysis of the inhibition by α-cyano-β-(1-phenylindol-3-yl)-acrylate of pyruvate-dependent oxygen uptake by heart mitochondria required a consideration of the consequences of the K_i for inhibition being considerably less than the concentration of the carrier molecules. Thus the free concentration of inhibitor was less than the total added concentration.

Let $[E_t]$ = carrier concentration/ml, K_i = dissociation constant for inhibitor, [I] = total concentration of inhibitor, [EI] = concentration of carrier–inhibitor complex, V = rate of pyruvate transport, and Diff = carrier-independent rate of pyruvate transport. Then

$$K_i = \frac{([E_t]-[EI])([I]-[EI])}{[EI]} \quad (1)$$

and

$$V = k([E_t]-[EI]) + \text{Diff} \quad (2)$$

From (1):

$$K_i[EI] = [E_t][I]-[EI][I]-[E_t][EI]-[EI]^2$$

Therefore

$$0 = [EI]^2 + (K_i + [E_t] + [I])[EI] - [E_t][I] \quad (3)$$

At any value of [I], V can be calculated from eqns. (2) and (3) and estimated values of K_i, $[E_t]$, k and Diff. Diff is estimated by the rate of pyruvate transport at high inhibitor concentration (100 μM), and the other parameters are determined by computer reiteration to give the best fit of calculated values with observed values of V. This was achieved by the procedure of Jones (1970). From the value of $[E_t]$ and the concentration of mitochondrial protein/ml, the concentration of carrier molecules per mg of protein can be calculated.

Results and discussion

Inhibition of mitochondrial pyruvate oxidation by α-cyano-β-(1-phenylindol-3-yl)acrylate

In Fig. 1 data are presented comparing the inhibition of α-cyano-4-hydroxycinnamate (Fig. 1*a*) and α-cyano-β-(1-phenylindol-3-yl)acrylate (Fig. 1*b*) of ADP-stimulated pyruvate-dependent oxygen uptake by heart mitochondria. Dixon plots for α-cyano-4-hydroxycinnamate are linear and give the same K_i values (2 μM) at all protein

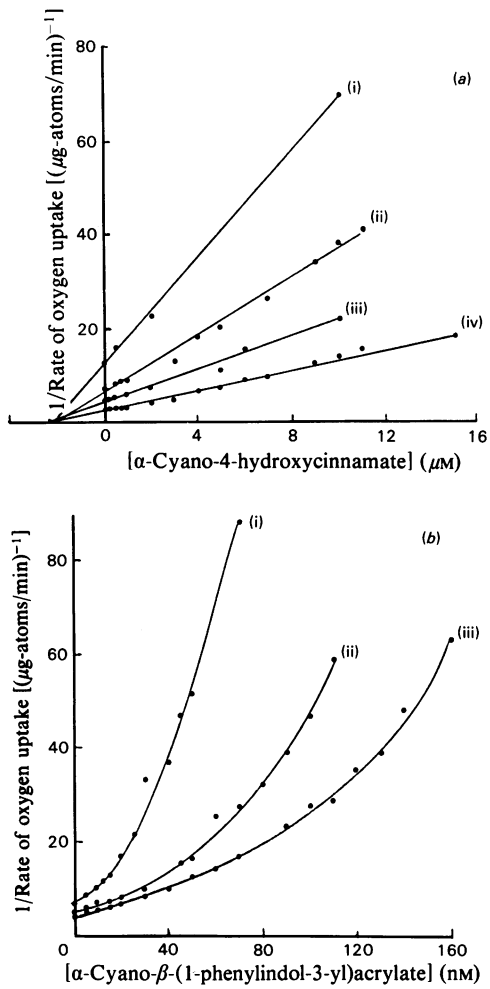

Fig. 1. *Dixon plots of the inhibition by α-cyano-4-hydroxycinnamate and α-cyano-β-(1-phenylindol-3-yl)acrylate of pyruvate oxidation by rat heart mitochondria*

Heart mitochondria were incubated in a Clark-type oxygen electrode at 37°C as described in the Experimental section. Inhibitors were added at the concentrations shown and preincubated with the mitochondria for 1min before addition of 1mM-pyruvate and 2mM-ADP and measurement of the rate of oxygen uptake. The concentrations of heart mitochondrial protein (mg/ml) were in (*a*) 0.21 (i), 0.38 (ii), 0.59 (iii), 1.0 (iv), and in (*b*) 0.37 (i), 0.53 (ii) and 0.62 (iii). All rates of oxygen uptake have been corrected for the rate of inhibitor-insensitive transport determined after addition of 100 μM-α-cyano-β-(1-phenylindol-3-yl)acrylate.

concentrations tested. However, the plots for α-cyano-β-(1-phenylindol-3-yl)acrylate are clearly not linear, and the deviation from linearity depends on the protein concentration. This can be explained if the inhibitor has a K_i much lower than the concentration of binding sites, as explained in the Experimental section. In Fig. 2 the data for two of the plots in Fig. 1(*b*) are replotted as velocity against [inhibitor], and the theoretical curves derived by computer fitting to the equation given in the Experimental section are drawn. The fit is good, and the derived values of K_i and concentration of binding sites per mg of protein are given in Table 1. Examination of Fig. 2 indicates that, until about 65% inhibition is reached, the inhibition of transport is linear with respect to [inhibitor]. This allows extrapolation to give [inhibitor] which produces the total inhibition of carrier-dependent pyruvate metabolism (indicated by the dotted line). If it is assumed that the inhibitor is binding to and inhibiting the transporter stoichiometrically, this value allows an independent calculation of the number of binding sites as given in Table 1. This procedure has also been successfully applied to inhibition of the Cl^-/HCO_3^- transporter of the human erythrocyte by isothiocyanostilbenesulphonate derivatives (Halestrap, 1976*b*). Comparison of the two methods of calculating the concentration of carrier molecules suggests that the extrapolation overestimates the true values by about 20%.

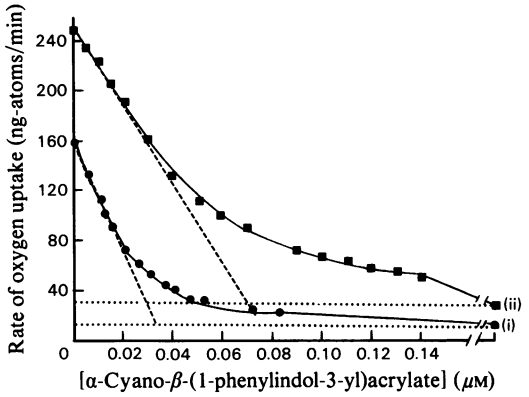

Fig. 2. *Inhibition by α-cyano-β-(1-phenylindol-3-yl)-acrylate of pyruvate oxidation by rat heart mitochondria*
The experimental procedure is the same as given in the legend to Fig. 1. The mitochondrial protein concentration was 0.37mg/ml (i) and 0.62mg/ml (ii). The dashed lines represent extrapolations of the linear portion of the plots and the dotted lines the inhibitor-insensitive rate of pyruvate oxidation. The continuous line was calculated by regression analysis as described in the Experimental section, and the derived parameter values are shown in Table 1.

A similar experimental approach was used for liver mitochondria, as shown in Fig. 3. To obtain maximal rates of pyruvate-induced oxygen uptake, it was necessary to employ hypo-osmotic solutions and uncoupler, as outlined in the Experimental section. Fig. 3 shows that the first additions of α-cyano-β-(1phenylindol-3-yl)acrylate had little effect on oxygen uptake. Higher inhibitor concentrations produced inhibition of oxygen uptake, which over a small range showed proportionality before the plot once again deviated from linearity at high inhibitor concentrations. The differences between the plots for liver and heart mitochondria are best explained if it is assumed that pyruvate transport is the rate-limiting step for pyruvate oxidation by heart mitochondria, but not by liver mitochondria. Extrapolation of the linear region of the plot to 100% inhibition of carrier-mediated transport (dotted line) yields a value for the concentration of inhibitor required to give total inhibition. Once again this is dependent on the protein concentration. This is to be expected if the inhibitor is

Table 1. *Estimation of the concentration of mitochondrial transport molecules per mg of heart mitochondrial protein*
Data from the three experiments in Fig. 1(*b*) were analysed by least-squares fit for non-competitive inhibition as described in the Experimental section. Values in parentheses are the calculated 95% confidence limits. The extrapolated value for the number of carrier sites was estimated as shown in Fig. 2. In Expt. 4, this procedure alone was used, there being insufficient data points for computer analysis. Each experiment represents pooled heart mitochondria from at least four rats. The mean values are given ± S.E.M. for the numbers of individual values shown.

Expt. no.	Concn. of mitochondrial protein (mg/ml)	Carrier sites (pmol/mg of mitochondrial protein): Estimated by computer fit	Carrier sites (pmol/mg of mitochondrial protein): Estimated by linear extrapolation	Carrier-dependent $V_{max.}$ (ng-atoms of O/min per mg of protein)	K_i (nM)
1	0.37	66.0 (59.2–72.7)	91.9	389 (373–405)	4.3 (2.9–5.7)
2	0.53	105.2 (89.2–121.1)	118.9	345 (319–370)	6.1 (1.5–10.6)
3	0.62	89.4 (74.0–104.8)	111.3	353 (326–380)	10.4 (4.1–16.6)
4	0.79	—	96.7	382	—
	Mean ...	86.9 ± 11.4	104.7 ± 6.3	367 ± 10.8	6.9 ± 1.8

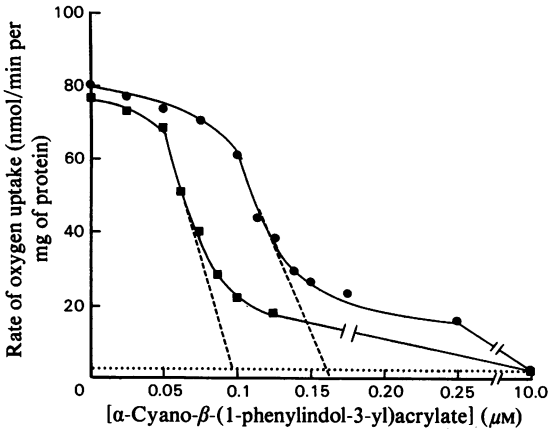

Fig. 3. *Inhibition by α-cyano-β-(1-phenylindol-3-yl)-acrylate of pyruvate oxidation by uncoupled rat liver mitochondria*

Pyruvate-dependent oxygen uptake was measured by hypo-osmotic medium in the presence of uncoupler and ADP as described in the Experimental section. Mitochondria from the liver of the same animals were incubated at 2.07 (●) and 1.12 (■) mg of protein/ml. The dashed and dotted lines represent extrapolation of the linear portion of the plot, and pyruvate-carrier-independent oxygen uptake, respectively. From the intercepts the estimated concentration of pyruvate carrier was 89 (■) and 83 (●) pmol/mg of protein.

titrating out the carrier in liver mitochondria, as it does in heart mitochondria. Thus division of this value by the concentration of mitochondrial protein/ml yields an estimate of the concentration of carrier per mg of protein in an analogous fashion to that used for heart mitochondria. The mean value ± S.E.M. for eight separate mitochondrial preparations was 107 ± 6.3 pmol/mg of mitochondrial protein. If it is assumed that liver mitochondria behave similarly to heart mitochondria, then this value may be an overestimate by about 20% as outlined above.

Previous estimates of 100 pmol/mg of protein for the concentration of the pyruvate carrier in liver mitochondria obtained by labelling the transporter with [^{3}H]*N*-phenylmaleimide (Thomas & Halestrap, 1981*a*) agree well with the data presented here. However, for heart mitochondria the value derived in those earlier studies was 450 pmol/mg of protein, substantially greater than reported here. The reason for this discrepancy could be that in the previous experiments smaller (200 g) male rats were used for the preparation of heart mitochondria, rather than the female rats used in the present studies. However, the $V_{max.}$ value for pyruvate-dependent oxygen uptake obtained in the previous studies was lower than that found here. This is not compatible with the larger number of transporter molecules determined in those experiments, unless the absence of CaEGTA and lower temperature produced considerably lower rates of pyruvate oxidation. It is quite possible that the previous studies over-estimated the concentration of carrier molecules for two reasons. Firstly the binding of [^{3}H]*N*-phenylmaleimide to a 15000-M_r protein, which was prevented by the presence of α-cyanocinnamate, was used to estimate the number of transporter molecules. Corrections had to be made relating to the efficiency of α-cyanocinnamate in preventing inhibition by *N*-phenylmaleimide. These measurements could have been subject to error. Secondly, a correction was made relating the amount of mitochondrial membrane protein run on the sodium dodecyl sulphate/polyacrylamide gels to the initial mitochondrial protein concentration. Thomas & Halestrap (1981*a*) use the inhibition of pyruvate oxidation by α-cyano-β-(1-phenylindol-3-yl)acrylate to estimate the number of binding sites of the inhibitor per mg of protein, and again the estimated number of carrier molecules was about 400 pmol/mg of protein. However, their analysis of the data used assumed linear Dixon plots, which is incorrect, as demonstrated in the present paper. Re-analysis of the earlier data by using linear extrapolation gives a value of about 290 pmol/mg of protein.

The ability of α-cyano-β-(1-phenylindol-3-yl)-acrylate to titrate out the mitochondrial pyruvate transporter allows its use in assessing the regulatory role of the transporter in pyruvate metabolism. This is the subject of the accompanying paper (Halestrap & Armston, 1984).

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