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ON THE KINETICS AND SUBSTRATE SPECIFICITY OF THE PYRUVATE TRANSLOCATOR IN RAT LIVER MITOCHONDRIA

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SUMMARY

(1) The kinetics and substrate specificity of the pyruvate translocator in rat liver mitochondria have been studied by both influx and efflux experiments.

(2) Pre-exposure of mitochondria to unlabelled pyruvate or 2-oxobutyrate caused marked decrease of the K_m of pyruvate uptake but no change in the V.

(3) Mitochondrial [^{14}C]pyruvate exchanged with saturation kinetic with specific oxomonocarboxylates. The kinetic parameters of the various [^{14}C]pyruvate-monocarboxylate exchanges were determined. All the reactions exhibited the same V value but different K_m values.

(4) The initial rate of [^{14}C]pyruvate uptake by mitochondria was inhibited by the presence in the external medium of the oxomonocarboxylates which in the efflux experiments were found to exchange with mitochondrial [^{14}C]pyruvate. Dicarboxylates had, on the other hand, no effect on pyruvate transport. The inhibition of pyruvate uptake by oxomonocarboxylates was of competitive type. The K_i values for the various monocarboxylic oxoacids competing for pyruvate uptake were determined and found to be practically equal to the K_m values measured in the exchange experiments.

(5) Besides oxomonocarboxylates, the initial rate of pyruvate uptake was inhibited by various halogenated monocarboxylic acids. Also these acids behaved as purely competitive inhibitors of pyruvate uptake.

(6) The nature of the inhibition of pyruvate transport by α-cyanocinnamate has been studied. α-Cyanocinnamate acted as a purely competitive inhibitor of pyruvate uptake by both unloaded and 2-oxobutyrate preloaded mitochondria.

(7) The results presented provide evidence for the existence in the pyruvate translocator of a single substrate-binding site. On the basis of the structural requirements of the binding site a possible chemical mechanism for the interaction between the substrate and the functional groups of the translocator is proposed. The mechanism of the inhibition of pyruvate transport by α-cyanocinnamate and other SH-reagents is discussed in terms of two types of functional thiol groups in the translocator.

INTRODUCTION

Evidence has been obtained in this and other laboratories showing the existence in the inner membrane of mitochondria of a system which mediates the translocation of pyruvate and other monocarboxylates [1–9]. The existence of a pyruvate translocator has been also demonstrated in the plasma membrane of human erythrocytes [3, 10] and isolated epithelial cells [11–12] and in mitochondria isolated from yeast [13] and Ehrlich tumor cells [14].

The mitochondrial pyruvate translocator catalyzes exchange diffusion of pyruvate with hydroxyl ions (or pyruvate-H^+ symport) [1, 2, 5] or with other monocarboxylates [4, 5]. These exchange reactions are characterized by saturation kinetics and sensitivity to thiol group reagents [2] and to α-cyanocinnamate [5].

For a better understanding of the physiological role of the pyruvate translocator as well as for an insight into its catalytic mechanism, detailed kinetic studies are needed.

Previous work on the translocation of pyruvate in rat liver mitochondria has revealed some differences in the kinetic constants of pyruvate-influx and pyruvate-monocarboxylate exchange [2]. In addition, it was recently found that the activity of pyruvate translocator, measured as initial rate of pyruvate uptake, was differently affected by pre-exposure of mitochondria to various substituted monocarboxylates [7]. In this paper, a comprehensive study of the kinetic properties of the pyruvate transporting system in rat liver mitochondria and its substrate specificity is presented. The nature of the inhibition by α-cyanocinnamate has also been investigated.

MATERIALS AND METHODS

Rat liver mitochondria were prepared as described by Myers and Slater [15]. 0.25 M sucrose was used for homogenization and washing.

Pyruvate uptake

The initial rate of pyruvate uptake by rat liver mitochondria was measured by the centrifugation filtration technique as follows (see also refs. 16, 2, 7). Mitochondria were preincubated at 20 °C in a reaction medium containing: 150 mM sucrose, 30 mM Tris · HCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 1 mM arsenite, 10 μg/ml oligomycin, 1.4 μg/ml rotenone, 0.34 μg/ml antimycin. After 2 min preincubation, mitochondria were layered on top of a second incubation layer, at 4 °C, and then spun down through this layer by rapid centrifugation. $HClO_4$ was immediately added to the mitochondrial pellet. The second incubation layer was of the same composition of the preincubation mixture and in addition contained [^{14}C]pyruvate at the concentration shown in the tables and figures. A discontinuous density gradient increasing toward the bottom of the centrifuge tube was made by addition of dextran to the second layer. The exposure time of the mitochondria to the 2nd incubation layer was estimated to be about 15 s by measuring the oxidation of 3-hydroxybutyrate to acetoacetate.

Pyruvate efflux

The initial rate of pyruvate efflux from mitochondria was measured by the

procedure described above (see also ref. 4). Mitochondria were loaded with [^{14}C]-pyruvate in the sucrose medium described above, at 20 °C. After preincubation, pyruvate-loaded mitochondria were layered on the top of a second incubation layer at 4 °C, and then spun down through this layer by rapid centrifugation. The exchange diffusion of mitochondrial [^{14}C]pyruvate with various anions was followed by adding these substrates to the second layer. Pyruvate was measured both in $HClO_4$ extracts of the mitochondrial pellet and in supernatant. The substrate content of the matrix space was calculated by correcting the amount in the mitochondrial extract with that in the sucrose-permeable space plus adherent supernatant. This was determined with [^{14}C]sucrose. The total H_2O of the mitochondrial pellet was determined with ^3H_2O.

Assays

The mitochondrial level of pyruvate was determined either using [^{14}C]pyruvate or enzymatically [17]. Oxygen uptake was measured polarographically with a Clark electrode. Mitochondrial proteins were determined by the usual biuret method.

Materials

All radiochemicals were obtained from the Radiochemical Centre, Amersham, England, α-cyanocinnamate was obtained from R. Emmanuel Wembley, Middx, U. K. Enzymes, coenzymes and substrates were purchased from Sigma Chemical Co. or Boehringer Mannheim Corp.

RESULTS

Kinetic parameters of pyruvate translocator

The experiment of Fig. 1A illustrates the kinetics of pyruvate uptake by mitochondria and the effect of preincubation with unlabelled pyruvate or 2-oxobutyrate on this process. Double reciprocal plots of the initial rate of pyruvate uptake by normal mitochondria vs. external pyruvate concentration gave saturation kinetics with a K_m of 640 μM and V of 20.1 nmol · min^{-1} · mg protein^{-1}. Preincubation of mitochondria with pyruvate or 2-oxobutyrate resulted in a marked decrease of the K_m of pyruvate uptake, being respectively 120 μM after preincubation with pyruvate and 185 μM after preincubation with 2-oxobutyrate. There was no change in the V of pyruvate uptake. Separate controls showed that the preincubation time (3 min) of mitochondria with monocarboxylates used in this and the following experiments was long enough to approach full equilibrium distribution of substrates across the mitochondrial membrane.

Fig. 1B illustrates the kinetics of [^{14}C]pyruvate$_{in}$/pyruvate$_{out}$ and [^{14}C] pyruvate$_{in}$/2-oxobutyrate$_{out}$ exchange diffusion reactions in mitochondria. Double reciprocal plots show saturation kinetics for both exchanges. The two reactions gave the same V (12.1 nmol · min^{-1} per mg protein), the affinity of pyruvate was however higher than that exhibited by 2-oxobutyrate (K_m, 115 μM for pyruvate and 170 μM for 2-oxobutyrate).

Table I summarizes the kinetic parameters of pyruvate translocation reactions in mitochondria, described in Fig. 1, with a statistical analysis of the data. It can be noted that the V for the exchange of mitochondria [^{14}C]pyruvate with externally added monocarboxylate is significantly lower than that measured for pyruvate uptake

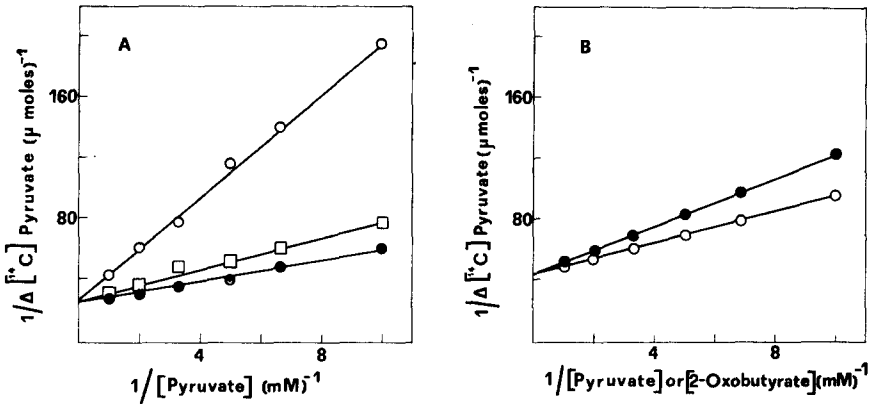

Fig. 1. Kinetic analysis of pyruvate influx (A) and pyruvate efflux (B) reactions by rat liver mitochondria. (A) Double reciprocal plots of the effect of preincubation of mitochondria with unlabelled pyruvate and 2-oxobutyrate on the pyruvate uptake. Mitochondria (7.5 mg of protein) were preincubated in the sucrose-medium described under Methods. Final pH 7.2. Temperature 20 °C. 3 min later, mitochondria were centrifuged through a second incubation layer at 4 °C containing the same components as the preincubation medium, except unlabelled pyruvate and 2-oxobutyrate, and in addition [^{14}C]pyruvate at the concentrations indicated in the figure. Symbols: ○—○, control; ●—●, 0.5 mM pyruvate or □—□, 2 mM 2-oxobutyrate added in the preincubation phase. (B) Double reciprocal plots of the [^{14}C]pyruvate-pyruvate and [^{14}C]pyruvate-2-oxobutyrate exchange reactions. Mitochondria (7.8 mg protein) were preincubated in the sucrose medium described under Methods in the presence of 2 mM [^{14}C]pyruvate. Final pH 7.2. Temperature 20 °C. 2 min later, [^{14}C]pyruvate-loaded mitochondria were centrifuged through a second layer, at 4 °C, containing the same components as the preincubation mixture, except labelled pyruvate, and in addition different concentrations of unlabelled pyruvate or 2-oxobutyrate as indicated. Δ[^{14}C]Pyruvate is the difference in the amount of pyruvate remaining in the matrix after passage of the mitochondria through the second layer in the absence and presence of cold pyruvate or 2-oxobutyrate. Symbols: ○—○, unlabelled pyruvate; ●—●, 2-oxobutyrate. For other details, see under Methods.

by mitochondria. It has however to be stressed that the initial rate of [^{14}C]pyruvate influx into mitochondria gives a measure of the total activity of the pyruvate translocator. This does not hold for the efflux of mitochondria [^{14}C]-pyruvate in exchange with external monocarboxylates. This exchange represents only part of the activity of the system. In fact, when [^{14}C]pyruvate-loaded mitochondria are exposed to an anion-free medium, there occurs a net efflux of pyruvic acid (pyruvate-OH^- exchange (see ref. 1, 2)) and the rate of pyruvate-monocarboxylate exchange is measured on the basis of the extra efflux of [^{14}C]pyruvate induced by the addition of monocarboxylates in the medium (cf. ref. 2). It should be noted that the V of net pyruvate uptake measured in the present work is considerably higher than that measured by Halestrap [5]. This difference is probably due to the different experimental procedure used to measure transport. The centrifugation filtration technique used in the present work avoids use of inhibitors which might affect measurement of transport rates. Furthermore, as shown in Table V, the centrifugation filtration technique gave kinetic parameters for other anion transport reactions practically equal to those generally reported in literature [21–23]. The inhibitor stop technique, as used by Halestrap, could cause underestimation of transport rate in that the inhibitor could display some of the pyruvate bound to the carrier which, in the short incubation time used to measure initial transport rates, might represent a substantial part of the overall uptake.

TABLE I

KINETIC CONSTANTS OF PYRUVATE TRANSLOCATION REACTIONS IN RAT LIVER MITOCHONDRIA

The uptake of [^{14}C]pyruvate by mitochondria was followed as described in the legend to Fig. 1A. Mitochondria (7–9 mg protein) were preincubated in the sucrose medium described under Methods. Final pH 7.2. Temperature 20 °C. 3 min later, mitochondria were centrifuged through a second incubation layer at 4 °C containing the same components as the preincubation medium and in addition [^{14}C]pyruvate. Where indicated, unlabelled pyruvate (0.5 mM) or 2-oxobutyrate (2 mM) were added in the preincubation medium. The efflux of [^{14}C]pyruvate by mitochondria was followed as described in the legend to Fig. 1B. After 3 min of preincubation in the standard sucrose medium in the presence of 2 mM [^{14}C]pyruvate, mitochondria (7–9 mg protein) were centrifuged through a second incubation layer at 4 °C containing the same components as the preincubation (except [^{14}C]pyruvate) and in addition various concentrations of unlabelled pyruvate or 2-oxobutyrate. For other details, see the legend to Fig. 1 and under Methods.

Translocation reactions		Number of expts.	K_m (mM)	V (nmol/min per mg protein)
Preincubation layer (in)	II layer (out)			
None	[^{14}C]Pyruvate	22	0.640±0.016	20.1±0.8
Pyruvate	[^{14}C]Pyruvate	4	0.124±0.011	20.4±2.1
2-Oxobutyrate	[^{14}C]Pyruvate	14	0.185±0.016	21.2±1.5
[^{14}C]Pyruvate	Pyruvate	28	0.115±0.004	12.8±0.2
[^{14}C]Pyruvate	2-Oxobutyrate	8	0.170±0.007	12.6±0.5

TABLE II

EFFECT OF VARIOUS MONOCARBOXYLIC OXOACIDS ON THE INITIAL RATE OF PYRUVATE UPTAKE BY 2-OXOBUTYRATE-PRELOADED MITOCHONDRIA

Mitochondria (9.1 mg protein) were preincubated with 2 mM 2-oxobutyrate in the standard sucrose medium described under Methods. Final pH 7.2. 3 min later, mitochondria were centrifuged through a second incubation layer at 4 °C containing the same components as the preincubation medium and in addition 200 μM [^{14}C]pyruvate and the various oxoacids at the concentration of 2 mM as indicated in the table. Pyruvate uptake is expressed as nmol/15 s/total amount of protein. All results are expressed as means±S.E.M. of 4 separate observations. The K_i values for the oxomonocarboxylates competing for pyruvate uptake were calculated according to the Dixon method [18]. The competing oxomonocarboxylic acid was added simultaneously with [^{14}C]pyruvate at concentrations of 0.25–3.0 mM. For further experimental details, see the legend to Fig. 2 and under Methods.

Substrate	Pyruvate uptake±S.E.M. (nmol)	Inhibition (%)	K_i (mM)
None	18.60±0.91		
2-Oxobutyrate	6.62±0.71	64.4	0.17
Oxamate	7.22±0.87	61.2	0.21
3-Fluoropyruvate	7.48±0.83	59.4	0.22
2-Oxovalerate	8.05±0.63	56.7	0.25
3-Hydroxypyruvate	8.97±0.42	51.7	0.70
Acetoacetate	11.76±0.46	36.8	0.82
2-Oxocaproate	13.05±0.94	29.8	1.20
2-Oxoisovalerate	13.81±1.12	25.8	2.80
Phenylpyruvate	14.67±1.06	21.1	3.80
2-Oxoisocaproate	16.08±1.18	13.5	5.20

Exchange diffusion of mitochondrial pyruvate with various oxomonocarboxylates and their inhibitory action on pyruvate uptake

In a previous paper, it was shown that intramitochondrial [^{14}C]pyruvate exchanged with various externally added oxomonocarboxylates [4] (see Fig. 1B). All the exchanges studied followed saturation kinetics exhibiting the same V (12.2±0.42 nmol/min per mg protein). However the K_m values for the various exchange-diffusion reactions changed with the structure of the oxomonocarboxylate used as counteranion going from 0.115±0.004 for pyruvate to 0.170±0.007 for 2-oxobutyrate, 0.292±0.018 for 2-oxovalerate, 0.610±0.021 for acetoacetate, 0.960±0.0046 for 2-oxocaproate, 1.35±0.09 for 2-oxoisovalerate, 3.85±0.19 for 2-oxoisocaproate (see also ref. 4) and 2.85±0.11 for phenylpyruvate.

The effect of a variety of oxoacids on the initial rate of pyruvate uptake by 2-oxobutyrate loaded mitochondria is reported in Table II. When added externally, simultaneously to [^{14}C]pyruvate, many of the monocarboxylates tested inhibited pyruvate uptake. The most effective in this respect were 2-oxobutyrate, oxamate, 2-oxovalerate, 3-hydroxypyruvate and 3-fluoropyruvate. Significant inhibition was also obtained with 2-oxocaproate, phenylpyruvate and acetoacetate whereas 2-oxoisovalerate and 2-oxoisocaproate were slightly effective in this respect. 4-Oxovalerate, 2-oxomalonate and 2-oxoglutarate did not show any effect on the initial rate of pyruvate uptake.

The inhibition of pyruvate uptake by oxomonocarboxylates was analyzed by Lineweaver-Burk plots. A typical experiment illustrating the inhibitory pattern of 2-oxobutyrate on the kinetics of pyruvate uptake by rat liver mitochondria is reported in Fig. 2. 2-Oxobutyrate behaved as a purely competitive inhibitor of pyruvate uptake. Qualitatively similar results were obtained with other monocarboxylates listed in

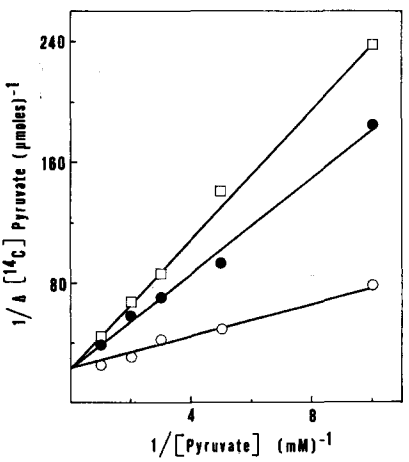

Fig. 2. Double reciprocal plots of the effect of 2-oxobutyrate on the uptake of pyruvate by mitochondria preincubated with 2-oxobutyrate. Mitochondria (8 mg protein) were preincubated in the presence of 2 mM 2-oxobutyrate in the sucrose medium described under Methods. Final pH 7.2. 3 min later, mitochondria were centrifuged through a second incubation layer containing [^{14}C]pyruvate, at the concentrations indicated in the figure, and 2-oxobutyrate as indicated below. Symbols: ○—○, control; ●—●, 0.5 mM 2-oxobutyrate; □—□, 2 mM 2-oxobutyrate in the second incubation layer. For other experimental details see under Methods.

Table II. The inhibition constant K_i values for oxomonocarboxylates competing for pyruvate uptake, calculated by the Dixon method [18], are presented in Table II.

It is worth noting that the K_i values for the inhibition of pyruvate uptake by oxomonocarboxylates are practically similar to the K_m values measured for their exchange reaction with intramitochondrial [^{14}C]pyruvate (ref. 4).

Effect of halogenated monocarboxylates on pyruvate transport

Various halogenated monocarboxylates have been shown to exchange with mitochondrial pyruvate [5]. Table III shows the effect of several halogenated carboxylic acids on the initial rate of pyruvate uptake by mitochondria. Monochloroacetate, dichloroacetate, monofluoroacetate and 2-chloropropionate were found to be strong inhibitors of pyruvate uptake. 3-Chloropropionate and 2,2-dichloropropionate were also effective in this respect. A slight inhibition was obtained with propionate, whereas acetate and trichloroacetate did not affect pyruvate uptake at all. As previously reported [2] it is likely that acetic acid and propionic acid cross the mitochondrial membrane by simple passive diffusion.

The inhibition of pyruvate uptake by halogenated carboxylic acids was of a purely competitive type. The K_i values are listed in Table III.

Effect of miscellaneous compounds

It has been reported that malate promotes efflux of pyruvate from mitochondria and inhibits pyruvate uptake when added simultaneously to the labelled substrate [6, 19]. In addition, Mowbray [6] has reported that palmitoylcarnitine acts as a pyruvate counteranion, in that it promotes pyruvate efflux from mitochondria. In the experiment reported in Table IV, the effect of inorganic phosphate, malate, succinate, citrate, carnitine and palmitoylcarnitine, added simultaneously to [^{14}C]pyruvate, on pyruvate uptake by 2-oxobutyrate loaded mitochondria was tested. In conditions

TABLE III

EFFECT OF SEVERAL HALOGENATED MONOCARBOXYLYC ACIDS ON THE INITIAL RATE OF PYRUVATE UPTAKE BY 2-OXOBUTYRATE-PRELOADED MITOCHONDRIA

Mitochondria (8.6 mg protein) were preincubated with 2 mM 2-oxobutyrate in the standard sucrose medium. 3 min later, mitochondria were centrifuged through a second incubation layer at 4 °C containing the same components as the preincubation medium and in addition 200 μM [^{14}C]pyruvate. The various anions listed in the table were added in the second incubation layer at the concentration of 2 mM. Pyruvate uptake is expressed as nmol/15 s/total amount of protein. All results are expressed as means$\pm$S.E.M. of 4 separate observations. The K_i values were determined as described in the legend to Table II. For other experimental details, see the legend to Table II and under Methods.

Substrate	Pyruvate uptake$\pm$S.E.M. (nmol)	Inhibition (%)	K_i (mM)
None	17.63$\pm$1.04		
Chloroacetate	7.90$\pm$0.93	55.2	0.25
Fluoroacetate	8.63$\pm$0.96	51.1	0.36
Dichloroacetate	8.87$\pm$0.84	49.7	0.46
2-Chloropropionate	10.14$\pm$0.98	42.5	0.59
3-Chloropropionate	12.93$\pm$1.16	26.7	2.20
2,2-Chloropropionate	14.96$\pm$1.33	15.2	5.60

TABLE IV

EFFECT OF MISCELLANEOUS COMPOUNDS ON THE INITIAL RATE OF PYRUVATE UPTAKE BY 2-OXOBUTYRATE-PRELOADED MITOCHONDRIA

Mitochondria (9.3 mg protein) were preincubated with 2 mM 2-oxobutyrate in the standard incubation medium described under Methods. Final pH 7.2. 3 min later, mitochondria were centrifuged through a second incubation layer at 4 °C containing the same components as the preincubation layer and in addition 200 μM [^{14}C]pyruvate and the various substrates at the concentration of 2 mM. Pyruvate uptake is expressed as nmol/15 s/total amount of proteins. For other experimental details, see the legend to Table II and III and under Methods. All results are expressed as means ± S.E.M. of 4 separate observations.

Substrate	Pyruvate uptake ± S.E.M. (nmol)	Inhibition (%)
None	18.43 ± 1.81	
L-Lactate	15.64 ± 1.06	15.4
3-Hydroxybutyrate	16.06 ± 1.14	14.9
2-Hydroxybutyrate	18.40 ± 0.96	0.1
2-Hydroxyvalerate	18.42 ± 1.64	0.1
Phosphate	17.56 ± 1.16	4.8
Malate	17.99 ± 1.48	2.4
Succinate	17.83 ± 1.96	3.3
Citrate	17.58 ± 1.46	4.7
DL-Carnitine	18.33 ± 2.01	0.6
Palmitoyl-L-Carnitine	17.97 ± 1.68	2.5

under which other substrates (see Table II and III) were able to inhibit pyruvate uptake, none of the above reported anions had an appreciable effect on pyruvate uptake. Furthermore, none of these anions promoted pyruvate efflux from pyruvate preloaded mitochondria (cf. ref. 2).

It should be noted that in our experiments the effect of the anions was tested on the initial rate of pyruvate uptake at low temperature (4 °C). This eliminates the possibility that ΔpH collapse, caused by uptake of the anions examined, could indirectly depress pyruvate uptake by mitochondria.

These results indicate that there is no direct relationship between the transport of pyruvate and that of inorganic phosphate di- and tricarboxylates (see also refs. 20–23), carnitine and palmitoyl-carnitine (cf. ref. 24).

Table IV illustrates the effect of 3-hydroxybutyrate, L-lactate, 2-hydroxybutyrate and 2-hydroxyvalerate on the rate of pyruvate uptake by mitochondria. Whilst 2-hydroxybutyrate and 2-hydroxyvalerate had no effect on pyruvate uptake, a slight but significant inhibition was obtained with L-lactate and 3-hydroxybutyrate. These results would suggest that both these monocarboxylates could utilize the pyruvate carrier. We have previously shown that 3-hydroxybutyrate exchanged with mitochondrial pyruvate but that its affinity for the carrier was very low [4]. The occurrence of a pyruvate-3-hydroxybutyrate exchange across the inner membrane of rat brain mitochondria has been recently reported [25, 26].

Effect of pre-exposure of mitochondria to different anionic substrates on the rate of pyruvate uptake

The initial rate of pyruvate uptake by mitochondria is differently affected by pre-exposure of mitochondria to certain anionic substrates [7].

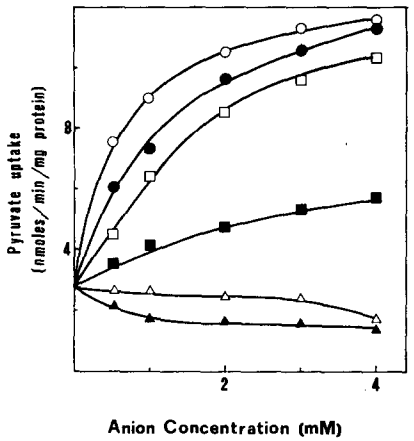

Fig. 3. Concentration-dependence effect of preincubation of mitochondria with various oxomonocarboxylates on the initial rate of pyruvate uptake. Mitochondria (8.7 mg protein) were preincubated in a sucrose medium described under Methods. The various oxomonocarboxylates were added, at the concentration shown in the figure, in the preincubation phase. Final pH 7.2. 3 min later, mitochondria were centrifuged through a second incubation layer containing the same components as the preincubation medium, except cold oxomonocarboxylates, and in addition [^{14}C]pyruvate (200 μM). Symbols: ○—○, unlabelled pyruvate; ●—●, 2-oxobutyrate; □—□, oxamate; ■—■, acetoacetate; △—△, phenylpyruvate; ▲—▲, 2-oxocaproate. The initial rate of pyruvate uptake in the control amounted to 2.65 nmol/min/mg protein. For other experimental details, see under Methods.

Fig. 3 illustrates the effect of preincubation of rat liver mitochondria with various concentrations of several oxomonocarboxylates on the initial rate of pyruvate uptake. Pyruvate was used at the concentration of 0.2 mM which is around the physiological concentration of this substrate in the cell [27]. Preincubation of mitochondria with pyruvate, 2-oxobutyrate, oxamate and acetoacetate led to a marked enhancement

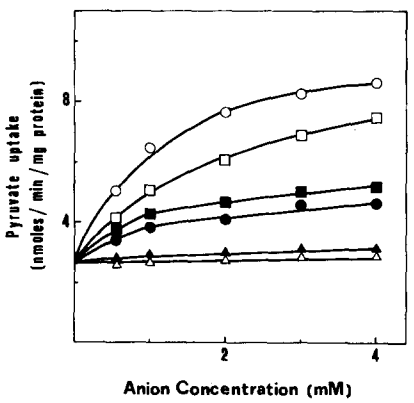

Fig. 4. Concentration-dependence effect of preincubation of mitochondria with acetate, propionate and several halogenated carboxylic acids on the initial rate of pyruvate uptake. The experimental conditions are similar to those reported in Fig. 3. Mitochondrial protein, 8.3 mg. Symbols: △—△, acetate; ▲—▲, propionate; ●—●, dichloroacetate; ■—■, 3-chloro-propionate; □—□, fluoroacetate; ○—○, chloroacetate. The initial rate of pyruvate uptake in the control amounted to 2.70 nmol/mg protein.

of the rate of pyruvate uptake. The concentration dependence of the stimulation of pyruvate uptake brought about by pre-exposure of mitochondria to these substrates shows that pyruvate exhibited the highest stimulatory effect, giving 100 % stimulation at a concentration of about 0.2 mM. 2-Oxobutyrate and oxamate were less effective giving 100 % stimulation at concentrations of 0.4 and 0.64 mM respectively. Phenylpyruvate and 2-oxocaproate did not stimulate pyruvate uptake. Among the other oxomonocarboxylates tested (not shown) 2-oxovalerate produced a stimulation of pyruvate uptake comparable to that given by acetoacetate; 2-oxoisovalerate and 2-oxoisocaproate had respectively a slight and no effect on pyruvate uptake.

The initial rate of pyruvate uptake was significantly enhanced by preincubation of mitochondria with various halogenated carboxylic acids. The concentration dependence effect of preincubation of mitochondria with halogenated monocarboxylic acids is presented in Fig. 4. Monochloroacetate and monofluoroacetate strongly stimulated pyruvate uptake, whereas 3-chloropropionate and dichloroacetate were less effective in this respect. Acetate and propionate had no effect on pyruvate uptake.

Inhibition of pyruvate transport by α-cyanocinnamate

α-Cyanocinnamate is a powerful inhibitor of the mitochondrial pyruvate carrier [3, 5]. The nature of the inhibition of pyruvate translocation in mitochondria by α-cyanocinnamate was analyzed in the experiments reported in Fig. 5, where the effect of this inhibitor on the kinetics of pyruvate uptake by unloaded and 2-oxobutyrate-loaded mitochondria was tested. α-Cyanocinnamate behaved as a purely competitive inhibitor of pyruvate uptake by both normal and 2-oxobutyrate loaded mitochondria. However, the K_i was 12 μM for unloaded and 80 μM for 2-oxobutyrate-loaded mitochondria. Competitive inhibition by α-cyanocinnamate of the [^{14}C]-

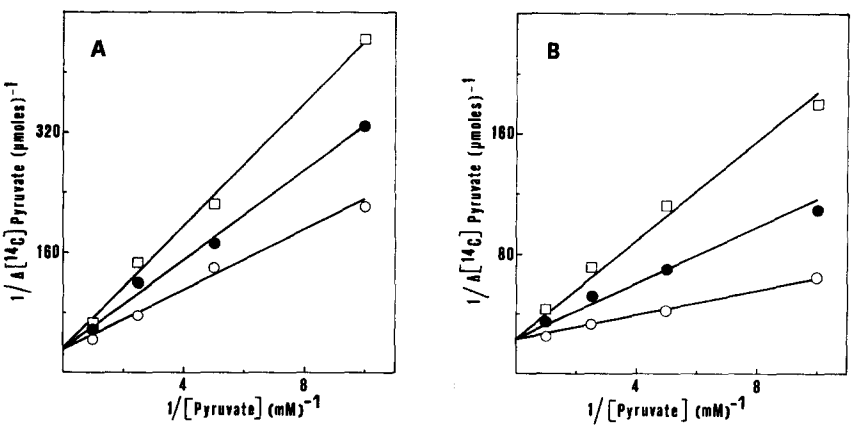

Fig. 5. Kinetic analysis of the inhibition of pyruvate uptake by α-cyanocinnamate in normal (A) and 2-oxobutyrate loaded mitochondria (B). Mitochondria (Expt. A, 8.0 mg protein and Expt. B, 8.8 mg) were preincubated in the sucrose medium described under Methods in absence (A) or in presence (B) of 2 mM 2-oxobutyrate. α-Cyanocinnamate, at the concentrations indicated below, was added after 1 min of preincubation. 2 min later, normal and 2-oxobutyrate loaded mitochondria were centrifuged through a second incubation layer containing [^{14}C]pyruvate at the concentrations indicated in the figure. Symbols: (A), ○—○, control; ●—●, α-cyanocinnamate 5 μM; □—□, α-cyanocinnamate 20 μM; (B), ○—○, control; ●—●, α-cyanocinnamate 50 μM; □—□, α-cyanocinnamate 200 μM.

TABLE V

KINETIC ANALYSIS OF THE EFFECT OF α-CYANOCINNAMATE ON THE TRANSLOCATION OF PYRUVATE, PHOSPHATE, 2-OXOGLUTARATE AND CITRATE IN RAT LIVER MITOCHONDRIA

Mitochondria were preincubated with 2 mM 2-oxobutyrate (Expt. 1) and 2 mM malate (Expts. 2, 3 and 4) in the sucrose medium described under Methods. After 3 min, 2-oxobutyrate-loaded mitochondria (Expt. 1) and malate-loaded mitochondria (Expts. 2, 3 and 4) were centrifuged through a second incubation layer containing different concentrations of [^{14}C]pyruvate (Expt. 1), $^{32}P_i$ (Expt. 2), 2-[^{14}C]oxoglutarate (Expt. 3) and [^{14}C]citrate (Expt. 4). α-Cyanocinnamate (0.5 mM in all experiments) was added to the preincubation medium 1 min after the addition of 2-oxobutyrate or malate respectively. Mitochondrial protein ranged from 8.2 to 9.4 mg.

Expts.	Substrate	Km (μM)		V (nmol/min per mg protein)	
		Control	+ α-Cyanocinnamate	Control	+ α-Cyanocinnamate
1	Pyruvate	183	1250	20.2	20.2
2	Phosphate	476	476	120.2	120.2
3	2-Oxoglutarate	57	57	58.0	58.0
4	Citrate	111	111	49.5	49.5

pyruvate-pyruvate and [^{14}C]pyruvate-acetoacetate exchanges has been previously reported ([4]; see however [5]).

The specificity of the inhibition of α-cyanocinnamate for the monocarboxylate translocator is illustrated by the data of Table V. At a concentration of 0.5 mM, this compound strongly inhibited pyruvate uptake by 2-oxobutyrate-loaded mitochondria, but had, on the contrary, no effect on the uptake of inorganic phosphate, 2-oxoglutarate and citrate by malate-loaded mitochondria.

To further investigate the nature of the inhibition of pyruvate transport by α-cyanocinnamate, the effect of this compound on the pyruvate-supported respiration in

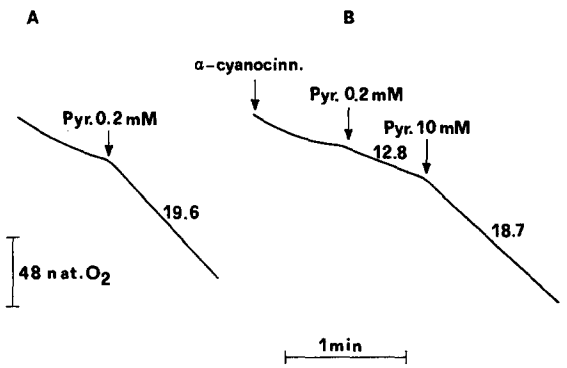

Fig. 6. Removal of the α-cyanocinnamate inhibition of pyruvate oxidation in mitochondria by high concentration of substrate. Mitochondria (4.3 mg protein) were incubated in a reaction medium containing in 1 ml: 75 mM sucrose; 30 mM Tris · HCl; 50 mM KCl; 1 mM $MgCl_2$; 0.5 mM EDTA; 1 mM P_i and 0.5 μM FCCP (carboxylcyanide *p*-trifluoromethoxyphenyl hydrazone). Final pH 7.2. Temperature 20 °C. Where indicated α-cyanocinnamate, at the concentration of 1 μM, was added 30 s before the addition of pyruvate. The numbers on the traces indicate the respiratory rates as natom O_2/min per mg protein.

intact rat liver mitochondria was examined. The polarographic traces of Fig. 6 show that the inhibitory effect of α-cyanocinnamate on pyruvate oxidation could be overcome by raising pyruvate concentration. This confirms the competitive nature of the inhibition.

DISCUSSION

The mitochondrial pyruvate translocator has been shown to mediate net transport of pyruvic acid and acetoacetic acid (anion-OH^- antiport or anion-H^+ symport) [1, 2] as well as exchange diffusion of mitochondrial pyruvate with specific oxomonocarboxylates [4]. The monocarboxylates found to exchange with mitochondrial pyruvate act also as purely competitive inhibitors of pyruvic acid uptake by mitochondria. The inhibitory potency of monocarboxylates is directly related to the affinity they exhibit in the exchange process, i.e. the K_i values of inhibition are quite close, if not identical, to the K_m values these compounds exhibit in the exchange-diffusion with mitochondrial pyruvate. It is thus conceivable that the pyruvate translocator possesses a single substrate binding-site which is directly involved in the transport process.

The exchange-diffusion experiments and the inhibitory pattern allow the definition of the affinity requirements of the substrate binding site of the pyruvate (or monocarboxylate) translocator.

The distance between the carbonyl and the carboxylic groups, both to be present in the substrates of the translocator, is very critical. In fact monocarboxylates with a carbonyl group in C_2 position exhibit much lower K_m and K_i values than their isomers with the carbonyl group in C_3 or C_4 position. 4-Oxovalerate is apparently not transported, neither does it inhibit pyruvic acid uptake. The length of the aliphatic chains is also important, as shown by the observation that K_m and K_i values increase as the chain length increases, going from pyruvate to 2-oxocaproate. Finally, the affinity of the substrate for the transporting system depends upon the spatial arrangement of the carbon chain. Thus the exchange and the competitive activity of 2-oxoisovalerate and 2-oxoisocaproate are much lower than those of 2-oxovalerate and 2-oxocaproate respectively.

An important aspect of the structural requirements of the pyruvate translocator is that the carbonyl group in C_2 can be replaced by an halogen. In fact chloroacetate, fluoroacetate and 2-chloropropionate exchange with intramitochondrial pyruvate and inhibit competitively pyruvate uptake with an affinity very close to that of pyruvate.

Taken all together, the structural requirements exhibited by the pyruvate translocator suggest that the substrate interacts with a polar region of the carrier protein, where two functional groups of amino acid residues find place at a few Å distance, one capable of forming hydrogen bond with the carbonyl or halogen in the C_2 position of the substrate, the other interacting with the carboxylic group. The hydrogen bond-forming group could be represented by an amino group, but also by an imidazole, indole or hydroxyl substituent. Involvement of amino groups in the transport of pyruvate is suggested by the observation that fluorodinitrobenzene inhibits pyruvate transport [28].

The lower affinity observed with 2-oxomonocarboxylates with aliphatic chains longer than four carbon atoms, branched hydrocarbon chains or a phenol ring could be due to hydrophobic shielding of the substrate polar groups and consequent hindrance of the reaction with the polar functional groups of the carrier.

It should be recalled that the substrate specificity, the kinetic parameters and the inhibition by SH-reagents differentiate the transport reactions described here from the diffusion of monocarboxylic acids which has been shown by Bakker and Van Dam to take place across artificial lecithin membranes at high substrate concentration [29].

The affinity for pyruvate is enhanced when the carrier is occupied by its substrates. This is shown by the finding that preincubation of mitochondria with a low K_m substrate (pyruvate, 2-oxobutyrate, chloroacetate and fluoroacetate) induces a stimulation of pyruvate uptake, which is characterized by a decrease of the K_m of pyruvate with no change of the V.

The substrate-induced activation of the translocator suggests that occupation of the carrier by the substrate, by bringing together at a few Å distance the two functional groups of the translocator, results in a conformation more favourable for binding of successive substrate molecules. The amount of the translocator which is brought into the active configuration by preexposure to substrates will be directly related to the equilibrium concentration of the substrate · carrier complex and is thus inversely related to their K_m and K_i values. In fact low K_m substrates produce a significant activation of the carrier; high K_m substrate, on the other hand, have small or no stimulatory effect on the translocator.

When added pyruvate finds the unoccupied carrier in the membrane, net pyruvate uptake takes place. In this case, the affinity of the carrier for pyruvate is lower and binding of the substrate is accompanied by a stoichiometric uptake of protons (or hydroxyl ion release). It is conceivable that the proton (or hydroxyl ion) transfer reaction takes place directly at the functional group interacting with the carboxylic group of the substrate.

Substrate-dependent activation of the translocator can also explain the observation that the K_m for the efflux of [^{14}C]pyruvate, from preloaded mitochondria, in exchange with externally-added unlabelled pyruvate is lower than that for [^{14}C]-pyruvate uptake by unloaded mitochondria [2] but equal to that of [^{14}C]pyruvate uptake by mitochondria preincubated with cold pyruvate or 2-oxobutyrate (see Table I).

α-Cyanocinnamate and its analogues are powerful [3, 5] and highly specific inhibitors (see Table V) of pyruvate transport in mitochondria as well as in the plasma membrane [3]. Halestrap has provided evidence that α-cyanocinnamate compounds inhibit pyruvate transport by attacking thiol groups of the carrier [28]. Pyruvate transport is also inhibited by less specific SH-reagents such as mersalyl and *N*-ethylmaleimide [2].

Our results show that α-cyanocinnamate behaves as a purely competitive inhibitor of pyruvate uptake by mitochondria as well as of exchange-diffusion of mitochondrial [^{14}C]pyruvate with external unlabelled substrates (see ref. 4). A competitive type of inhibition of pyruvate transport by α-cyanocinnamate has been recently shown to be present also in yeast mitochondria [13].

It is therefore conceivable that α-cyanocinnamate and its analogues react specifically with thiol groups closely related to the substrate binding site of the monocarboxylate carrier.

At difference with what found with α-cyanocinnamate, the inhibition of pyruvate uptake by NEM and mersalyl was previously found to be non-competitive [2]. This suggests that there exists in the pyruvate translocator a second type of

functional SH-groups which are not directly related to monocarboxylate binding to the carrier. Preincubation of mitochondria with monocarboxylates antagonizes interaction of α-cyanocinnamate with the SH-groups related to the substrate binding site (K_i is enhanced) and prevents interaction of NEM and mersalyl with the SH-groups unrelated to substrate binding. In fact, in the case of the exchange of mitochondrial pyruvate with external monocarboxylates, the inhibition by mersalyl was found to change from non-competitive to purely competitive and the inhibition by NEM disappeared [2]. These observations provide further evidence that substrate binding to the translocator modifies its conformation.

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