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The effect of phenylglyoxal on the translocation of pyruvate in rat-heart mitochondria

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The effect of phenylglyoxal, an arginine-specific reagent, on the translocation of pyruvate and on the binding of α-cyanocinnamate by rat-heart mitochondria has been studied. It has been found that both the uptake and the oxidation of pyruvate by mitochondria are inhibited by phenylglyoxal. The inhibitory potency increases with the increasing of the pH of the medium. Phenylglyoxal does not affect the transmembrane ΔpH. Phenylglyoxal also inhibits the binding of α-cyanocinnamate to mitochondria. Substrates of the carrier, such as pyruvate itself and monochloroacetate, partially prevent the inhibition of α-cyanocinnamate binding by phenylglyoxal, whilst acetate has no effect in this respect. Phenylglyoxal affects only the affinity of the α-cyanocinnamate binding site(s), without changing their total number. The results obtained indicate that arginine residues are involved in the mechanism of pyruvate translocation and of α-cyanocinnamate binding in rat-heart mitochondria.

Introduction

Pyruvate is transported into mitochondria from various biological sources by a transporting system which is specifically inhibited by α-cyanocinnamate derivatives [1–5]. A considerable amount of work has been published on the properties of this translocator [6–12]. However, little is known about its molecular mechanism. Recently, by using the radiolabelled α-cyanocinnamate, the binding-site of this inhibitor in rat-heart mitochondria has been well characterized [13,14]. Furthermore partial purification of the monocarboxylate carrier from bovine heart mitochondria has been reported [15,16].

On the basis of the inhibition by α-cyanocinnamate and other SH group reagents, sulphydryl groups have been shown to be the only functional groups of the pyruvate carrier [2,5,6]. In order to get further information on the molecular mechanism of this translocator in mitochondria, it is important to investigate the involvement of essential functional groups, other than SH groups. Possible candidates could be cationic aminoacids such as arginine residues which could participate in the binding of negatively charged group present in the pyruvate anion. There is now considerable evi-

Abbreviations: EDTA, ethylenediaminetetraacetic acid; DMO, 5,5-dimethyloxazoline-2,4-dione; TMPD, tetramethylphenylenediamine; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone.

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dence that phenylglyoxal is a very specific reagent for arginine residues [17]. It has been shown that this reagent is a strong inhibitor of several enzymes [18] and of the anion translocators in erythrocytes [19] and in mitochondrial membrane [20,21].

In this work the effect of phenylglyoxal on both the translocation of pyruvate and on the binding of α-cyanocinnamate by rat-heart mitochondria has been investigated. It has been found that both these functions are inhibited by phenylglyoxal.

Materials and Methods

Materials. Phenylglyoxal was obtained from Sigma. Solutions of this reagent were freshly prepared in ethanol and used daily. The radioactive [2-^{14}C]pyruvate, [^{14}C]acetate and [6,6′-^{3}H]sucrose, were obtained from the Radiochemical Centre, Amersham. Radioactive pyruvate was treated as follows. It was dissolved in water, divided into 5 μCi samples, freeze dried and stored in sealed tubes at −20 °C. α-Cyanocarboxyl [^{14}C]cinnamic acid was synthesized in the Radiochemical Centre, Amersham. Its specific activity was 19.2 mCi/mmol and its purity was 98%. All other reagents were of reagent grade purity and were purchased from Sigma.

Biological preparations. Rat-heart mitochondria and inside-out submitochondrial particles were prepared as described in Refs. 13 and 14.

The standard medium used in the measurements of respiratory activity, pyruvate transport, and in the binding experiments usually contained: 100 mM sucrose; 50 mM KCl; 20 mM Tris-HCl; 1 mM $MgCl_2$ and 0.5 mM EDTA.

Pyruvate transport. The initial rate of pyruvate transport by mitochondria was measured at 18 °C by the Halestrap inhibitor method, using α-cyanocinnamate as inhibitor [2]. The reactions were conducted in plastic centrifuge tubes (1.5 ml capacity). Each reaction mixture contained in 1 ml of the reaction medium described above: 0.5 mM sodium arsenite, 5 μg/ml rotenone, 0.5 μg/ml antimycin, 3 mM ascorbic acid, 0.05 mM TMPD and 0.7–1.5 mg of mitochondrial protein. After 5 min of preincubation of mitochondria, radiolabelled pyruvate was added and 15 s later the reaction was stopped by the addition of 2mM α-cyanocinnamate. The tubes were rapidly centrifuged at 20000 × *G* for 5 min at 4 °C. The pellets were washed with sucrose 0.25 M and dissolved in $HClO_4$. The vials were then recentrifuged. Solubilized mitochondria were transferred to 10 ml scintillation fluid and [^{14}C]pyruvate retained by mitochondria counted in a scintillation counter. The amount of [^{14}C]pyruvate, expressed as nmol per mg of mitochondrial protein, associated with the mitochondria was calculated from the amount of radioactivity in the mitochondrial pellet and the specific activity of the [^{14}C]pyruvate. The amount of [^{14}C]pyruvate present in the fluid outside the matrix or absorbed to the mitochondria was estimated in reactions in which α-cyanocinnamate was added before [^{14}C]pyruvate. The difference between the amount of pyruvate associated with the mitochondria in the absence of α-cyanocinnamate and that associated with the organelles in the reactions in which the inhibitor was added before the radioactive pyruvate was defined as pyruvate uptake.

Measurements of respiration. Rates of oxygen uptake by mitochondria were measured as previously described [13,14].

Measurements of binding. The binding of radioactive α-cyanocinnamate to rat-heart mitochondria and to inside-out submitochondrial particles was assayed essentially as described in Ref. 13 and 14.

pH measurements. The external pH was determined potentiometrically on the supernatant obtained after centrifugation of the mitochondrial suspension. The intramitochondrial pH (matrix space) was calculated on the basis of the distribution of 5,5-dimethyl[^{14}C]oxazoline-2,4-dione between the matrix space and the medium by the equation of Addanki and al. (Ref. 22; see also Ref. 6).

Mitochondrial protein were determined by the usual biuret method.

Results

In the experiment reported in Fig. 1 the effect of phenylglyoxal on the rate of pyruvate uptake by rat-heart mitochondria at both pH 7.0 and 7.6 has been studied. Preincubation of mitochondria with increasing concentrations of phenylglyoxal resulted in a concentration-dependent inhibition of pyruvate uptake. The inhibition was more pro-

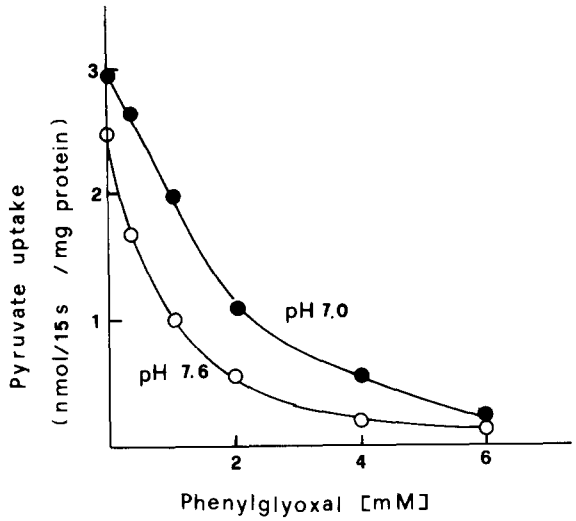

Fig. 1. The effect of increasing concentrations of phenylglyoxal on the rate of pyruvate uptake by rat-heart mitochondria and the effect of pH. The composition of the reaction medium and measurement of [^{14}C]pyruvate uptake by mitochondria were as described in Materials and Methods. Phenylglyoxal was added in the preincubation phase 5 min before adding pyruvate.

nounced at pH 7.6 with respect to pH 7.0, half maximal values of inhibition being obtained at concentrations of phenylglyoxal around 0.7 and 2 mM at pH values of 7.6 and 7.0, respectively.

The inhibition of pyruvate transport by phenylglyoxal was time-dependent. The time-dependence of the inhibition was more pronounced at pH 7.0 with respect to pH 7.6 (results not shown).

It has been well established that the uptake of pyruvate by mitochondria is driven by the transmembrane ΔpH [2,6]. Thus the inhibition of pyruvate transport by phenylglyoxal may not be due to a direct effect on the carrier itself, but rather a consequence of a decreased transmembrane ΔpH. In order to verify this possibility, the effect of phenylglyoxal on the accumulation of pyruvate and acetate, an anion which enters the mitochondria as free acid independently of pyruvate carrier was studied. The results reported in Table I demonstrate that whilst the uptake of pyruvate by mitochondria is strongly inhibited by phenylglyoxal, that of acetate is not affected at all. This clearly demonstrates that the inhibition by phenylglyoxal of pyruvate transport is not due to a decreased transmembrane ΔpH. This conclusion is further confirmed by a direct measurement of

TABLE I

THE EFFECT OF PHENYLGLYOXAL ON THE MITOCHONDRIAL PYRUVATE AND ACETATE UPTAKE AND ON THE TRANSMEMBRANE ΔpH

The uptake of pyruvate and acetate by mitochondria was measured as follows. Mitochondria (approx. 1 mg of protein/ml) were suspended in 1 ml of the standard medium described in Materials and Methods in the presence of inhibitors of the respiratory chain, $TMPD^+$ ascorbate and [6,6-^{3}H]sucrose as an extramitochondrial space marker. The medium was at pH 7.0 and maintained at 18°C. After 3 min of preincubation radiolabelled pyruvate or acetate, both at 0.5 mM concentration, were added; 1 min later the mitochondria were separated from the medium by rapid centrifugation. The pellets and the supernatants were acidified with $HClO_4$ and the acids extracts were analyzed for the radioactivity. Mitochondrial substrate content was corrected for substrate in the extramitochondrial space. The transmembrane ΔpH was determined as described in Materials and Methods. The experimental conditions were essential identical to those described above with the only exception that 5,5-dimethyl[^{14}C]oxazoline-2,4-dione was added instead of labelled pyruvate and acetate. The results are expressed as means of $\pm$S.E. of four separated observations.

Additions	Mitochondrial uptake of carboxylate (nmol/mg protein)		ΔpH
	pyruvate	acetate	
None	8.7 $\pm$ 0.5	7.4 $\pm$ 0.4	0.84 $\pm$ 0.03
Phenylglyoxal (4 mM)	2.5 $\pm$ 0.2	7.0 $\pm$ 0.4	0.84 $\pm$ 0.03

the transmembrane ΔpH measured by the distribution of 5,5-dimethyl[^{14}C]oxazoline-2,4-dione. In fact as shown in Table I, phenylglyoxal did not disturb the mitochondrial pH gradient.

The transport of pyruvate in mitochondria can be followed also by measuring the rate of pyruvate-dependent oxygen uptake in the presence of ADP. Under this experimental condition the transport of pyruvate has been shown to be a rate-limiting step for its oxidation [12]. The results reported in Table II show the effect of phenylglyoxal on the rate of pyruvate oxidation by rat-heart mitochondria. In the same experiment the effect of α-cyanocinnamate, a well known and specific inhibitor of pyruvate transport, has been tested. As expected, α-cyanocinnamate 1 μM almost completely inhibited the rate of pyruvate oxidation. This inhibition was totally prevented by the addition, in the preincubation phase, of di-

TABLE II

THE EFFECT OF α-CYANOCINNAMATE AND PHENYLGLYOXAL ON THE OXIDATION OF PYRUVATE BY RAT-HEART MITOCHONDRIA

The pyruvate-dependent oxygen uptake by mitochondria was measured with a Clark-type electrode essentially as described in Refs. 13 and 14. Mitochondria (1 mg protein/ml) were preincubated in the standard medium described under Materials and Methods, in the presence of 2 mM ADP and 2 mM P_i. Final pH 7.0; temperature, 25 °C. When a steady state of oxygen consumption was obtained, 0.5 mM pyruvate was added. The rate of respiration that followed within 30 s of pyruvate addition was used for calculating the rate of pyruvate-dependent oxygen uptake. When present, the various reagents were added in the preincubation phase 5 min prior the addition of pyruvate, at concentration of 1 μM α-cyanocinnamate, 5 mM dithioerythritol, 5 mM phenylglyoxal. The results are expressed as means of ± S.E. of four separate observations.

Additions	Pyruvate oxidation (ngatoms O/min per mg protein)	Percentage of inhibition
Control	220 ± 12	
α-Cyanocinnamate	12 ± 2	95
Dithioerythritol	220 ± 16	0
α-Cyanocinnamate + dithioerythritol	218 ± 18	1
Phenylglyoxal	40 ± 8	82
Phenylglyoxal + dithioerythritol	42 ± 9	81

thioerythritol, which reacts chemically with α-cyanocinnamate [23]. This result confirms that α-cyanocinnamate inhibits the transport of pyruvate by reacting with SH groups which are essential for the transport activity. Similarly to α-cyanocinnamate, phenylglyoxal was also able to inhibit the oxidation of pyruvate strongly. However, the addition of dithioerythritol in the preincubation phase did not prevent the inhibition, thus demonstrating that, by contrast to what was found with α-cyanocinnamate, the inhibition of pyruvate oxidation by phenylglyoxal does not involve the interaction of this aminoacid reagent with SH groups of the pyruvate carrier.

In the experimental conditions under which phenylglyoxal inhibited the oxidation of pyruvate by more than 85%, it inhibited the oxidation of acetoacetate (this substrate can utilize the pyruvate carrier) by 50%, whilst having only a slight inhibitory effect on the oxidation of succinate. In addition, phenylglyoxal inhibited the pyruvate-dependent oxygen uptake even when this process was followed with mitochondria incubated with an uncoupler (FCCP) rather than coupled with ADP. Furthermore, at concentrations of up to 5 mM phenylglyoxal had little or no effect on the activity of isolated pyruvate dehydrogenase (results not shown). These data indicate that the inhibition of pyruvate oxidation by phenylglyoxal is primarily due to the impairment of pyruvate uptake by mitochondria.

In the experiment reported in Fig. 2 the effect of pH on the inhibition of pyruvate oxidation by phenylglyoxal has been studied. As can be seen, the inhibition increases by increasing the pH of the medium from 7.0 to 7.6. Half maximal inhibition was obtained at concentrations of phenylglyoxal of 2 and 0.8 mM at pH values of 7.0 and 7.6, respectively. It was not possible to use higher pH values, since this led to a significant decrease of the rate of pyruvate oxidation in the controls.

Recently, the binding of α-cyanocinnamate by rat-heart mitochondria has been well characterized by using the radiolabelled inhibitor [13,14]. In the

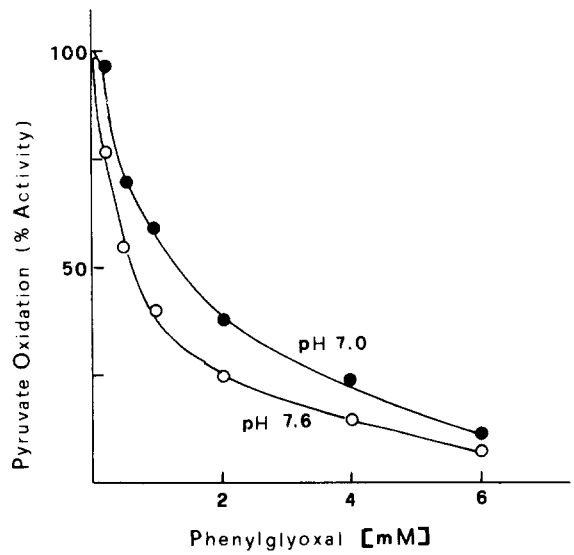

Fig. 2. The effect of pH on the inhibition of pyruvate oxidation by phenylglyoxal in rat-heart mitochondria. The pyruvate-dependent oxygen uptake by mitochondria was measured as described in Materials and methods and under the legend to Table I. Mitochondrial protein, 1 mg/ml. Phenylglyoxal, at the concentrations indicated in figure, was added in the preincubation phase 5 min before adding pyruvate.

experiment reported in Table III the influence of phenylglyoxal on the binding of α-cyanocinnamate to mitochondria was studied. At concentrations of 5 mM phenylglyoxal decreased the binding of α-cyanocinnamate by 45%. The presence in the preincubation phase of substrates of the pyruvate carrier, such as pyruvate itself and monochloroacetate, partially prevented the inhibition of α-cyanocinnamate binding by phenylglyoxal, whilst acetate, which is not a substrate of this carrier, had no effect.

The experiment reported in Table III was repeated, under the same experimental conditions, with inside-out submitochondrial particles. The results obtained (not shown) were practically similar to those obtained with intact mitochondria. This demonstrates that the inhibition by phenylglyoxal of α-cyanocinnamate binding in intact mitochondria is not due to damage of the mitochondrial membranes.

In order to explore better the interaction of phenylglyoxal with the α-cyanocinnamate binding site, the effect of this aminoacid reagent on the

TABLE III

THE EFFECT OF PHENYLGLYOXAL ON THE BINDING OF α-CYANOCINNAMATE BY RAT-HEART MITOCHONDRIA

The binding of α-cyanocinnamate to rat-heart mitochondria was measured essentially as described in Refs. 13 and 14. Mitochondria (1.2 mg of protein/ml) were preincubated at pH 7.0 and at 25°C in the standard medium described under Materials and Methods. After 10 min of preincubation 0.5 μM radioactive α-cyanocinnamate was added at 10 min later mitochondria were separated from the medium by rapid centrifugation. Phenylglyoxal (5 mM) and the various monocarboxylic acids (2 mM) were added in the preincubation phase. The results are expressed as means of S.E. of four separate observations.

Additions	α-Cyanocinnamate binding (pmol/mg protein)	Percentage of inhibition
Control	40.5 ± 5	
Phenylglyoxal	22.4 ± 3	45
Phenylglyoxal + pyruvate	34.1 ± 4	16
Phenylglyoxal + monochloroacetate	30.1 ± 4	26
Phenylglyoxal + acetate	22.3 ± 5	45

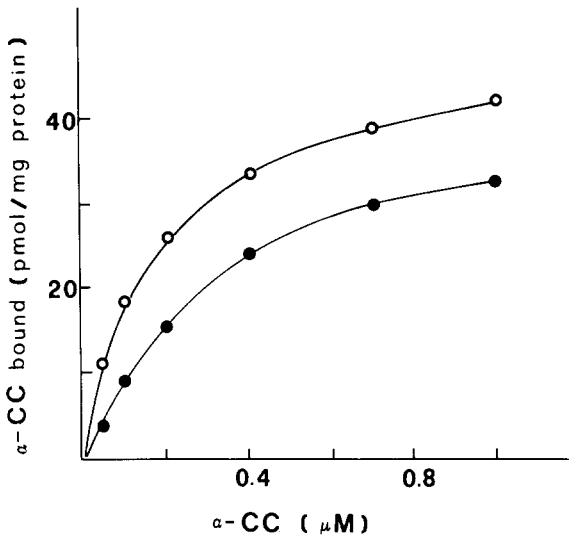

Fig. 3. The effect of phenylglyoxal on the binding of increasing concentrations of α-cyanocinnamate (α-CC) by rat-heart mitochondria. The binding of radioactive α-cyanocinnamate was measured as described in the legend of Table II (see also Refs. 13 and 14). Mitochondria (1 mg of protein) were preincubated in the standard medium described in Materials and Methods. After 10 min of preincubation, increasing concentrations of radioactive α-cyanocinnamate were added and 10 min later mitochondria were separated from the medium by rapid centrifugation. Phenylglyoxal (5 mM) was added in the preincubation phase. ○, control; ●, phenylglyoxal in the preincubation phase.

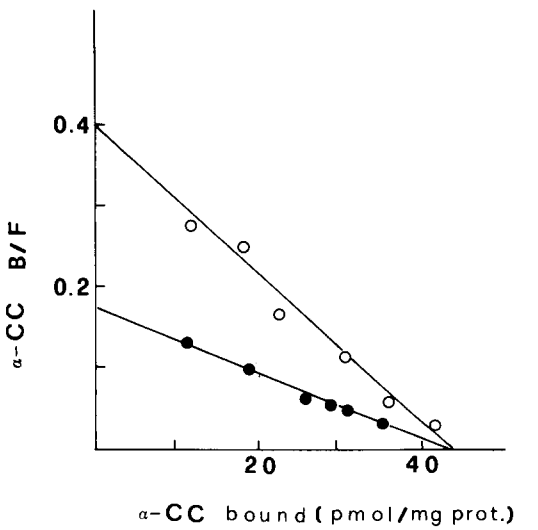

Fig. 4. Scatchard plots of α-cyanocinnamate (α-CC) binding to rat-heart mitochondria and the effect of phenylglyoxal. Data from Fig. 3.

binding of increasing concentrations of labelled inhibitor was examined. The results of this experiment are reported in Fig. 3. As previously reported [13,14] the binding of α-cyanocinnamate by rat-heart mitochondria was saturable. The saturation plateau of the binding curve corresponds to around 50 pmol of high affinity sites per mg of mitochondrial and the K_d was around 100 nM. Phenylglyoxal decreased the binding capacity of mitochondria for α-cyanocinnamate.

The data of Fig. 3 were analyzed by Scatchard plots and the results are reported in Fig. 4. It can be observed that phenylglyoxal modifies the affinity of α-cyanocinnamate binding sites, without changing their total number.

Discussion

The results reported in the present investigation demonstrate that both the translocation of pyruvate and the binding of α-cyanocinnamate by rat-heart mitochondria are inhibited by phenylglyoxal. This compound is well known to react specifically with arginyl residues of proteins [17]. Thus the most probable candidates for phenylglyoxal inactivation of the pyruvate carrier and α-cyanocinnamate binding are arginyl residues in an hydrophobic environment, although some unexpected reactivity of this reagent with other components of the pyruvate carrier cannot be excluded. In fact some reactivity of phenylglyoxal with sulphydryl groups has been reported [17]. The pyruvate carrier contains very reactive SH groups [5,6]. However, the following considerations exclude the possibility that the inhibition of pyruvate translocation by phenylglyoxal could be due to an interaction of this reagent with reactive SH groups of the carrier molecule. In fact, as shown in Fig. 2, the inhibition of pyruvate oxidation by phenylglyoxal exhibits a pH-dependence exactly opposite to that found previously with α-cyanocinnamate on the same process [14]. More important, whilst the inhibition of pyruvate oxidation by α-cyanocinnamate is totally prevented by dithioerythitol, that by phenylglyoxal is not affected by this reductant of thyol groups (see Table II). In addition phenylglyoxal has been reported to have the highest selectivity for arginine residues at pH 7.4 [23]. Thus the present data suggest that arginyl residues are primarily modified by phenylglyoxal. Butanedione is another high selective arginine modifying reagent. However, it was not possible to use this reagent in the present experiments, since the drastic conditions required for its use as arginine reagent, such as borate buffer at high pH values, cause a high degree of alteration of mitochondrial membrane.

Previous studies in our laboratory [13,14] have demonstrated that mitochondria contain a specific site at which α-cyanocinnamate binds, which is directly involved in the inhibition of pyruvate transport. It is shown here (see Table III) that phenylglyoxal also inhibits the binding of α-cyanocinnamate to mitochondria. Substrates of the pyruvate carrier such as pyruvate itself and monochloroacetate can partially protect the binding of α-cyanocinnamate against the inactivating effect of phenylglyoxal, whilst acetate, which is not a substrate of the carrier, has no protective effect. The partial protection afforded by substrates of the carrier against the inhibitory effect of phenylglyoxal suggests a partial overlapping of pyruvate sites and α-cyanocinnamate sites on the pyruvate carrier protein. Interestingly enough, phenylglyoxal modifies only the affinity of α-cyanocinnamate binding to mitochondria without changing the total number of the binding sites.

Thus the present data suggest that, besides SH groups, arginine residues also seem to be involved in the mechanism of pyruvate translocation in mitochondria. The exact role of arginine residues and probably of other aminoacids residues in the functioning of the pyruvate carrier will surely better ascertained once the carrier molecule has been isolated and purified to homogeneity.

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