Volume 205, number 2 FEBS 4010 September 1986

Inhibition of the mitochondrial tricarboxylate carrier by arginine-specific reagents

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Received 30 June 1986

The effect of arginine-specific reagents on the activity of the partially purified and reconstituted tricarboxylate carrier of the inner mitochondrial membrane has been studied. It has been found that 1,2-cyclohexanedione, 2,3-butanedione, phenylglyoxal and phenylglyoxal derivatives inhibit the reconstituted citrate/citrate exchange activity. The inhibitory potency of the phenylglyoxal derivatives increases with increasing hydrophilic character of the molecule. Citrate protects the tricarboxylate carrier against inactivation caused by the arginine-specific reagents. Other tricarboxylates, which are not substrates of the carrier, have no protective effect. The results indicate that at least one essential arginine residue is located at the substrate-binding site of the tricarboxylate carrier and that the vicinity of the essential arginine(s) has a hydrophilic character.

Tricarboxylate carrier *Arginine-specific reagent* *Mitochondria* *Membrane transport* *(Rat liver)*

1. INTRODUCTION

The inner mitochondrial membrane contains a specific carrier system for the transport of citrate known as the tricarboxylate carrier (review [1]). This system catalyzes an electroneutral strict counterexchange of citrate (isocitrate or phospho*enol*pyruvate) and malate, which is important for metabolic processes such as fatty acid synthesis, gluconeogenesis and transfer of reducing equivalents across the membrane [1,2]. Kinetic studies have shown that the activity of the tricarboxylate carrier is high in liver compared to that in heart and brain [3–5] and that the carrier has a single binding site for all its substrates [3]. Furthermore, the tricarboxylate carrier is inhibited specifically and competitively by the impermeable substrate analogue 1,2,3-benzenetricarboxylate [3].

In a previous paper we have partially purified the tricarboxylate carrier from rat liver mitochondria and reconstituted its activity in liposomes [6,7]. As in the case of the phosphate carrier [8–10], the citrate carrier also has an essential requirement for cardiolipin [6,11].

To obtain further information on the molecular mechanism of citrate transport in mitochondria, it is important to investigate the role of the essential functional groups of the carrier protein. Here, we report the effect of phenylglyoxal and other arginine-specific reagents on the activity of the partially purified and reconstituted tricarboxylate carrier. It is known that the α-dicarbonyl reagents specifically bind to arginyl residues and therefore are potent inhibitors of several enzymes [12] and of the anion translocator in erythrocyte membranes [13–15].

We have found that the reconstituted citrate transport activity is inhibited by phenylglyoxal, 1,2-cyclohexanedione, 2,3-butanedione and more strongly by the phenylglyoxal chromophoric derivatives *p*-nitrophenylglyoxal, *p*-hydroxyphe-

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Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/86/$3.50

nylglyoxal and 4-hydroxy-3-nitrophenylglyoxal.

2. MATERIALS AND METHODS

Hydroxyapatite (Bio-Gel HTP) and AG1×8 (100–200 mesh, acetate form) were purchased from Bio-Rad, [1,5-^{14}C]citric acid from the Radiochemical Centre (Amersham), cardiolipin from Avanti Polar Lipids, egg yolk phospholipids and 2,3-butanedione from Fluka, phenylglyoxal from Aldrich and 1,2-cyclohexanedione from K&K Laboratories. *p*-Nitrophenylglyoxal, *p*-hydroxyphenylglyoxal and 4-hydroxy-3-nitrophenylglyoxal were synthesized by L.Z. All other reagents were obtained as reported [6].

Rat liver mitochondria were isolated by standard procedures. Solubilization of mitochondria, purification of the tricarboxylate carrier by hydroxyapatite chromatography and reconstitution of the hydroxyapatite eluate in liposomes prepared in the presence of 20 mM citrate were carried out as in [6]. After removal of the external citrate by an AG 1×8 column (acetate form), the proteoliposomes were incubated at pH 7.4 with the arginine-specific reagents. After 40 min at 30°C, citrate/citrate exchange was initiated by adding 0.6 M [^{14}C]citrate and stopped 10 min later by 10 mM 1,2,3-benzenetricarboxylate. Deviations from this procedure are indicated in the figure legends. In the control samples 1,2,3-benzenetricarboxylate was added together with [^{14}C]citrate. The citrate/citrate exchange activity was calculated by subtracting the control values from the experimental samples [3,6]. Protein was determined by the Lowry method modified for the presence of Triton [16].

3. RESULTS

3.1. *Inhibition of the citrate/citrate exchange activity in reconstituted liposomes by various arginine-specific reagents*

The effects of the α-dicarbonyl reagent phenylglyoxal and its chromophoric analogues *p*-nitrophenylglyoxal, *p*-hydroxyphenylglyoxal and 4-hydroxy-3-nitrophenylglyoxal on reconstituted citrate transport activity are shown in fig.1A. All these arginine-specific reagents inhibit the reconstituted exchange, although different concentrations are necessary for 50% inhibition. 4-Hydroxy-3-nitrophenylglyoxal is the most effective inhibitor. The half-maximal inhibition of citrate/citrate exchange activity in liposomes was found to be 0.7 mM for 4-hydroxy-3-nitrophenylglyoxal, 2.4 mM for *p*-hydroxyphenylglyoxal, 3.2 mM for *p*-nitrophenylglyoxal and 4.1 mM for phenylglyoxal. In addition, two other reagents which are very specific for arginine residues in proteins, 1,2-cyclohexanedione and 2,3-butanedione (in the dark), were found to inactivate the reconstituted citrate transport activity. As compared with the phenylglyoxal derivatives, however, higher concentrations were necessary for inhibition (fig.1B). The concentrations required to achieve 50% inhibition were 15 and 23 mM for 1,2-cyclohexanedione and 2,3-butanedione, respectively. Almost identical inhibition curves (not shown) were obtained when the tricarboxylate carrier was titrated with arginine reagents directly in the hydroxyapatite eluate before its reconstitution into liposomes.

The time course of the inhibition of the reconstituted citrate transport activity by different concentrations of phenylglyoxal is shown in fig.2. The inhibition is time-dependent and increases in relation to the concentration of the inhibitor.

3.2. *Effect of pH on the inhibition of the reconstituted citrate transport activity by phenylglyoxal*

As shown in fig.3, the pH has a significant influence on the inhibition of the reconstituted citrate/citrate exchange activity by phenylglyoxal. The inhibition increases with increasing pH from 6.2 to 8.0 indicating that the pK of the reacting group(s) is rather high [12]. However, the experiments reported here were mainly carried out at pH 7.4 because the activity of the tricarboxylate carrier shows a maximum at about pH 7.0 [3,6]. The pH optimum of the modified carrier is the same as for the unmodified carrier (not shown).

3.3. *Protection of the reconstituted citrate/citrate exchange activity against inactivation caused by arginine-specific reagents*

The protective effect of citrate with respect to inactivation of the tricarboxylate carrier by 5 mM phenylglyoxal is shown in fig.4. The concentrations of citrate applied during the incubation of the proteoliposomes with the inhibitor were 0.6 and

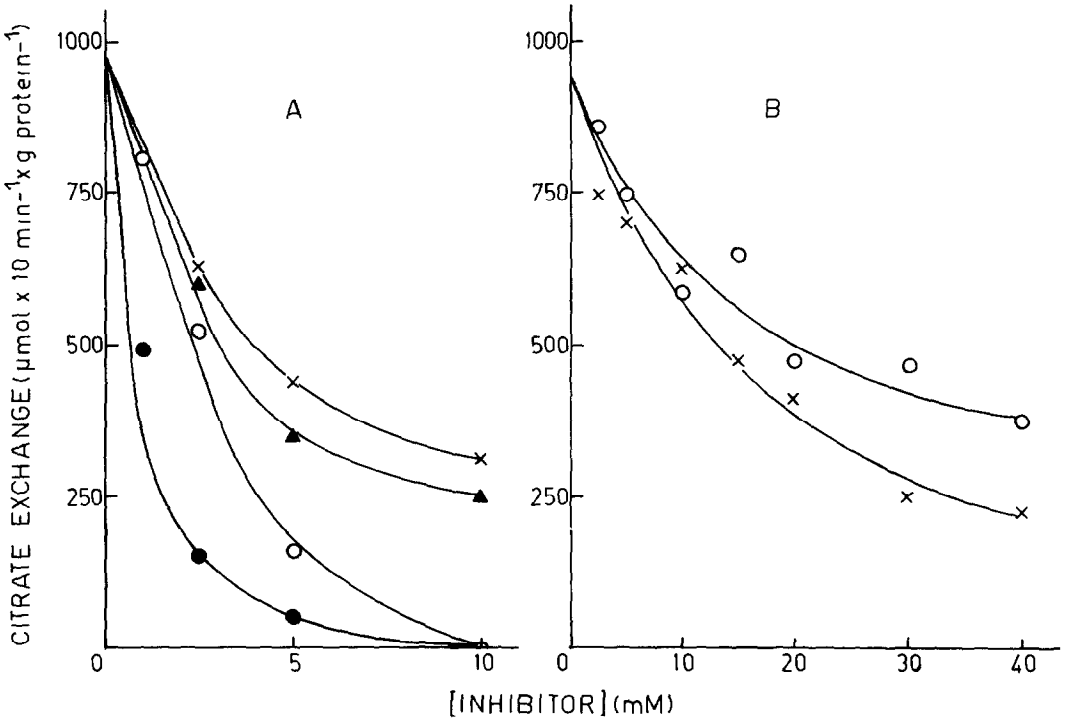

Fig.1. Effect of various arginine-specific reagents on the reconstituted citrate/citrate exchange activity. The reconstituted liposomes were incubated with the indicated amounts of phenylglyoxal (×), *p*-nitrophenylglyoxal (▲), *p*-hydroxyphenylglyoxal (○) and 4-hydroxy-3-nitrophenylglyoxal (●) in A and of 1,2-cyclohexanedione (×) and 2,3-butanedione (○) in B.

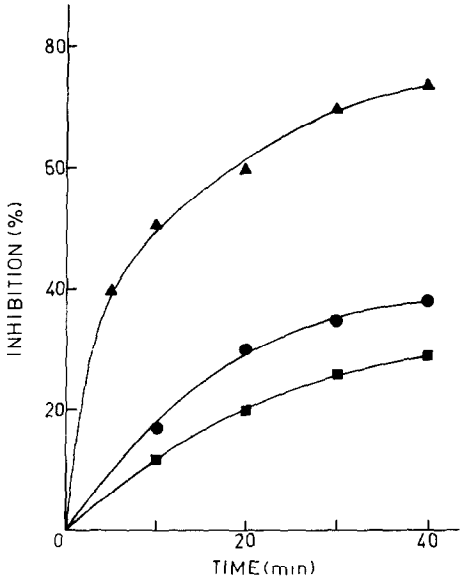

Fig.2. Time course of the inhibition of the reconstituted citrate/citrate exchange by phenylglyoxal. The time of incubation with the inhibitor was varied as indicated. Inhibitor concentration was 1.5 mM (■), 3 mM (●) and 9 mM (▲). The activity of the untreated carrier was 1030 μmol citrate · 10 min^{-1} · g protein^{-1}.

2.0 mM, i.e. about 2- and 7-times higher than the K_m value [6]. The activity was measured in all cases in the presence of 2 mM external [^{14}C]citrate. The results show that the protection is dependent on citrate concentration, reaching 64% protection in the presence of 2 mM citrate. When, on the other hand, 1,3,5-pentanetricarboxylate, which is chemically very similar to citrate, but which is no substrate of the carrier [3], is added to the proteoliposomes instead of citrate, no protection against inhibition by phenylglyoxal is achieved (fig.4). In agreement with this observation citrate/citrate exchange is not inhibited by adding 1,3,5-pentanetricarboxylate alone. In other experiments (not shown) it was found that citrate also protects the reconstituted citrate/citrate exchange activity against inactivation by 4-hydroxy-

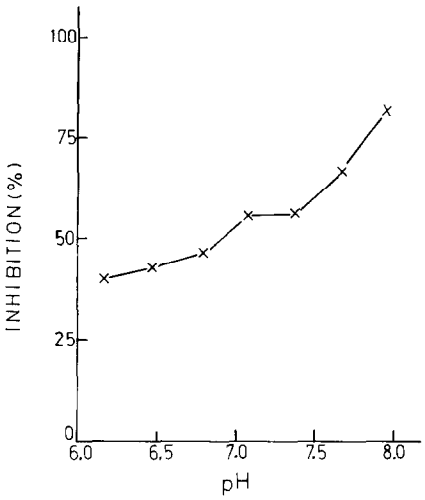

Fig.3. pH dependence of the inhibition of the reconstituted citrate transport by phenylglyoxal. The proteoliposomes were incubated at the indicated pH values with and without 7 mM phenylglyoxal.

3-nitrophenylglyoxal, which is the most potent inhibitor among the α-dicarbonyl reagents tested (see fig.1). In contrast, other tricarboxylates such as 1,3,5-pentanetricarboxylate, 1,3,5-benzenetricarboxylate and *trans*-aconitate, which are neither substrates nor inhibitors of the tricarboxylate carrier [3], do not exhibit any protection.

4. DISCUSSION

The reconstituted tricarboxylate carrier is inhibited by arginine-specific reagents such as phenylglyoxal, phenylglyoxal derivatives and two other α-dicarboxyl compounds 1,2-cyclohexanedione and 2,3-butanedione.

Among all the arginine-specific reagents tested, the phenylglyoxal derivative 4-hydroxy-3-nitrophenylglyoxal is the most effective inhibitor. *p*-Hydroxyphenylglyoxal is less effective than 4-hydroxy-3-nitrophenylglyoxal, but more than *p*-nitrophenylglyoxal and phenylglyoxal (fig.1). It is interesting that the inhibitory potency of the phenylglyoxal derivatives on the mitochondrial tricarboxylate carrier correlates well with the hydrophilicity of these compounds, as determined by Zaki [17]. This suggests that the vicinity of the essential arginine(s) has a hydrophilic character. In contrast, it should be noted that the same correlation has not been found with the anion-transporting system of erythrocytes [17]. It is known that 2,3-butanedione is highly selective for modification of arginyl residues in proteins when used in the dark. This reagent is usually used in the presence of borate buffer which stabilizes the adduct between the guanidino group and the inhibitor [12]. However, it was not possible to use borate buffer in the present experiments since it caused a high degree of inactivation of the reconstituted citrate/citrate exchange activity. This may explain why rather high concentrations of

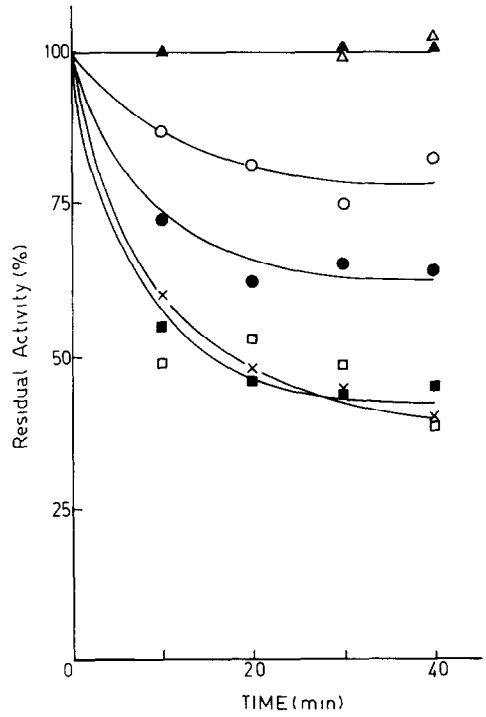

Fig.4. Protection of the reconstituted citrate transport activity against inhibition by phenylglyoxal. The proteoliposomes were incubated for the times indicated with 5 mM phenylglyoxal in the absence (×) or presence of 0.6 mM (●) and 2 mM (○) citrate, and 0.6 mM (□) and 2 mM (■) 1,3,5-pentanetricarboxylate. In parallel experiments the proteoliposomes were incubated for the indicated times with no addition (▲) or with 2 mM 1,3,5-pentanetricarboxylate (△). The citrate/citrate exchange was initiated by adding carrier-free [^{14}C]citrate (○), 2 mM [^{14}C]citrate (×, □, ■, ▲, △) or 1.4 mM [^{14}C]citrate (●) and stopped 5 min later with 20 mM 1,2,3-benzenetricarboxylate. The control activity was 715 μmol citrate · 5 min^{-1} · g protein^{-1}.

2,3-butanedione are required for inhibition of the tricarboxylate carrier. As shown in fig.1, the degree of inhibition of the reconstituted citrate/citrate exchange activity by 1,2-cyclohexanedione is higher than that caused by 2,3-butanedione. It should be noted that the arginine-specific reagents do not induce leakage of the proteoliposomes since the loss of internal radioactivity after application of the maximal concentration of reagents was less than 10–15% (not shown). Although the inhibitory effect of phenylglyoxal increases with increasing pH, all experiments were performed at pH 7.4 to avoid inhibition of citrate transport by high pH. On the other hand, phenylglyoxal has been reported to have the highest selectivity for arginine residues at pH 7.4 [18].

Citrate, the substrate of the tricarboxylate carrier, can protect the reconstituted transport activity against the inactivation caused by arginine-specific reagents. In contrast, other tricarboxylates which are not substrates of the carrier show no protective effect.

These results indicate that the tricarboxylate carrier possesses one (or more than one) essential arginine residue present at the substrate-binding site and located in a hydrophilic environment.

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