Journal of Bioenergetics and Biomembranes, Vol. 31, No. 6, 1999

Inactivation of the Reconstituted Oxoglutarate Carrier from Bovine Heart Mitochondria by Pyridoxal 5′-Phosphate[1,2]

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Received June 22, 1999; accepted July 12, 1999

The effect of pyridoxal 5′-phosphate and some other lysine reagents on the purified, reconstituted mitochondrial oxoglutarate transport protein has been investigated. The inhibition of oxoglutarate/oxoglutarate exchange by pyridoxal 5′-phosphate can be reversed by passing the proteoliposomes through a Sephadex column but the reduction of the Schiff's base by sodium borohydride yielded an irreversible inactivation of the oxoglutarate carrier protein. Pyridoxal 5′-phosphate, which caused a time- and concentration-dependent inactivation of oxoglutarate transport with an IC_{50} of 0.5 m*M*, competed with the substrate for binding to the oxoglutarate carrier (K_i = 0.4 m*M*). Kinetic analysis of oxoglutarate transport inhibition by pyridoxal 5′-phosphate indicated that modification of a single amino acid residue/carrier molecule was sufficient for complete inhibition of oxoglutarate transport. After reduction with sodium borohydride [^{3}H]pyridoxal 5′-phosphate bound covalently to the oxoglutarate carrier. Incubation of the proteoliposomes with oxoglutarate or L-malate protected the carrier against inactivation and no radioactivity was found associated with the carrier protein. In contrast, glutarate and substrates of other mitochondrial carrier proteins were unable to protect the carrier. Mersalyl, which is a known sulfhydryl reagent, also failed to protect the oxoglutarate carrier against inhibition by pyridoxal 5′-phosphate. These results indicate that pyridoxal 5′-phosphate interacts with the oxoglutarate carrier at a site(s) (i.e., a lysine residue(s) and/or the amino-terminal glycine residue) which is essential for substrate translocation and may be localized at or near the substrate-binding site.

KEY WORDS: Oxoglutarate carrier; pyridoxal 5′-phosphate; transport; proteoliposomes; mitochondria.

INTRODUCTION

The inner mitochondrial membrane contains a specific carrier system that catalyzes a counterexchange between oxoglutarate and malate (for reviews, see LaNoue and Schoolwerth, 1979; Meijer, 1981; Krämer and Palmieri, 1992). The oxoglutarate carrier (OGC) plays an important role in several metabolic processes such as gluconeogenesis from lactate and the malate–aspartate shuttle. In our laboratory, the OGC from heart and liver mitochondria has been isolated and functionally reconstituted into liposomes (Bisaccia *et al.*, 1985, 1988; Indiveri, *et al.*, 1987). The kinetic mechanism of reconstituted OGC purified from bovine heart mitochondria has been studied

[1] This paper is dedicated to the memory of Prof. Giacomino Randazzo.

[2] Key to abbreviations: DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonate; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; GME, glycine methyl ester; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; $NaBH_4$, sodium borohydride; OGC, oxoglutarate carrier; PIPES, 1,4-piperazine-diethanesulfonic acid; PLP, pyridoxal 5′-phosphate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SITS, 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonate.

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0145-479X/99/1200-0535$16.00/0

(Indiveri *et al.*, 1991). The amino acid sequence of the OGC has been determined by cDNA sequencing (Runswick *et al.*, 1990). The topography of the OGC in the mitochondrial membrane and in proteoliposomes has been studied by employing impermeable reagents, such as proteases and peptide-specific antibodies (Bisaccia *et al.*, 1994).

The identification of specific residues within the substrate-binding site of the carrier molecule is of importance for understanding its molecular mechanism. Amino acid-specific reagents have been used to identify functionally active sites in the OGC (Stipani *et al.*, 1995; 1996; Zara and Palmieri, 1988). Recently, we have studied the effect of α-dicarbonyl compounds, known to be highly selective for arginine residues, on oxoglutarate carrier activity (Stipani *et al.*, 1996).

In this paper we have investigated the effect of pyridoxal 5′-phosphate (PLP) and some other lysine-specific reagents on oxoglutarate transport in proteoliposomes. Our results show that the OGC contains one or more lysine groups and/or an amino-terminal glycine, which are essential for its activity and may be located within or near the substrate-binding site.

MATERIALS AND METHODS

Materials

Hydroxyapatite (Bio-Gel HTP) and AG1-X8 were purchased from Bio-Rad; celite 535 from Roth, 2-keto[1-^{14}C]glutaric acid from Dupont De Nemours. PIPES, Triton X-114, glycine methyl ester, and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide from Sigma; pyridoxal 5′-phosphate, 4,4′-diisothiocyanostilbene-2,2′-disulfonate and 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonate from Serva; amberlite from Fluka; egg yolk phospholipids from Merck; and Sephadex G-75 from Pharmacia. All other reagents were of analytical grade.

Isolation and Reconstitution of the Oxoglutarate Carrier

The oxoglutarate carrier was purified from bovine heart mitochondria (Bisaccia *et al.*, 1985) and reconstituted into liposomes by cyclic removal of the detergent with a hydrophobic column (Palmieri *et al.*, 1995). The composition of the initial mixture used for reconstitution was: 300 μl of the purified oxoglutarate carrier (about 0.5 μg protein), 45 μl of 10% Triton X-114, 100 μl of 10% egg yolk phospholipids in the form of sonicated liposomes, 10 m*M* oxoglutarate, and 10 m*M* PIPES (pH 7.0) in a final volume of 700 μl. After vortexing, this mixture was recycled ten times through an Amberlite column (0.5 × 5 cm) preequilibrated with 10 m*M* PIPES (pH 7.0).

Assay of Oxoglutarate Transport Activity

The external substrate was removed by chromatography on Sephadex G-75 column (0.8 × 15 cm) preequilibrated with 50 m*M* NaCl and 10 m*M* PIPES (pH 7.0). The first 600 μl of the turbid eluate from the Sephadex column were collected and distributed in reaction vessels (150 μl each). Transport was initiated by the addition of 0.3 m*M* [^{14}C]oxoglutarate and stopped, after the desired time, by adding 2 m*M* mersalyl or 20 m*M* pyridoxal 5′-phosphate. In control samples, the inhibitor was added together with the labeled substrate. The assay temperature was 25°C. The external radioactivity was removed by passing each sample of proteoliposomes (150 μl) through a Dowex AG1-X8 column, 100–200 mesh, chloride form. The proteoliposomes eluted with 1 ml of 50 mM NaCl were collected in 4 ml of scintillation mixture, vortexed, and counted. The transport activity was calculated by subtracting the control from the experimental values. The control value of uninhibited oxoglutarate/oxoglutarate exchange (i.e., 0.3 m*M* external [^{14}C]oxoglutarate, 10 m*M* internal oxoglutarate, in the absence of modifying reagents) was 5323 ± 338 μmol × min^{-1} × g protein^{-1} (24 experiments). This value was not dependent on the preincubation time from 0 to 20 min at 25°C.

Chemical Modification of the Oxoglutarate Carrier

The external substrate was removed by gel filtration on Sephadex G-75 as described above. The modification of the OGC was carried out by incubating proteoliposomes with the reagents in the dark at 25°C and at pH 8.0. In protection experiments, the substrates were added prior to the reagent. The modification reactions were stopped by adding GME (EDC) or DTE (DIDS and SITS). Where indicated, a second gel filtration was rapidly performed before assaying oxoglutarate transport. The modifying reagents used were

prepared fresh daily; PLP was prepared for each experiment at pH 8.0 and stored in the dark. Sodium borohydride ($NaBH_4$) was prepared at pH 8.0 with NaOH. EDC and succinate were mixed at pH 6.2 3 min before addition. For protein labeling, 1 m*M* [^{3}H]PLP was used. After precipitation by acetone, detergent, and lipids were extracted with organic solvent, and the labeled protein was then applied to a polyacrylamide slab gel electrophoresis performed in the presence of 0.1% SDS according to Laemmli (1970). Fluorographic detection of radioactivity was performed as in Stipani *et al.*, 1995.

Other Methods

Protein was measured by the biuret method or by a modified Lowry procedure (Dully and Grieve, 1975). Pyridoxal 5′-phosphate was tritiated by reduction with [^{3}H] $NaBH_4$ and reoxidation with freshly prepared MnO_2 (Stock *et al.*, 1966; Raibaud and Goldberg, 1974). The concentration of PLP was determined by measuring the absorbance at 324 nm in 100 m*M* NaOH (Bogner *et al.*, 1986). The specific activity obtained was $2.2–3.4 \times 10^6$ dpm/nmol.

RESULTS

Inhibition of the Reconstituted Oxoglutarate Carrier Protein by Lysine-Specific Reagents

Table I shows that when proteoliposomes were incubated with 5 mM PLP for 5 min, reconstituted oxoglutarate/oxoglutarate exchange activity was completely inhibited. Since at nonalkaline pH the modification of protein by PLP is reversible (Rippa *et al.*, 1967; Anderson *et al.*, 1966; Dierks *et al.*, 1992), we passed the PLP-treated proteoliposomes through a gel filtration column. By this procedure, the OGC activity was nearly completely restored (Table I). The inhibition became irreversible after reduction with $NaBH_4$. In the same table, the effect of other lysine reagents on reconstituted oxoglutarate transport activity is shown. When proteoliposomes were incubated with other lysine reagents such as 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) in the presence of succinate, or 4,4′-diisothiocyanostilbene-2,2′-disulfonate (DIDS), or 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonate (SITS), a large amount of oxoglutarate transport activity was inhibited. The results reported in Fig. 1A show that incubating proteoliposomes with PLP in the dark at 25°C and pH 8.0 for 10 min caused a concentration-dependent inhibition of reconstituted oxoglutarate transport activity. The concentration of PLP was varied from 0.25 to 10 m*M* in the absence of external oxoglutarate. From the data in Fig. 1A, the concentration required for half-maximal inhibition of oxoglutarate transport (IC_{50}) of 0.5 m*M* was deduced. The time course of the inhibition of reconstituted transport activity caused by 5 m*M* PLP is shown in Fig. 1B. The inhibition of transport activity was dependent on the incubation time of the proteoliposomes with PLP. At pH 8.0, the inactivation of the OGC was very rapid and maximum inhibition (about 90%) was reached within 5 min; the $t_{1/2}$ was 1 min (Fig. 1B).

Table I. Inactivation of the Reconstituted Oxoglutarate Carrier After Chemical Modification by Lysine-Specific Reagents[a]

Reagent	Concentration (m*M*)	Inhibition (%)
PLP[b]	5	96 ± 3 (n = 7)
PLP	5	5 ± 1 (n = 7)
EDC/succinate	2/5	84 ± 4 (n = 7)
DIDS	0.1	96 ± 5 (n = 5)
SITS[b]	0.25	91 ± 6 (n = 5)
SITS	0.25	11 ± 4 (n = 5)

[a] After removal of the external substrate, the proteoliposomes were incubated with PLP, EDC/succinate, DIDS, or SITS at the indicated concentrations for 5 min in the dark at 25°C and pH 7.6. After a second gel filtration, the residual exchange activity was measured by adding 0.3 m*M* [^{14}C] oxoglutarate. The reaction was stopped by 2 m*M* mersalyl after 1 min.

[b] Activity assay without previous removal of reagent.

Kinetic Analysis and Substrate Protection of the Oxoglutarate Carrier against Inactivation by Lysine-Specific Reagents

The inhibition of the oxoglutarate/oxoglutarate exchange by PLP is analyzed in Fig. 2. The addition of 1 or 2 mM PLP simultaneously with [^{14}C]oxoglutarate increased K_m without changing the V_{max} of oxoglutarate transport, in agreement with a competitive type of inhibition. A K_i of 0.4 m*M* was evaluated. The competitive interaction between oxoglutarate and PLP was further supported by a Dixon analysis of the data in Fig. 2 (not shown).

The specific protection of a protein against an inhibitor in the presence of its substrate is taken as evidence for the interaction of the inhibitor at the active substrate binding-site. Figure 3 demonstrates that when

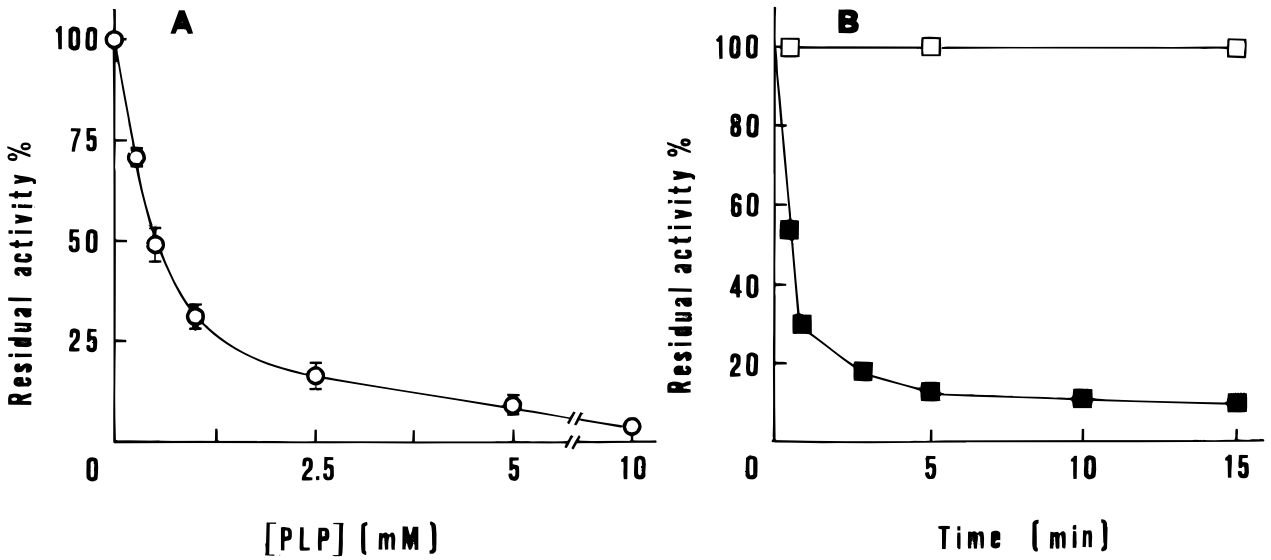

Fig. 1. (A) Inhibition of the reconstituted oxoglutarate transport activity by PLP. After gel filtration to remove the external oxoglutarate, proteoliposomes were incubated with the indicated concentrations of PLP for 10 min in the dark at 25°C and pH 8.0. Following the addition of 20 m*M* $NaBH_4$, oxoglutarate/oxoglutarate exchange activity was measured after a second gel filtration by adding 0.3 m*M* [^{14}C] oxoglutarate. (B) Time course of the inhibition of the reconstituted oxoglutarate carrier by PLP. After removal of the external oxoglutarate, the proteoliposomes were incubated for the times indicated with (■) or without (□) 5 mM PLP in the dark at 25°C and pH 8.0. The reaction was stopped by adding 20 m*M* $NaBH_4$, pH 8.0. The transport of oxoglutarate was measured as in (A) by adding 0.3 m*M* [^{14}C] oxoglutarate. In (A), the data represent means ± SD obtained from an average of four experiments in duplicate. In (B) the data are from a representative experiment.

the proteoliposomes were incubated for the times indicated with 2 m*M* PLP in the presence of 2.5 or 5 m*M* oxoglutarate, the oxoglutarate transport activity was largely protected against inhibition by 2 m*M* PLP. In contrast, a substrate analog such as glutarate, that is known not to be transported by the OGC, did not cause any protective effect (Fig. 3). In other experiments, we found that the other substrate of the OGC, i.e., L-malate, as well as EDC in the presence of succinate, protected the OGC against inhibition by PLP, but not against DIDS or SITS (data not shown).

Stoichiometry of Inactivation by PLP

At nonlimiting concentrations of PLP, the plots of the logarithm of transport activity versus the time of PLP treatment are linear (Fig. 4A), showing that the inactivation follows pseudo-first-order kinetics with respect to the remaining activity. At low concentrations of PLP, there is a linear relationship between the apparent rate constant of inactivation (K_{app}) and the concentration of the inactivator

$$K_{app} = K[\text{PLP}]^n \quad (1)$$

where n is the reaction order with respect to PLP concentration and K is the proportionality constant (Levy *et al.*, 1963). Thus

$$\log K_{app} = \log K + n \log [\text{PLP}] \quad (2)$$

Applying the analysis described by Levi *et al.* (1963), K_{app} values could be derived from the slope of the inactivation kinetics depicted in Fig. 4A. When the values of Fig. 4A were replotted according to Eq. (2), n was calculated to be about 0.9 (Fig. 4B). This value is close to one suggesting that the modification of a single amino acid residue per functional carrier molecule was sufficient for complete inactivation of oxoglutarate/oxoglutarate exchange. The modification of the OGC by PLP increased with increasing pH in the incubation mixture, reaching a maximum at pH 8.0 (not shown). It was, therefore, feasible that besides amino groups, SH groups of cysteine residues (p*K* 8.4) could react with PLP. Since OGC possesses highly reactive cysteines (Zara and Palmieri, 1988; Quagliariello and Palmieri, 1972; Capobianco *et al.*, 1996; Bisaccia *et al.*, 1996a, b), we thought that it would be

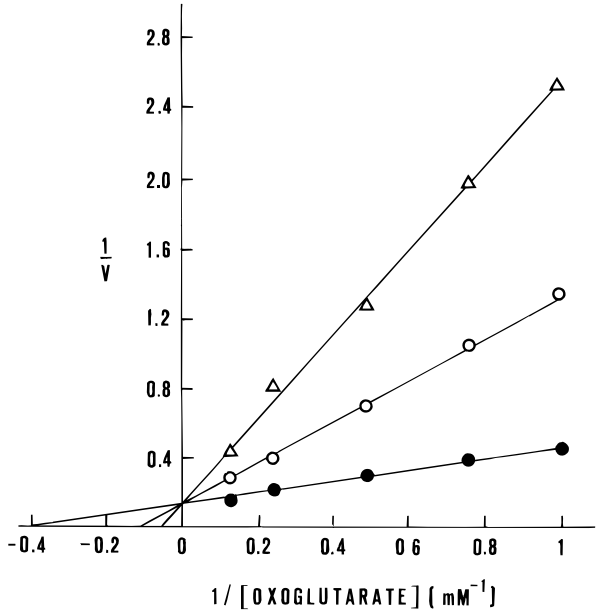

Fig. 2. Competitive inhibition of the oxoglutarate transport activity by PLP. The oxoglutarate/oxoglutarate exchange activity was measured in proteoliposomes after removal of the external substrate. [^{14}C] oxoglutarate was added at the concentrations indicated. With the exception of the controls (●), 1 m*M* (○) or 2 m*M* (△) PLP was added simultaneously with the [^{14}C] oxoglutarate. *V* is expressed in μmol/min × mg protein.

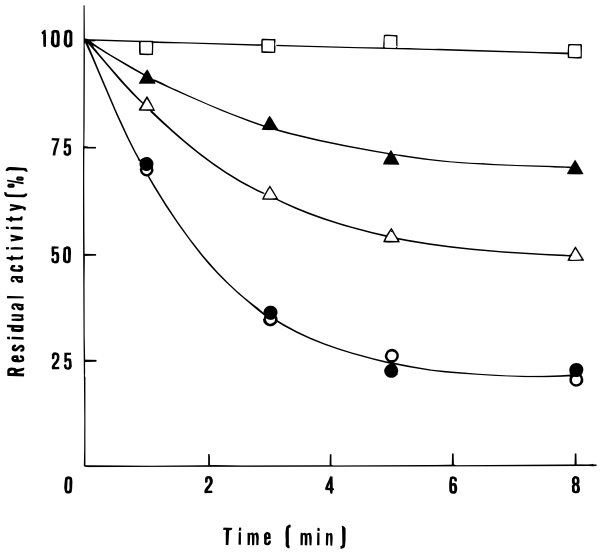

Fig. 3. Substrate protection against inhibition of the reconstituted oxoglutarate carrier by PLP. After removal of external substrate, proteoliposomes were incubated for the times indicated without (□) or with 2 m*M* PLP in the absence (●) or in the presence of 2.5 m*M* (△) or 5 m*M* (▲) oxoglutarate or 10 m*M* glutarate (○). At the times indicated, the reaction was stopped by adding 20 m*M* $NaBH_4$, pH 8.0. After gel filtration, oxoglutarate/oxoglutarate exchange was measured by adding 0.3 m*M* [^{14}C] oxoglutarate. The data are from a representative experiment; similar results were obtained in three different experiments in duplicate.

interesting to investigate whether SH reagents are able to protect the OGC against inactivation by PLP. As shown in Table II, the incubation of proteoliposomes with mersalyl before addition of PLP did not change the inhibition by PLP of the oxoglutarate transport activity, as measured after removal of the SH reagent by dithioerythritol. This means that the inhibition of OGC by pyridoxal 5′-phosphate is not caused by a reaction of this reagent with sulfhydryl groups of the carrier protein.

Labeling of the Oxoglutarate Carrier by [^{3}H] PLP

In the experiment illustrated in Fig. 5, the oxoglutarate carrier was extracted from proteoliposomes incubated with 1 m*M* [^{3}H]PLP and subjected to SDS-polyacrylamide gel electrophoresis and fluorography. Lane 1 of Fig. 5 shows that [^{3}H]PLP in the presence of $NaBH_4$ covalently bound to the oxoglutarate carrier protein. The labeling of the OGC by [^{3}H]PLP was prevented when oxoglutarate (lane 3), L-malate (lane 6) or EDC/succinate (lane 4) were present during the incubation of the proteoliposomes with the inhibitor. In contrast, the labeling of the oxoglutarate carrier protein was not influenced (lanes 2 and 5 of Fig. 5) when the proteoliposomes were incubated with [^{3}H]PLP in the presence of glutarate and DIDS, respectively. These results are in agreement with the substrate protection of the carrier against the inhibition by PLP.

DISCUSSION

Pyridoxal 5′-phosphate modifies the ε-amino group of lysine residues and the α-amino group of the amino terminus of a protein with high specificity (Lundblad, 1991) and reversibly (Rippa *et al.*, 1967; Anderson *et al.*, 1966; Dierks *et al.*, 1992 and Table I). The results presented here show that PLP inhibits the purified and reconstituted oxoglutarate carrier competitively. Its inhibition constant (K_i = 0.4 m*M*) is slightly higher than that exhibited on the mitochondrial citrate carrier (Gremse *et al.*, 1995) and not significantly different from that exhibited on the mitochon-

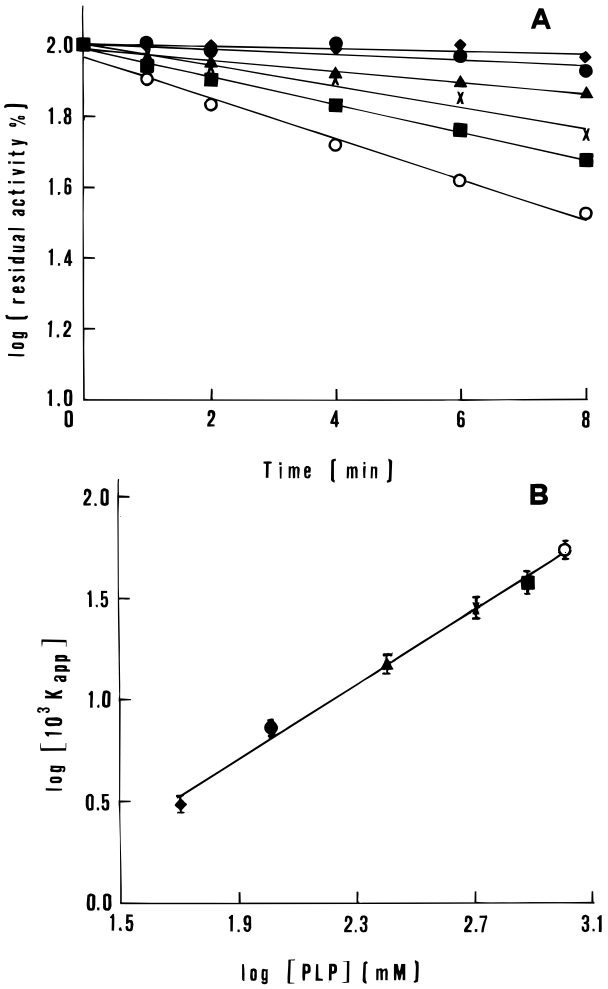

Fig. 4. Kinetics of inactivation of the reconstituted OGC by PLP. After removal of the external substrate, the proteoliposomes were incubated in the dark at 25°C and pH 8.0 with different PLP concentrations (◆, 0.05 m*M*; ●, 0.1 m*M* ▲, 0.25 m*M*; ×, 0.5 m*M*; ■, 0.75 m*M*; ○, 1 m*M*). The reaction was stopped at the times indicated by addition of 20 m*M*. $NaBH_4$, pH 8.0. After a second gel filtration, the remaining oxoglutarate transport activity was measured. In (A) the residual activity is given as a percentage of controls; the means of four experiments are reported. In (B), the double-logarithmic secondary plot of the means ± SD of the apparent inactivation constant K_{app} (2.3 × slope of the straight lines of A) versus the applied PLP concentration is shown.

drial aspartate/glutamate carrier (Dierks *et al.*, 1992). Furthermore, it is consistent with the apparent IC_{50} (concentration leading to half-maximal inhibition) of 0.5 m*M* determined after 10-min incubation of the reconstituted OGC with PLP. Our results further show that after reduction with sodium borohydride, the OGC becomes irreversibly modified, as shown by the irreversible inhibition of the carrier activity (Table II) and

Table II. Lack of Protection by Mersalyl on the Pyridoxal 5′-Phosphate Inhibition of the Oxoglutarate Carrier[a]

Addition	Inhibition (%)
Control	0
PLP, $NaBH_4$	76
Mersalyl	84
Mersalyl, dithioerythritol	4
Mersalyl, PLP	81
Mersalyl, PLP, dithioerythritol	8
Mersalyl, PLP, $NaBH_4$, dithioerythritol	79

[a] After gel filtration, proteoliposomes were incubated with buffer or 0.25 mM mersalyl (2 min) before addition of 2 mM PLP (10 min). Where indicated, 20 m*M* $NaBH_4$ (5 min) and/or 10 m*M* dithioerythritol (5 min) was added. After a second gel filtration, the residual oxoglutarate/oxoglutarate exchange activity was measured. The activity of the unmodified carrier did not change with or without dithioerythritol. The results are means of three experiments.

by the radioactivity associated with the carrier (Fig. 5). Most importantly, the substrate protection of the OGC against the inactivation caused by PLP and its binding to the carrier protein indicates that PLP modifies a lysine residue(s) and/or the amino-terminal glycine residue, which may reside either within or near the substrate-binding site of the oxoglutarate carrier. Other lysine reagents inhibit the reconstituted OGC. However, among these reagents, EDC protects the carrier against inactivation and binding by PLP, whereas

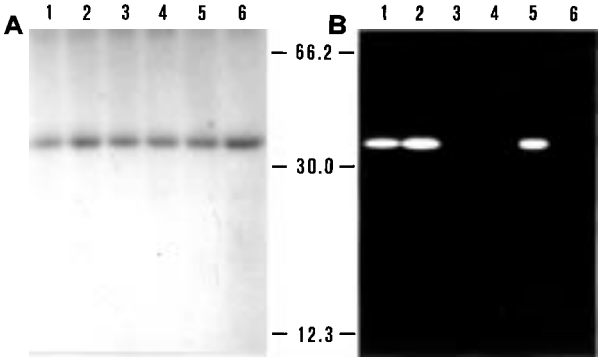

Fig. 5. Labeling of oxoglutarate carrier in proteoliposomes by [^{3}H]PLP. After gel filtration the proteoliposomes were incubated for 20 min in the dark at 25°C with 1 m*M* [^{3}H]PLP in the absence (lane 1) or in the presence of 10 m*M* glutarate (lane 2), 5 m*M* oxoglutarate (lane 3), 2.5/5 m*M* EDC/succinate (lane 4), 0.5 m*M* DIDS (lane 5) or 5 m*M* L-malate (lane 6). The reaction was stopped by adding 20 m*M* $NaBH_4$, pH 8. After removal of the unbound [^{3}H]PLP, lipids were removed from the oxoglutarate carrier protein and subjected to SDS-gel electrophoresis (A) and fluorography (B).

DIDS and SITS do not. These results can be interpreted to show that the oxoglutarate carrier possesses two classes of lysine residues, the first inhibited by PLP and EDC at or near the substrate-binding site and the second inhibited by DIDS and SITS located at a site(s) distant from the oxoglutarate-binding site. We have previously found that the transport of oxoglutarate reconstituted in liposomes with purified OGC is inhibited by α-dicarbonyl reagents (Stipani *et al.*, 1996) and diethylpyrocarbonate (unpublished results). Taken together, these results suggest that the oxoglutarate carrier contains positively charged amino acids that are essential in the binding and in translocation of the negatively charged substrates. It is remarkable that the OGC possesses 16 lysine and 20 arginine residues, respectively. Experiments are in progress to identify the lysine residue(s) of the oxoglutarate carrier modified by pyridoxal 5′-phosphate.

ACKNOWLEDGMENTS

The scientific contact with Dr. F. Palmieri (Bari) is greatly appreciated. This work was supported by grants from the MURST-PRIN 1997, the European Social Fund and the CNR.

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