FEBS Letters 392 (1996) 54–58 FEBS 17391

The mitochondrial oxoglutarate carrier protein contains a disulfide bridge between intramembranous cysteines 221 and 224

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Received 19 June 1996; revised version received 2 July 1996

Abstract The oxoglutarate carrier (OGC) purified from bovine heart mitochondria was treated, both in its active and in its SDS-denatured state, with the fluorescent *N*-(1-pyrenyl)maleimide and other SH reagents before and after reduction with dithioerythritol or β-mercaptoethanol. The number of SH groups per OGC polypeptide chain was found to be about 1 for the oxidized carrier and 3 for the reduced carrier. The bovine oxoglutarate carrier contains three cysteines: Cys-184, Cys-221 and Cys-224. Sequencing of BrCN cleavage products of oxoglutarate carrier showed that *N*-(1-pyrenyl)maleimide binds to only Cys-184 of the oxidized protein and also to Cys-221 and Cys-224 after reduction of the protein. These results show the presence of a disulfide bridge between the latter two cysteines of the purified carrier. The oxidized and the reduced forms of the oxoglutarate carrier exhibited different V_{max} but virtually the same K_m values for oxoglutarate.

Key words: Oxoglutarate carrier; Disulfide bridge; *N*-(1-Pyrenyl)maleimide; Reducing reagent; Transport; Mitochondria

1. Introduction

The oxoglutarate carrier (OGC) catalyzes the transport of 2-oxoglutarate across the inner mitochondrial membrane in an electroneutral exchange for malate or other dicarboxylic acids [1], and plays an important role in several metabolic processes, including the malate-aspartate shuttle, the oxoglutarate-isocitrate shuttle, gluconeogenesis from lactate and nitrogen metabolism [2,3]. It has been purified [4–6], kinetically characterized in reconstituted liposomes [5,7] and its amino acid sequence determined [8–10]. There is only one gene for this protein in man and cow [9]. Furthermore, the oxoglutarate carrier has been expressed in *E. coli* and refolded in reconstitutively active form [11]. On the basis of both structural and functional properties, this transport protein belongs to the mitochondrial carrier family (for review see [12–14]). Like for the other members of this family, a model has been proposed for the arrangement of the OGC in the inner mitochondrial membrane in which the polypeptide chain consists of six transmembrane α-helices connected by hydrophilic loops [15].

Disulfide bridges are the most important covalent bonds involved in the stabilization of the tertiary and quaternary structures of proteins. The characterization of sulfhydryl groups in protein and the assignment of disulfide bridges provide valuable information for the determination of protein structure. The OGC protein contains three cysteines: Cys-184 located in the fourth transmembrane segment [15], and Cys-221 and Cys-224 located in the fifth transmembrane segment [15]. In a previous paper [16], using liposomes reconstituted with purified OGC, we have demonstrated that mercurials and maleimides interact only with Cys-184 and that this reaction is responsible for the inhibition of the OGC transport activity.

In this report we give conclusive evidence that Cys-221 and Cys-224 of the OGC purified from bovine heart mitochondria are linked by a disulfide bridge.

2. Materials and methods

PM, FM, mersalyl and DTNB were obtained from Sigma; egg yolk phospholipids (lecithin from eggs) and Dowex AG1-X8 (50–100 mesh) from Fluka; α[1-^{14}C]ketoglutarate and *N*-[ethyl-1-^{14}C]maleimide from Dupont; L-[1,4(2,3)-^{14}C]malate from Amersham; polyvinylidene difluoride (PVDF) membranes from Applied Biosystems. Phthalonic acid was a gift of Prof. G. Randazzo. All other reagents were of the highest purity commercially available.

The OGC from bovine heart mitochondria was purified in Triton X-114 as described previously [4,17]. Modification of the functionally active Triton X-114-solubilized OGC was performed in the same medium in which the protein was purified (3% Triton X-114 (w/v), 20 mM Na_2SO_4, 2 mM EDTA and 10 mM PIPES, pH 7.0). The purified OGC was denatured with 1% SDS after precipitation with a 20-fold excess of cold acetone for 4 h at −20°C and removal of the lipids by the method of Wessel and Flügge [18]. The SDS-denatured protein was incubated with the modifying reagents in 1% SDS. The other incubation conditions are reported in the legends to figures and Table 1. At the end of the incubations (active Triton X-114-solubilized OGC) or after each incubation (SDS-denatured OGC), OGC samples were acetone-precipitated as described above, washed with cold acetone 3–4 times and then dissolved in the sample buffer for SDS-polyacrylamide slab gel electrophoresis [16] or in 80% formic acid for BrCN cleavage. BrCN cleavage was performed in the dark with an excess of BrCN (30 mg/ml). After 13 h, the solution was diluted 10 times with water and then freeze-dried. The dried material was dissolved in SDS-sample buffer [16], loaded onto an SDS-slab gel and separated by the system of Schägger and von Jagow [19]. PM labelling of the OGC protein and of the BrCN cleavage products was visualized by exposing the gel immediately after electrophoresis to UV light. Fluorographs were obtained by photographing the fluorescent emission with the help of cutoff filters. For protein sequencing, the separated peptides were transferred to PVDF membranes, detected by staining with Coomassie brilliant blue dye, excised and subjected to Edman degradation in an Applied Biosystems 477A pulse liquid protein sequencer.

The purified OGC was reconstituted into liposomes in the presence of 20 mM oxoglutarate by the cyclic detergent removal procedure [17]. External oxoglutarate was removed from the resulting proteoliposomes by chromatography on a Sephadex G-75 column. Transport was started by adding [^{14}C]oxoglutarate or [^{14}C]malate to the eluted proteoliposomes and terminated after 60 s by the addition of 30 mM phthalonate according to the inhibitor stop method [17]. In control

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Abbreviations: DTE, dithioerythritol; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); FM, *N*-phenylmaleimide; ME, β-mercaptoethanol; NEM, *N*-ethylmaleimide; OGC, oxoglutarate carrier; PM, *N*-(1-pyrenyl)maleimide

PII S0014-5793(96)00784-3

samples, the inhibitor was added together with the labelled substrate [17,20]. The incubation temperature was 25°C. Each quenched sample was then passed over a Dowex AG1-X8 in order to remove extraliposomal oxoglutarate or malate and the eluted radioactivity was measured by liquid scintillation counting [17]. The transport activity was calculated by subtracting the respective control from the experimental values.

The content of -SH groups in the SDS-denatured OGC, before and after reduction with DTE or ME, was measured by the Ellman procedure [21,22] (at 412 nm after addition of 1 mM DTNB and using $\varepsilon_{412} = 13600\ M^{-1}\ cm^{-1}$) and by [^{14}C]NEM alkylation (after reaction with a 300-fold excess of labelled NEM for 30 min at 25°C, precipitation with cold acetone, washing 4 times with acetone, resuspension in SDS and counting).

3. Results and discussion

As shown in Fig. 1A, the labelling of purified and Triton X-114-solubilized OGC by the fluorescent sulfhydryl reagent PM was completely abolished by 2 mM FM. Obviously, all the free sulfhydryl groups of the solubilized carrier, which can be alkylated by maleimides under these conditions (presumably Cys-184 according to [16]), were blocked by FM and were therefore no longer available to PM, although this reagent was used at a concentration 5 times higher than FM. If, however, the solubilized carrier was reduced by ME, after having been treated with FM, the subsequent addition of PM again led to labelling of the protein (Fig. 1A). This labelling (after the treatments with FM and ME) was also due to the presence of free sulfhydryl groups since it was abolished by specific SH reagents such as mersalyl (Fig. 1A). Furthermore, when ME was added to the solubilized protein without previous addition of FM, the PM labelling of the carrier was much greater than the control (i.e. direct labelling by PM in the absence of ME), indicating that treatment with ME makes more PM-reacting sites available per protein molecule (e.g. by breaking a possible disulfide bridge between Cys-221 and Cys-224). These results suggest that PM binds only to Cys-184 when the protein is in the oxidized state and also to Cys-221 and Cys-224 when the protein is reduced by the addition of ME. In the experiments described in Fig. 1A, the solubilized OGC protein was successively treated with several reagents at 0°C for rather long times for a total of 3 h. It was therefore important to check that the carrier was not damaged by incubations as long as 3–4 h at 0°C. Measurements of the carrier activity performed in proteoliposomes, as exchange of external 0.1 mM [^{14}C]oxoglutarate with intraliposomal 20 mM oxoglutarate, at the beginning and after 4 h incubation of the solubilized protein were virtually identical, showing that the OGC is rather stable in the solubilized state as previously found in the reconstituted state [15].

In the experiments of the type reported in Fig. 1A the modifying reagents were not removed after each treatment and, consequently, rather high concentrations of reagents had to be applied (for example 10 mM PM after 2 mM FM to be sure of the complete protection of PM binding by FM). We therefore decided to perform experiments similar to that reported in Fig. 1A using the purified protein after denaturation in SDS. In this case, the excess of each reagent was removed after its incubation with the OGC by precipitation of the protein and several washes. The results obtained with the SDS-denatured OGC protein, reported in Fig. 1B–D, confirmed and extended the information obtained with the solubilized and functionally active protein. NEM almost totally abolished, and FM or mersalyl completely abolished, the PM labelling of the denatured protein in the oxidized state. When the denatured carrier was first treated by NEM or FM (in concentrations sufficient to abolish the PM labelling of the oxidized carrier) and then by DTE, the subsequent addition of PM led again to labelling of the protein, suggesting that DTE makes available further SH groups to alkylation by the fluorescent probe. The hypothesis that DTE makes available free sulfhydryl groups on the carrier protein is supported by the fact that not only FM (Fig. 1C) but also mersalyl (Fig. 1D) and other specific SH reagents, such as *p*-hydroxymercuribenzoate and *p*-chloromercuriphenylsulfonate (not shown), when added after FM and DTE, abolished the subsequent labelling by PM. In line with this interpretation, Fig. 1C,D also shows that the amount of PM bound to the reduced carrier was greater than that bound to the oxidized carrier, as shown by the more intense fluorescence of the fourth lane in Fig. 1C and of the fifth lane in Fig. 1D as compared to the respective first lanes in each figure.

The amount of -SH groups of the oxidized and the reduced OGC after denaturation with SDS was determined by means of [^{14}C]NEM and DTNB (Table 1). Using NEM, one finds a reaction of 29.6 ± 3.8 μmol -SH groups/g protein for the oxidized carrier (without ME), 97.8 ± 5.1 μmol for the reduced carrier (with ME) and 57.8 ± 4.0 μmol for the carrier protein treated with FM and then with ME. This extent of incorporation of NEM into the OGC corresponded to about one -SH group per polypeptide chain (with the molecular weight of 34 172 [8]) for the oxidized carrier (without ME), three -SH groups per polypeptide chain for the reduced carrier (with ME) and two -SH groups per polypeptide chain for the carrier protein treated with FM and then reduced by ME. With the more specific -SH group reagent DTNB (Ellman's reagent) about the same number of -SH groups were found for the oxidized and the reduced OGC protein. These results demonstrate that two cysteines of the purified and oxidized carrier protein (out of a total of three) failed to react with NEM or DTNB unless the protein was reduced with ME.

Table 1
Reactive -SH groups of SDS-denatured OGC after reduction by β-mercapthoethanol

Incubation with	μmol -SH groups/g protein			
	[^{14}C]NEM	No. Expts	DTNB	No. Expts
–	29.6 ± 3.8	4	30.3 ± 2.1	6
ME	97.8 ± 5.1	3	85.4 ± 4.2	7
FM	0	2	0	2
FM and then ME	57.8 ± 4.0	3	64.8 ± 4.5	3

The purified SDS-denaturated OGC was incubated with each indicated reagent for 30 min at 25°C. The concentrations of the modifying reagents were 0.5 mM β-mercaptoethanol (ME) and 2 mM *N*-phenylmaleimide (FM). After each incubation the carrier protein was acetone-precipitated and washed 4 times. At the end, OGC samples were redissolved in SDS and reacted with [^{14}C]NEM or DTNB.

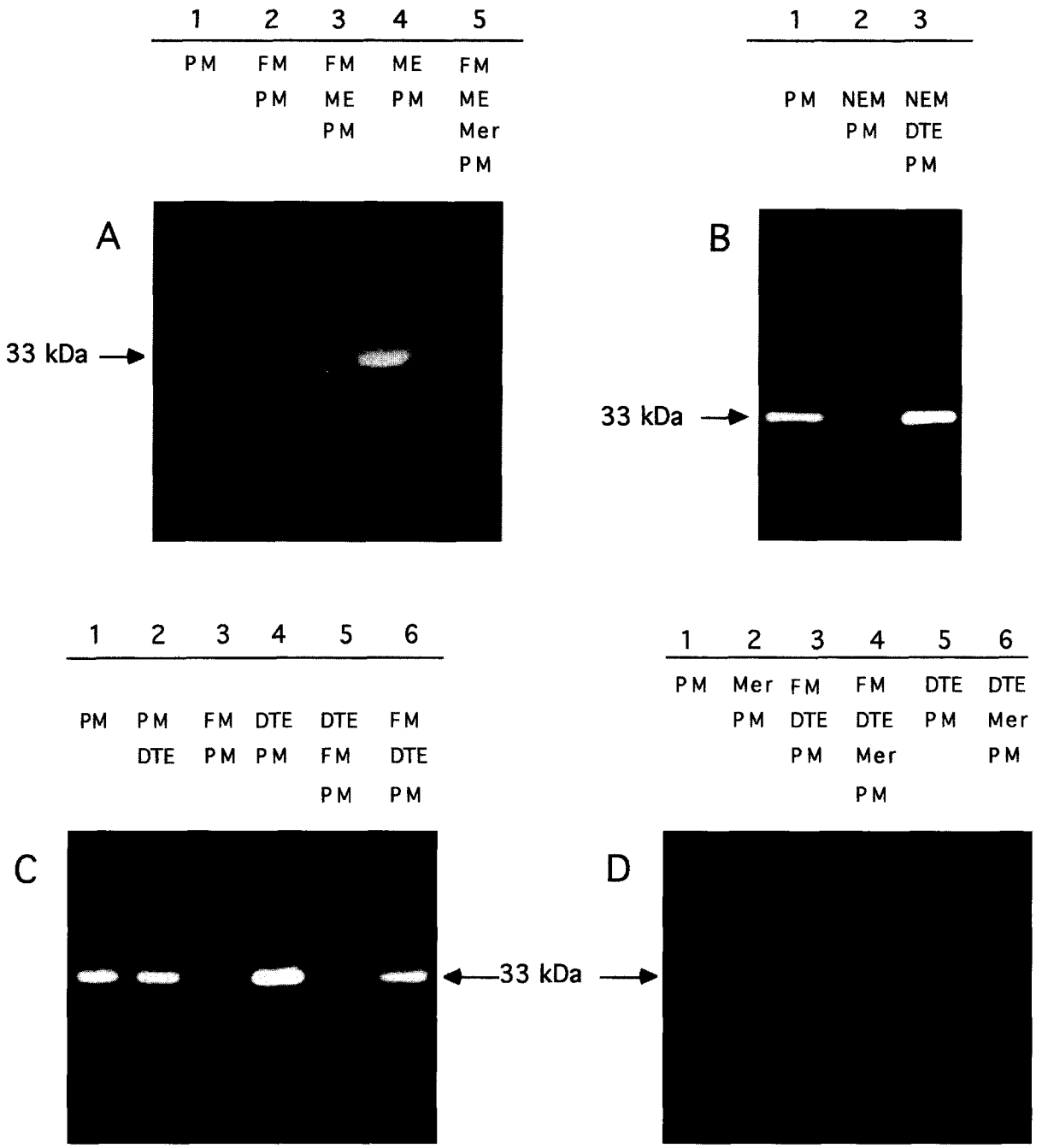

Fig. 1. Influence of reducing reagents and SH reagents on PM labelling of the OGC in the Triton X-114-solubilized state or in the SDS-denatured state. (A) The purified and Triton X-114-solubilized OGC was successively incubated with the indicated reagents for 30 min (ME or Mer) or 60 min (PM or FM) at 0°C. The concentrations of the modifying reagents were 10 mM *N*-(1-pyrenyl)maleimide (PM), 2 mM *N*-phenylmaleimide (FM), 4 mM β-mercapthoethanol (ME), and 5 mM mersalyl (Mer). At the end of incubation, OGC samples were acetone-precipitated, washed 3 times, solubilized in SDS-sample buffer and run on SDS-gel electrophoresis. (B–D) The purified and SDS-denatured OGC was incubated with the indicated sequences of reagents (for 30 min at 25°C with each reagent). The concentrations of the reagents were 1 mM PM, 2 mM NEM, 2 mM FM, 100 μM mersalyl, and 10 mM DTE. After each incubation, the carrier protein was acetone-precipitated and washed 4 times. At the end, the OGC was redissolved in SDS-sample buffer and run on SDS-gel electrophoresis.

Direct evidence that PM binds to only Cys-184 of the oxidized protein and that the same reagent also binds to Cys-221 and/or Cys-224 of the reduced protein is provided in Fig. 2. In these experiments the oxidized protein (lane 1), the reduced protein (lane 2) and the protein treated with FM and then reduced by DTE (lane 3) were labelled with PM and subsequently cleaved by BrCN. In all three cases 13 peptides were obtained (lanes 1–3 of Fig. 2A). Among these, only one peptide with an apparent M_r of 4 kDa for the oxidized protein (lane 1 of Fig. 2B), two peptides with an apparent M_r of 4.5 and 4 kDa for the reduced protein (lane 2 of Fig. 2B), and only one peptide with an apparent M_r of 4.5 kDa for the protein treated with FM and then reduced by DTE (lane 3 of Fig. 2B) were found to be fluorescent. In parallel experiments the BrCN cleavage products were blotted to a PVDF membrane and those which were fluorescent were sequenced.

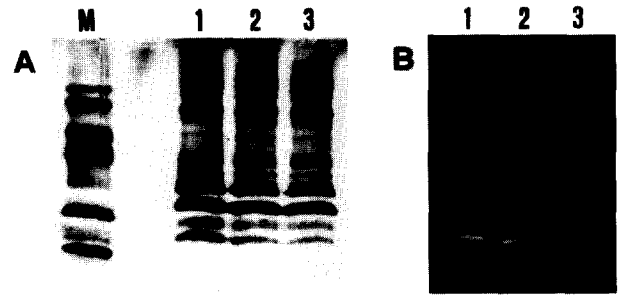

Fig. 2. BrCN cleavage of the SDS-denatured OGC after its modification by PM in the oxidized and in the reduced forms. The purified SDS-denatured OGC was incubated with the following sequences of reagents (for 30 min at 25°C with each reagent): PM (lane 1); DTE and then PM (lane 2); FM, DTE and then PM (lane 3). After each incubation the carrier protein was acetone-precipitated and washed 4 times. At the end, OGC samples were dissolved in formic acid, digested with BrCN and subjected to SDS-gel electrophoresis according to Schägger and von Jagow [19]. The concentrations of the modifying reagents were 1 mM PM, 10 mM DTE, and 2 mM FM. (A) Coomassie blue staining; (B) fluorography. Lane M, molecular mass markers (SDS-17 kit from Sigma).

The only fluorescent peptide of the oxidized protein had the N-terminal sequence TADGRLPVDQRRG. This peptide, on the basis of its sequence and its molecular mass, corresponds to the BrCN cleavage product between Met-147 and Met-188 and contains only Cys-184, which is therefore the only cysteine of the oxidized protein to be labelled by PM, as found for the OGC reconstituted into liposomes [16]. The two fluorescent peptides of the reduced protein (with M_rs of 4.5 and 4 kDa) showed the N-terminal sequence ARAVVVNAAQLA and TADGRLPVDQRRG, respectively. Of these two peptides, the one with the higher molecular mass thus corresponds to the BrCN cleavage fragment between Met-188 and Met-238 and contains Cys-221 and Cys-224. This finding provides evidence that these residues are made available to alkylation by PM after reduction of the protein with DTE. This conclusion is further supported by the observation that the only fluorescent peptide of the protein treated with FM and then reduced by DTE (with a M_r of 4.5 kDa) showed the N-terminal sequence ARAVVVNAAQLA and hence contains only Cys-221 and Cys-224.

Transport measurements were performed to investigate whether there are any functional differences between the oxidized and reduced forms. To this end, the purified OGC was incubated in the presence and absence of 0.5 mM ME for 30 min at 0°C, and then the reduced and oxidized forms of the transport protein were independently reconstituted into liposomes containing 20 mM oxoglutarate. Lineweaver-Burk plots (Fig. 3) of the [^{14}C]oxoglutarate/oxoglutarate and [^{14}C]malate/oxoglutarate exchanges measured in proteoliposomes clearly show that the apparent transport affinity constants (K_m) of the oxoglutarate carrier for both external substrates (oxoglutarate or malate) were virtually the same, independently of whether the carrier protein was in the oxidized or in the reduced state. In both cases the K_m values of the carrier for its two substrates (0.26 ± 0.05 mM for oxoglutarate and 0.88 ± 0.07 for malate in 4 experiments) were not significantly different from those reported previously for the OGC purified from bovine heart mitochondria [7]. In contrast, the maximal rates of the [^{14}C]oxoglutarate/oxoglutarate and [^{14}C]malate/oxoglutarate exchanges were about 2-fold higher in proteoliposomes reconstituted with the reduced carrier as compared to those reconstituted with the oxidized form. The V_{max} values obtained with the oxidized OGC (8.93 ± 0.14 and 8.63 ± 0.20 mmol min^{-1} g protein^{-1} for the oxoglutarate/oxoglutarate exchange and the malate/oxoglutarate exchange in 4 experiments, respectively) agreed well with those reported previously for the carrier purified from bovine

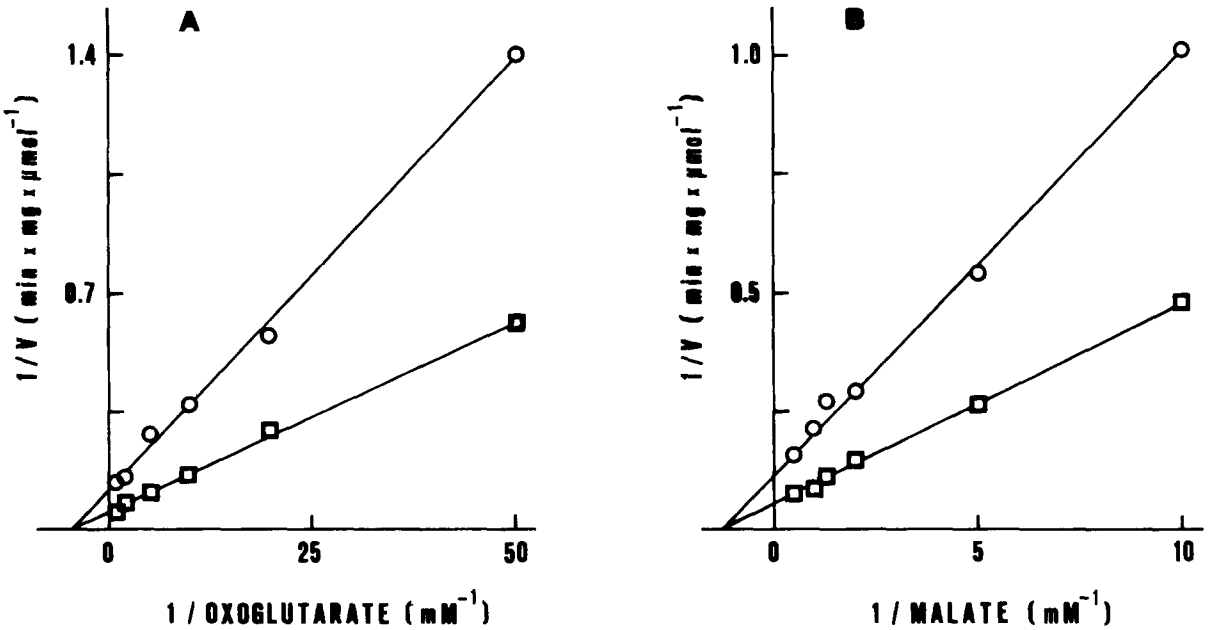

Fig. 3. Lineweaver-Burk plots of the [^{14}C]oxoglutarate/oxoglutarate and [^{14}C]malate/oxoglutarate exchanges in proteoliposomes reconstituted with the oxidized and the reduced forms of OGC. Purified Triton X-114-solubilized OGC, incubated with (□) and without (○) 0.5 mM β-mercaptoethanol, was incorporated into liposomes. [^{14}C]Oxoglutarate (A) or [^{14}C]malate (B) was added at the concentrations indicated to proteoliposomes containing 20 mM oxoglutarate.

mitochondria [7], indicating that the purified OGC is essentially found in the oxidized form.

The labelling of Cys-221 and Cys-224 of the purified OGC by PM only after reduction with ME or DTE demonstrates that the OGC protein, both in its active and in its SDS-denatured state, contains a disulfide bridge between Cys-221 and Cys-224. This finding explains the previous observation [16] that maleimides and mercurials interact only with Cys-184 of the reconstituted OGC (out of the three cysteines present in the carrier protein). Moreover, the presence of a disulfide bridge between Cys-221 and Cys-224 is consistent with the proposal [15] that the fifth hydrophobic segment of the OGC polypeptide chain is folded in an α-helix, which must therefore exhibit these two cysteines on the same face. The observation that the oxidized and the reduced forms of OGC exhibit the same K_m for the substrates oxoglutarate and malate indicates that the region of Cys-221 and Cys-224 is not involved in the binding of substrates and hence, most probably, does not reside within the substrate binding site. The fact that V_{max}, on the other hand, is significantly higher for the reduced form than for the oxidized form suggests that the fifth α-helix portion, where Cys-221 and Cys-224 are located, is involved in the translocation step. It is interesting to note that computer graphics studies indicate a significant conformational change of α-helix V associated to the transition between the oxidized and the reduced forms of OGC. It is possible that Cys-221 and Cys-224 line the translocation channel formed by some of the six transmembrane α-helices of the carrier protein and, therefore, the conformation of this portion of helix V has a marked influence on the substrate translocation through the carrier. Since the OGC is a component of the aspartate/malate shuttle [23], which catalyzes the transfer of reducing equivalents from the cytosol to the mitochondrial matrix, it is tempting to suggest that the change in the oxido-reduction state of the OGC may be a mechanism by which the aspartate/malate shuttle is regulated. It should be noted, however, that there is no evidence at present that this mechanism occurs in the inner mitochondrial membrane.

Acknowledgements: This work was supported by grants from the Ministero dell'Università e della Ricerca Scientifica e Tecnologica (MURST) and from the Consiglio Nazionale delle Ricerche (CNR). We are indebted to Dr. A. Green for helping to improve the English in the manuscript.

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