The Mitochondrial Oxoglutarate Carrier: Sulfhydryl Reagents Bind to Cysteine-184, and This Interaction Is Enhanced by Substrate Binding[†]

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ABSTRACT: The interaction of sulfhydryl reagents with the oxoglutarate carrier (OGC) of bovine heart mitochondria was investigated in proteoliposomes reconstituted from purified carrier and lipids. Incubation of the proteoliposomes with maleimides or mercurials led to inhibition of the oxoglutarate carrier protein. The inhibition of oxoglutarate transport by mercurials was removed by dithioerythritol (DTE), whereas inhibition by maleimides was not. Preincubation of the proteoliposomes with mercurials protected the carrier protein against inactivation by the fluorescent sulfhydryl reagent *N*-(1-pyrenyl)maleimide (PM) and decreased the fluorescence associated with the carrier, indicating that mercurials bind to the same cysteine which is modified by PM. The presence of the substrates oxoglutarate and malate increased the binding of PM to the reconstituted carrier as well as the degree of inhibition of the reconstituted transport activity caused by PM, other maleimides, and mercurials. This result is consistent with the assumption that substrate binding causes a change in the tertiary structure of the carrier protein. The primary sequence of the oxoglutarate carrier contains three cysteines (Cys-184, Cys-221, and Cys-224). We provide evidence that PM labels only Cys-184, whereas Cys-221 and Cys-224 are linked by a disulfide bridge.

The oxoglutarate carrier (OGC)[1] is a transport protein of the inner membrane of mitochondria which plays a central role in several metabolic processes, including the malate−aspartate shuttle, the oxoglutarate−isocitrate shuttle, gluconeogenesis from lactate, and nitrogen metabolism [for a review, see Meijer and van Dam (1981) and Palmieri et al. (1993a)]. This carrier catalyzes the transport of oxoglutarate in an electroneutral exchange for some other dicarboxylates, among which malate is bound with the highest affinity (Palmieri et al., 1972). The mitochondrial OGC has been isolated from heart and liver and reconstituted into liposomes in a functionally active state (Bisaccia et al., 1985, 1988). Similarly to the majority of the mitochondrial metabolite carriers [for a review, see Palmieri (1994)], the OGC functions according to a simultaneous (sequential) reaction mechanism, which implies that one internal and one external substrate molecule form a ternary complex with the carrier before the transport event occurs (Sluse et al., 1972; Indiveri et al., 1991). The amino acid sequence of the carrier from different mammals has been determined by cDNA sequencing (Runswick et al., 1990; Iacobazzi et al., 1992; Dolce et al., 1994), showing that it belongs to the carrier protein family of the mitochondrial inner membrane, which includes the ADP/ATP carrier, the phosphate carrier, the tricarboxylate carrier, and the uncoupling protein, as well as some other proteins whose functions are not yet known [for a review, see Walker (1992), Kuan and Saier (1993), and Palmieri (1994)]. The oxoglutarate carrier in man, cow, and rat has been found to be coded by a single gene (Iacobazzi et al., 1992; Dolce et al., 1994). Detailed topographic studies of the OGC polypeptide chain are consistent with its folding into six transmembrane α-helices in the inner mitochondrial membrane. These α-helices are connected by hydrophilic loops, and the N- and C-termini protrude toward the cytosol (Bisaccia et al., 1994). In addition, by using cross-linking reagents, it has been shown that the OGC exists as a homodimer (Bisaccia et al., 1996).

Although these results have extended our knowledge of the OGC toward the molecular level, the mechanism of oxoglutarate translocation through this transport protein is not yet understood. One approach for obtaining more information about the molecular mechanism of a carrier system is to investigate the functional groups and their role in substrate transport. The function of the OGC is inhibited by the mercurial reagents mersalyl and p-HMB but not by NEM (Quagliariello & Palmieri, 1972; Bisaccia et al., 1985, 1988). The OGC contains only three cysteine residues, one of which (Cys-184) is quite distant from the other two, which are located at positions 221 and 224.

In this paper, we studied the interaction of a variety of sulfhydryl reagents with purified OGC of bovine heart mitochondria reconstituted into liposomes and the effect of oxoglutarate and malate on this interaction. The reconstituted OGC is oriented inside-out as compared to its orientation in mitochondria (Bisaccia et al., 1994). We observed that both mercurials and maleimides interacted specifically with Cys-184. This reaction was associated with the inhibition of oxoglutarate and malate transport. The degree of inhibition

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[1] Abbreviations: DTE, dithioerythritol; FM, *N*-phenylmaleimide; NEM, *N*-ethylmaleimide; OGC, oxoglutarate carrier; Pipes, 1,4-piperazinediethanesulfonic acid; p-CMBS, *p*-(chloromercuri)benzenesulfonic acid; p-HMB, *p*-(hydroxymercuri)benzoic acid; PM, *N*-(1-pyrenyl)maleimide; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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of OGC activity and the extent of PM binding to the reconstituted carrier were enhanced by the presence of the substrates of the transport protein, suggesting that the substrate-induced conformational change of the carrier molecule leads to increased reactivity or accessibility of Cys-184 to sulfhydryl reagents. Moreover, evidence is provided that Cys-221 and Cys-224 of the reconstituted OGC became available to alkylation by PM after incubation of the proteoliposomes with the reducing reagents DTE or 2-mercaptoethanol, suggesting that these cysteines are linked by a disulfide bridge.

MATERIALS AND METHODS

Materials. Hydroxyapatite (Bio-Gel HTP) was obtained from Bio-Rad; Celite, Triton X-114, dithioerythritol (DTE), *N*-ethylmaleimide (NEM), acrylamide, *N*′,*N*′-methylenebis-(acrylamide), and pyridoxal 5′-phosphate from Serva; *N*-(1-pyrenyl)maleimide (PM), *N*-phenylmaleimide (FM), BrCN, *p*-(chloromercuri)benzenesulfonic acid (p-CMBS), and *p*-(hydroxymercuri)benzoate (p-HMB) from Sigma; egg yolk phospholipids (lecithin from eggs), Amberlite XAD-2, Dowex AG1-X8 (50−100 mesh), and bathophenanthrolinedisulfonic acid disodium salt from Fluka; cardiolipin from Serdary; α-[1-^{14}C]ketoglutarate from Dupont; L-[1,4(2,3)-^{14}C]malate from the Radiochemical Center, Amersham; Sephadex G-75 from Pharmacia; mersalyl acid from Aldrich; poly(vinylidene difluoride) (PVDF) membranes from Applied Biosystems. All other reagents were of analytical grade.

Purification of the OGC. The OGC was purified from bovine heart mitochondria by the procedure introduced by Bisaccia et al. (1985) with some modifications; 5 mL of extract, obtained by solubilization of the mitochondria with 3% Triton X-114 and subsequent centrifugation, was supplemented with cardiolipin (2 mg/mL) and applied on a dry hydroxyapatite/Celite (6 g, ratio 5:1) column. Elution was performed with the solubilization buffer [3% Triton X-114 (w/v), 50 mM NaCl, and 10 mM Pipes (pH 7.0)]. Four fractions of 1 mL each were collected. The second and the third fractions, containing pure oxoglutarate transport protein, were combined and used for the reconstitution experiments.

Reconstitution of the Purified OGC into Liposomes. The purified protein was reconstituted by removing the detergent with a hydrophobic ion-exchange column (Palmieri et al., 1995). In this procedure, the mixed micelles containing detergent, protein, and phospholipids were repeatedly passed through the same Amberlite XAD-2 column. The composition of the initial mixture used for reconstitution was as follows: 300 mL of purified oxoglutarate carrier (0.2−0.4 mg of protein), 40 μL of 10% Triton X-114, 9 mg of egg yolk phospholipids in the form of sonicated liposomes, prepared as described in Bisaccia et al. (1985), 20 mM oxoglutarate, and 10 mM Pipes (pH 7) in a final volume of 0.7 mL. After being vortexed, this mixture was passed 15 times through the same Amberlite XAD-2 column (0.5 × 4.5 cm) preequilibrated in a buffer containing 10 mM Pipes and 20 mM oxoglutarate (pH 7). All operations were performed at 4 °C, except the passage through Amberlite, which was carried out at room temperature. The substrate outside the proteoliposomes was removed by passing 600 μL of the liposomal suspension through a Sephadex G-75 column (0.7 × 15 cm) preequilibrated in a medium consisting of 50 mM NaCl and 10 mM Pipes (pH 7). The first 750 μL of the slightly turbid eluate from the Sephadex G-75 column containing the proteoliposomes was collected. These preparations were used for labeling experiments or for measurements of transport activity.

Measurements of Transport Activity. Aliquots of proteoliposomes (150 μL) were preincubated with sulfhydryl reagents in the presence and absence of substrates or other compounds (15 μL) or with water (15 μL) or, in the case of maleimides, 5 μL of DMSO plus 10 μL of water, under the conditions described in the legends to tables and figures. Transport was started by adding 10 μL of [^{14}C]oxoglutarate (to measure the [^{14}C]oxoglutarate/oxoglutarate exchange) or [^{14}C]malate (to measure the [^{14}C]malate/oxoglutarate exchange) at the concentrations indicated in the legends to tables and figures. This reaction was stopped by addition of 10 μL of a solution containing 555 mM pyridoxal 5′-phosphate and 185 mM bathophenanthroline (Palmieri et al., 1995). In control assays, the inhibitors were added together with the labeled substrate. The incubation temperature was 25 °C. The external [^{14}C]substrate was removed from each sample of proteoliposomes (160 μL) by chromatography on an anion-exchange column (Dowex AG-X8, acetate form, 0.5 × 5 cm). The proteoliposomes were eluted with 1 mL of 50 mM NaCl, and their radioactivity was determined by scintillation counting. The transport activity was evaluated as the difference between the experimental and the control values, which were measured after 30 and 60 s, i.e., within the initial linear range of [^{14}C]oxoglutarate or [^{14}C]malate uptake into the proteoliposomes. The pyridoxal 5′-phosphate and bathophenanthroline-insensitive radioactivity associated with the proteoliposomes was always less than 6% of the pyridoxal 5′-phosphate and bathophenanthroline-sensitive radioactivity taken up during the transport assay.

PM Labeling of the OGC Protein Reconstituted into Liposomes. Proteoliposomes were incubated with 2 mM pyrenylmaleimide (50 μL of 40 mM PM in DMSO/1 mL of proteoliposomes) for 30 min at 25 °C, and the reaction was stopped by adding 10 mM DTE. After treatment with PM, the proteoliposomes (divided into aliquots of 750 μL) were passed through a Sephadex G-75 column (0.7 × 15 cm) preequilibrated in a medium consisting of 50 mM NaCl, 10 mM Pipes (pH 7), and 1 mM DTE. The turbid eluates from the Sephadex column (700 μL) were pooled and precipitated with a 20-fold excess of cold acetone for 4 h at −20 °C. The sample was centrifuged at 44000*g* for 10 min at 0 °C. The lipids were removed essentially as described by Wessel and Flügge (1984). The pellet derived from 2 mL of proteoliposomes was suspended in 1 mL of distilled water. After the addition of 4 mL of methanol, 1 mL of chloroform, and 3 mL of water, the mixture was vortexed vigorously and centrifuged at 9000*g* for 10 min. The upper phase was removed and discarded. A further 2 mL of methanol was added to the remaining lower and intermediate phases, which contained the precipitated protein. The mixture was vortexed, and the protein was pelleted by centrifugation at 9000*g* for 4 min. The supernatant was removed, and the protein pellet was dried in a stream of nitrogen. The pellet was solubilized in the sample buffer for SDS−gel electrophoresis or in 80% formic acid for BrCN cleavage. In the kind of experiments illustrated in Figure 6, proteoliposomes were incubated with and without 4 mM FM for 30 min at 25 °C and then with and without 50 mM 2-mercaptoethanol or DTE for 23.5 h at 25 °C. After these treatments, the proteolipo-

somes were precipitated with cold acetone, and the carrier protein was delipidated as described above. The pellet was solubilized in 0.1% SDS, acetone-precipitated 3 times, and solubilized in 0.1% SDS again. The SDS-solubilized protein was incubated with 2 mM PM for 30 min at 25 °C. After this incubation, the excess of PM was removed by precipitation of the protein with a 20-fold excess of cold acetone for 4 h at −20 °C and by washing the pellet with cold acetone twice. At the end, the carrier protein was dissolved in the sample buffer for SDS−gel electrophoresis.

BrCN Cleavage and Protein Sequencing. The OGC, reconstituted into proteoliposomes (200 mL), labeled by PM, extracted from the vesicles, delipidated and dissolved in 80% formic acid (see above), was incubated with a 20-fold excess (mol/mol) of BrCN in the dark. After 13 h, the solution was diluted 10 times with water and then freeze-dried. The dried peptides were dissolved in SDS sample buffer, loaded onto an SDS slab gel, and separated by the system of Schägger and von Jagow (1987). For protein sequencing, the separated peptides were transferred to poly(vinylidene difluoride) (PVDF) membranes. After being stained with Coomassie blue dye, the fluorescent bands were excised and subjected to Edman degradation in a pulse liquid protein sequencer (Applied Biosystems 477A) equipped with an online PTH-amino acid analyzer.

Gel Electrophoresis. The sample buffer consisted of 7% SDS (w/v), 45% glycerol (v/v), 50 mM DTE, 225 mM Tris-HCl (pH 6.8), and traces of bromophenol blue. Polyacrylamide slab gel electrophoresis was performed in the presence of 0.1% SDS according to Laemmli (1970). The stacking gel contained 5% acrylamide, and the separation gel contained 17.5% acrylamide and an acrylamide/bis(acrylamide) ratio of 37.5 (30:0.8). Staining was performed with Coomassie Brilliant Blue R-250. For peptide analysis, the discontinuous Tricine system of Schägger and von Jagow (1987) was employed. We used 16.5% acrylamide with an acrylamide/bis(acrylamide) ratio of 15.5:1, with 13% glycerol included in the separation gel. The molecular mass markers used were a mixture of bovine serum albumin (66.2 kDa), carbonic anhydrase (29.5 kDa), and cytochrome *c* (12.5 kDa), or the molecular-mass SDS-17 kit from Sigma, containing cyanogen bromide fragments of myoglobin (17, 14.4, 10.7, 8.2, 6.2, 3.2, and 2.5 kDa).

Other Methods. PM labeling 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. Protein was determined by the Lowry method modified for the presence of Triton (Dulley & Grieve, 1975). All the samples used for protein determination were subjected to acetone precipitation and redissolved in 1% SDS (Indiveri et al., 1990).

RESULTS

Inhibition of the Reconstituted Oxoglutarate Carrier Protein by Maleimides. In previous investigations, the OGC was found to be inhibited by high concentrations of mersalyl and p-HMB, but not by NEM and other maleimides (Quagliariello & Palmieri, 1972; Bisaccia et al., 1985, 1988; Zara & Palmieri, 1988), irrespective of whether these studies were performed in mitochondria or in proteoliposomes. However, in all these investigations, relatively short incubation times of mitochondria or proteoliposomes with the sulfhydryl reagents were used (up to 3 min at 25 °C). Figure 1A of the present study demonstrates that NEM, PM, and FM were also able to inhibit the reconstituted OGC protein when the proteoliposomes were incubated with these reagents for 30 min at 25 °C. In these experiments, the concentration of the maleimides was varied from 0.5 to 5.0 mM in the absence of the external substrate. The inhibition of the oxoglutarate transport activity increased with increasing concentrations of the maleimides with an IC_{50} value (i.e., the concentration required for half-maximal inhibition) of approximately 1.25 mM for FM, 1.5 mM for PM, and 5.0 mM for NEM. It is noteworthy that the results shown in Figure 1A were obtained at pH 7.0. We found in other experiments that the degree of inhibition of OGC activity by maleimides increased with increasing pH from 6.5 to 8.0. This result agrees with the notion that ionization of the sulfhydryl groups favors their reaction with maleimides (van Iwaarden et al., 1992).

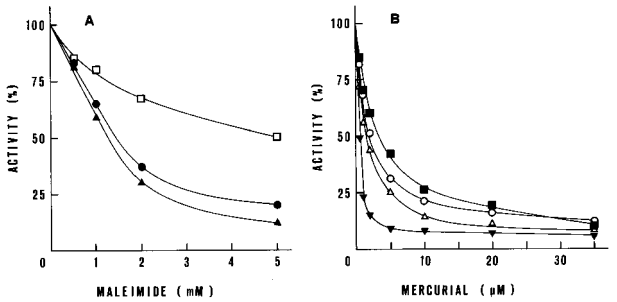

FIGURE 1: Inhibition of the reconstituted OGC by maleimides and mercurials. Proteoliposomes were incubated with NEM (□), PM (●), or FM (▲) for 30 min at 25 °C or with p-HMB (△), p-CMBS (○), mersalyl (■), or $HgCl_2$ (▼) for 5 min at 25 °C at the indicated concentrations. After incubation, oxoglutarate/oxoglutarate exchange activity was measured by adding 0.1 mM [^{14}C]oxoglutarate. The control value in the absence of maleimides (A) was 2245 μmol min^{-1} (g of protein)$^{-1}$ and in the absence of mercurials (B) 2130 μmol min^{-1} (g of protein)$^{-1}$.

In Figure 1B, the effectiveness of some mercurials in inhibiting the reconstituted oxoglutarate transport activity was compared. Because of the higher reactivity of the OGC toward mercurials as compared to maleimides, micromolar concentrations of mercurials were used, and the time of reaction was shortened to 5 min. Under these conditions, the IC_{50} value was about 3.3 μM for mersalyl, 1.7 μM for p-HMB, 2.0 μM for p-CMBS, and 1 μM for $HgCl_2$. Since the mercurials and the maleimides are known to react specifically with cysteines, these results show that the OGC possesses at least one functionally important sulfhydryl group, which is able to react not only with mercurials but also with maleimides.

Maleimides and Mercurials Are Competitive for Their Reaction with the Same Cysteine Residue(s). The primary sequence of the OGC contains three cysteines. It was not yet known whether the cysteine residue(s) modified by PM is (are) the one which also reacts with NEM, FM, and the mercurials. To answer this question, we took advantage of the fluorescent properties of PM, which can be used to label the OGC (see above). The proteoliposomes were preincubated with NEM or FM for 30 min or with p-HMB, p-CMBS, or mersalyl for 5 min before PM was added. After a further 30 min, the reaction was stopped by using DTE. Figure 2 shows the SDS−PAGE and the fluorography of

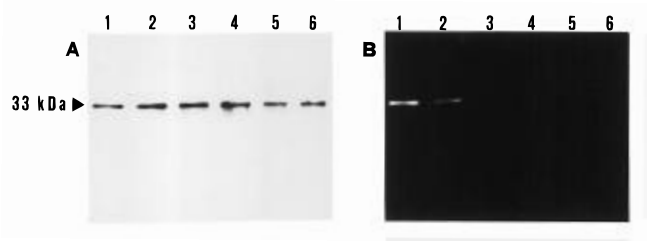

FIGURE 2: Labeling of the reconstituted OGC by PM in the presence and absence of mercurials or other maleimides. Proteoliposomes were preincubated for 30 min at 25 °C with 2 mM NEM (lane 2) or FM (lane 3), or for 5 min at 25 °C with 100 μM p-HMB (lane 4), p-CMBS (lane 5), or mersalyl (lane 6). After gel filtration, the proteoliposomes were incubated with 2 mM PM for 30 min at 25 °C. After removal of unbound PM, the OGC protein was delipidated and subjected to SDS−gel electrophoresis. (A) Coomassie Blue staining; (B) fluorography.

Table 1: Protection by Organic Mercurials against PM or FM Inhibition of the Reconstituted OGC[a]

addition at			activity [μmol min^{-1} (g of protein)$^{-1}$]
0 min	5 min	35 min	
			2287
mersalyl			115
mersalyl		DTE	2241
p-HMB			90
p-HMB		DTE	2172
	PM		800
	PM	DTE	816
mersalyl	PM	DTE	2127
p-HMB	PM	DTE	2149
	FM		617
	FM	DTE	599
mersalyl	FM	DTE	2104
p-HMB	FM	DTE	2160

[a] Proteoliposomes were incubated at 25 °C with different mercurials and maleimides, and DTE was added at the time indicated. The total incubation time was 37 min. After incubation, oxoglutarate/oxoglutarate exchange was measured by adding 0.1 mM [^{14}C]oxoglutarate. Reagents were used at the following concentrations: 100 μM mersalyl or p-HMB, 2 mM PM or FM, and 10 mM DTE.

the OGC extracted from the proteoliposomes that were incubated with PM after preincubation with or without the other SH-reagents. The results presented in Figure 2 demonstrate that the fluorescence associated with the OGC (lane 1) was markedly diminished when the proteoliposomes were preincubated with NEM (lane 2) and completely abolished when they were preincubated with FM (lane 3), p-HMB (lane 4), p-CMBS (lane 5), or mersalyl (lane 6). In other experiments, the proteoliposomes, treated with PM after preincubation with or without other SH-reagents, were tested for oxoglutarate/oxoglutarate exchange transport activity (Table 1). The data given in Table 1 show complete restoration of oxoglutarate transport when the proteoliposomes were preincubated with mersalyl, p-HMB, or (not shown) p-CMBS, successively treated with PM or FM and finally with DTE. When proteoliposomes were treated with mersalyl, p-HMB, or p-CMBS alone, the oxoglutarate transport was markedly inhibited, this inhibition being removed by the addition of DTE. However, when PM or FM alone was used, DTE was unable to restore the oxoglutarate transport activity at all. In further experiments using increasing concentrations of mersalyl, p-HMB, or p-CMBS, we showed that the degree of transport inhibition by these reagents correlated well with the extent of protection against inhibition by PM (data not shown). These results

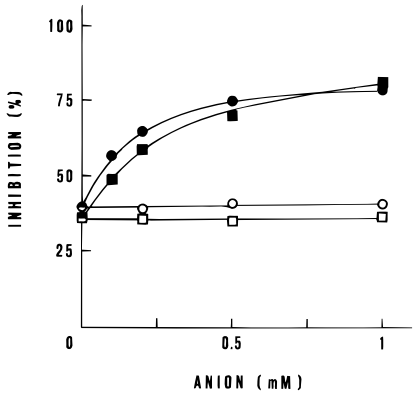

FIGURE 3: Influence of substrates on the inhibition of the reconstituted OGC by FM or mersalyl. Proteoliposomes were incubated for 30 min at 25 °C with 1 mM FM (■, □) or for 5 min at 25 °C with 2 μM mersalyl (●, ○) and the indicated concentrations of oxoglutarate (■, ●), α-ketoadipate (□), or glutarate (○). After this incubation, oxoglutarate/oxoglutarate exchange activity was measured by adding different concentrations of [^{14}C]oxoglutarate to reach the same external concentration of 1 mM in all the samples.

indicate that PM, FM, mersalyl, p-HMB, and p-CMBS do indeed react with the same cysteine residue(s) of the OGC.

OGC Substrates Malate and Oxoglutarate Increase the Binding of the SH-Reagents to the Carrier Protein. Considering the results described above, the question arose as to whether the OGC sulfhydryl group(s), which is (are) modified by the maleimides and the organic mercurials, is (are) localized at or near the substrate binding site of the OGC. To investigate this, we analyzed the effect of the substrates oxoglutarate and malate on the inhibition of carrier activity by the SH-reagents. Liposomes reconstituted with the purified OGC were incubated with different SH-reagents with and without the two substrates. In these experiments, the concentration of the sulfhydryl reagents was kept sufficiently low to obtain only partial transport inhibition. Under the conditions shown in Figure 3, the inhibition of the oxoglutarate/oxoglutarate exchange activity was increased from about 40% to about 80% by addition of 0.1−1 mM oxoglutarate during the incubation of the proteoliposomes with mersalyl or with FM. In both cases, a half-maximal increase in transport inhibition was obtained at an oxoglutarate concentration of approximately 0.25 mM, which is close to the transport affinity (K_m) of the reconstituted carrier for external oxoglutarate (Indiveri et al., 1991). Table 2 demonstrates that also the second substrate of the OGC, namely, L-malate, increased the extent of inhibition of the OGC not only by mersalyl or FM but also by various other SH-reagents. It is noteworthy that the enhancing effect of the substrates is specific, since other chemically related anions, such as α-ketoadipate and glutarate (Figure 3) as well as D-malate, oxomalonate, glutamate, phosphate, ADP, and citrate (not shown), which are neither substrates nor inhibitors of the OGC (Palmieri et al., 1972; Bisaccia et al., 1985), had no effect.

The substrate-dependent increase in the OGC inhibition by PM was due to an increase in the amount of maleimide bound to the carrier. This is demonstrated by the experiment illustrated in Figure 4, which shows the SDS−PAGE and the fluorography of the OGC recovered from proteoliposomes treated with PM with and without oxoglutarate. Clearly, the fluorescence associated with the carrier protein increased by raising the oxoglutarate concentration up to 1

Table 2: Effect of L-Malate and Oxoglutarate on the Extent of OGC Inhibition by Several SH-Reagents[a]

		% inhibition			
		expt 1		expt 2	
	concn	−malate	+malate	−oxoglutarate	+oxoglutarate
NEM	1 mM	19	43	21	51
	2 mM			39	65
FM	1 mM	42	65	39	71
	2 mM			68	84
	3 mM			79	94
PM	1 mM	41	72	40	76
mersalyl	2 μM			40	76
	5 μM	48	74		
	10 μM			65	85
	20 μM			81	95
p-HMB	5 μM	74	89		
p-CMBS	5 μM	64	82		

[a] Proteoliposomes were incubated with NEM, FM, or PM for 30 min at 25 °C or with mersalyl, p-HMB, or p-CMBS for 5 min at 25 °C in the presence and absence of 1 mM L-malate or 1 mM oxoglutarate. After this incubation, the OGC activity was measured by adding carrier-free [^{14}C]malate or 1 mM [^{14}C]malate to the samples with and without L-malate, respectively, and carrier-free [^{14}C]oxoglutarate or 1 mM [^{14}C]oxoglutarate to the samples with and without oxoglutarate, respectively.

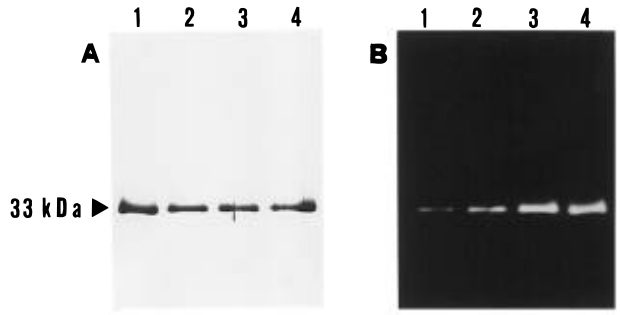

FIGURE 4: Influence of oxoglutarate on the labeling of the reconstituted OGC by PM. Proteoliposomes were incubated with 1 mM PM for 30 min at 25 °C in the presence of the following concentrations of oxoglutarate: 0 (lane 1), 0.5 mM (lane 2), 1 mM (lane 3), 5 mM (lane 4). After removal of unbound PM, the OGC protein was delipidated and subjected to SDS−gel electrophoresis. (A) Coomassie Blue staining; (B) fluorography.

mM (lane 3). Similar results were also obtained using L-malate, instead of oxoglutarate, except that in this case higher substrate concentrations (about 2 mM) were required for maximal increase in the labeling of the carrier by PM. This result agrees with the lower affinity of the reconstituted carrier for external malate as compared to oxoglutarate (Indiveri et al., 1991).

Identification of the Cysteine(s) of the OGC Labeled by PM in Proteoliposomes. OGC contains three cysteines: Cys-184, located in the fourth putative transmembrane segment close to the external surface of the proteoliposomal membrane (Bisaccia et al., 1994); and Cys-221 and Cys-224, located in the fifth putative transmembrane segment (Bisaccia et al., 1994). In order to identify the cysteine(s) of the OGC labeled by PM, the reconstituted liposomes were first incubated with PM; then the carrier protein was extracted from the vesicles and subjected to BrCN cleavage. Figure 5 demonstrates that the OGC recovered from the proteoliposomes was fluorescent (lane 1) and that one single BrCN fragment with an apparent molecular mass of 4 kDa was fluorescent (lane 2). In parallel experiments, the BrCN cleavage peptides shown in Figure 5A, lane 2, were

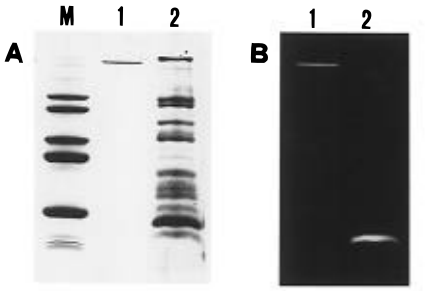

FIGURE 5: BrCN cleavage of the reconstituted OGC labeled by PM. After modification of the OGC with 2 mM PM in proteoliposomes, the carrier protein was delipidated, digested with BrCN, and subjected to SDS−gel electrophoresis according to Schägger and von Jagow. (A) Coomassie Blue staining; (B) fluorography. Lane M, molecular mass markers; lane 1, intact OGC; and lane 2, BrCN cleavage fragments of the OGC.

transferred onto a PVDF membrane for protein sequencing. The N-terminal sequence of the fluorescent peptide, TADGRLPVDQRRG, corresponded to a cleavage site at the level of methionine-147. Similar results, i.e., fluorescence of one single BrCN fragment of molecular mass 4 kDa and the same N-terminal sequence, were found when the incubation of the proteoliposomes with PM was performed in the presence of 1 mM oxoglutarate (not shown). The fluorescent peptide, as judged by its sequence and its molecular mass, contains only Cys-184, which is, therefore, the only cysteine labeled by PM in proteoliposomes.

Is There a Disulfide Bridge in the OGC between Cys-221 and Cys-224? The labeling of only Cys-184 in the reconstituted carrier by PM (Figure 5) suggested that the other two cysteines of the OGC (Cys-221 and Cys-224) either are linked by a disulfide bridge or are inaccessible to all sulfhydryl reagents used. We therefore tested whether addition of reducing reagents such as DTE, 2-mercaptoethanol, and ethanedithiol to proteoliposomes led to PM alkylation of cysteines other than Cys-184. To this end, the reconstituted OGC protein was treated first with FM (in order to protect Cys-184) and subsequently with a reducing reagent (in order to break the possible disulfide bridge between Cys-221 and Cys-224). Thereafter, the carrier protein was extracted from the vesicles and labeled with PM. The results of these experiments are shown in Figure 6. Labeling of OGC by PM was completely abolished by FM (lane 2), in agreement with the data reported in Figure 2. If, however, the reconstituted carrier was reduced by 2-mercaptoethanol after treatment with FM, the subsequent addition of PM again led to labeling of the protein (lane 3). This means that this labeling (after the treatments with FM and with 2-mercaptoethanol) was due to the presence of free sulfhydryl groups, because it was abolished by specific SH-reagents such as mersalyl (not shown). Moreover, when 2-mercaptoethanol was added to proteoliposomes without previous addition of FM (lane 4), the PM labeling of the carrier was much greater than the control, i.e., direct labeling by PM in the absence of 2-mercaptoethanol (lane 1), indicating that the addition of 2-mercaptoethanol made more PM-reacting sites available per protein molecule. Similar results were obtained using DTE instead of 2-mercaptoethanol, whereas the use of ethanedithiol was rendered impossible because this reagent destroyed the proteoliposomes at high concentrations. These results indicated both that PM binds to Cys-184 alone when the OGC protein is in the oxidized state, and that when the

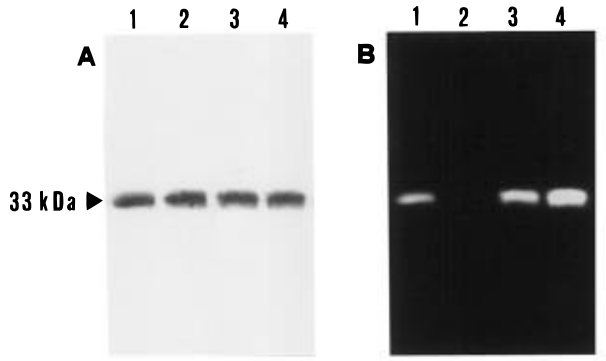

FIGURE 6: Effect of reducing agents on OGC labeling by PM. Proteoliposomes were incubated with 4 mM FM for 30 min at 25 °C (lanes 2 and 3) and then with 50 mM 2-mercaptoethanol for 23.5 h at 25 °C (lanes 3 and 4). After this incubation, all the samples were acetone-precipitated, and the OGC protein was delipidated and suspended in 0.1% SDS. Then the OGC was labeled with 2 mM PM for 30 min at 25 °C. At the end, the protein was acetone-precipitated, redissolved in SDS sample buffer, and run on SDS−gel electrophoresis. (A) Coomassie Blue staining; (B) fluorography.

carrier is reduced by the addition of 2-mercaptoethanol or DTE, PM also binds to at least one of the other cysteines (i.e., Cys-221 and Cys-224). It should be noted that high concentrations of reducing agents (50 mM DTE or 2-mercaptoethanol) and long incubation times (12−24 h) of these reagents with the reconstituted carrier were necessary to make Cys-221 and/or Cys-224 available to alkylation by the fluorescent probe. This may be due to the intramembranous location of the putative disulfide bridge and to its relative inaccessibility to hydrophilic reagents. Due to the long incubation times used in these experiments, we checked the stability of the OGC in the liposomal membrane over the course of the experiments. The carrier activity, measured as the exchange of extraliposomal 0.1 mM [^{14}C]oxoglutarate with intraliposomal 20 mM oxoglutarate, was virtually identical at the beginning and at the end of the incubation times, showing that the reconstituted OGC is stable at 25 °C over a very long time, as previously found (Bisaccia et al., 1994), in contrast to other mitochondrial carriers such as the phosphate carrier and the carnitine carrier (F. Bisaccia, C. Indiveri, and F. Palmieri, unpublished results).

DISCUSSION

For the characterization of sulfhydryl groups within proteins, maleimides have proven to be especially useful because their reaction with SH-groups of proteins is essentially irreversible, and because some of them are fluorescent or are available in radioactive form. In this paper, we have shown for the first time that maleimides react with the OGC and that the carrier−PM complex can be easily detected by fluorography. The use of the fluorescent PM allowed us to identify Cys-184 as the only sulfhydryl group of the OGC that is alkylated by this maleimide in the absence of reducing reagents. It is very likely that all the mercurials and all the maleimides tested in this study bind to Cys-184 alone out of the three cysteines present in the OGC. This is because we have shown here that these reagents, used under the same experimental conditions, effectively impair the binding, and thus the inhibitory effect, of PM to the OGC. In previous investigations in mitochondria and in the reconstituted system (Quagliariello & Palmieri, 1972; Bisaccia et al., 1985, 1988; Zara & Palmieri, 1988), the OGC has been reported to be insensitive to NEM and other maleimides, because rather short times of incubation were used. Here, using the reconstituted OGC carrier, whose activity remains essentially constant over a very long time (Bisaccia et al., 1994), it was possible to measure the inhibitory effect of maleimides on OGC transport activity after incubation times of usually 30 min (Figure 1). In addition, the low reactivity of Cys-184 to thiol reagents in mitochondria (Quagliariello & Palmieri, 1972) might be related to the location of this residue in the fourth transmembrane α-helix close to the inner face of the mitochondrial membrane, according to the recently proposed model of the transmembrane topography of the OGC (Bisaccia et al., 1994). Instead, in the reconstituted OGC, which is inside-out oriented compared to mitochondria (Bisaccia et al., 1994), Cys-184 is close to the external surface of the proteoliposomal membrane and probably displays higher accessibility and reactivity.

The results presented in this paper clearly rule out the possibility that Cys-184 is a component of the substrate binding site. This is demonstrated by the fact that the substrates of the OGC, oxoglutarate and malate, unlike other related organic anions, specifically increase the reactivity of Cys-184 toward maleimides and mercurials and hence enhance their inhibitory effect. It is noteworthy that this result agrees with the noncompetitive nature of the OGC inhibition by p-HMB and mersalyl, as deduced from kinetic studies performed in mitochondria (Quagliariello & Palmieri, 1972). The most likely explanation for the effect of the substrates on SH-reagent binding to the OGC is that substrate binding induces a change in the tertiary structure of the carrier protein which involves the region of Cys-184. As a result of the conformational change in the carrier, the reactivity or the accessibility of Cys-184 for SH-reagents is increased. There exist several potential explanations for this effect. It may be caused by a decrease in the p*K* of Cys-184, which may occur when the microenvironment surrounding the sulfhydryl group becomes more hydrophilic (van Iwaarden et al., 1992). In view of the putative location of Cys-184 at the border of the membrane, it is also possible that this residue may become more exposed to the water phase, or that a relative increase of positive charges close to the sulfhydryl group occurs. In the case of the ADP/ATP carrier, it has been proposed that the binding of substrates opens a gate located in the hydrophilic loop connecting the third and the fourth α-helix (Majima et al., 1993, 1995). If this proposal also applies to the OGC, it is clear that the microenvironment of Cys-184, located at the beginning of helix IV, could easily be influenced by the opening of the gate. It cannot be excluded, however, that the change in the microenvironment of Cys-184 reflects a more widespread alteration of the tertiary structure of the carrier induced by substrate binding. The alternative explanation, i.e., that the increased reactivity of Cys-184 is due to an increased accessibility of the sulfhydryl group to the SH-reagents, is not consistent with the finding that the increase in the inhibition induced by the presence of the substrates of the OGC is no greater for NEM or FM as compared to the bulkier PM. It should be recalled that changes in the reactivity of cysteines or other residues, as a consequence of gross conformational changes induced by substrate binding, have already been reported for other transport proteins. For example, in the case of the adenine nucleotide carrier, only in the presence of ADP or ATP can the residue Cys-

56, the only one out of a total of four, be alkylated by NEM (Aquila et al., 1982; Boulay & Vignais, 1984). Similarly, the reactivity of single cysteine residues at positions 269 or 322 of lactose permease for SH-reagents is increased by ligands of this transport system (Jung et al., 1994).

The effects of chemical modifications on transporters, i.e., the specific labeling of a particular amino acid residue of a transport protein in association with inhibition of its function, are usually taken as an indication of the involvement of that residue in the translocation mechanism. However, in view of recent site-directed mutagenesis studies of two other members of the mitochondrial carrier family, namely, the ADP/ATP carrier and the uncoupling protein (Arechaga et al., 1993; Klingenberg & Nelson, 1994), this assumption has to be taken with care. In these studies, in which each cysteine was replaced by a serine residue, none of the cysteine residues present in these two carriers was found to be essential for transport activity. It is possible that alkylation of Cys-184 of the OGC either has an obstructing effect on the oxoglutarate translocation path or disturbs some hydrogen bonding. Our results also allow for a second possibility, i.e., that the SH-group of Cys-184 in its free state would be a prerequisite for the substrate-induced conformational change to occur, and in its blocked state would sterically hinder this molecular motion. It could also be speculated that the conformational change outlined in this paper is a necessary intermediate step in the translocation process. However, the present data give no indication as to whether this structural change occurs in the catalytic step of the exchange, like that described by Sluse-Goffart et al. (1983). At present, it cannot be excluded that the alkylation of Cys-184 results in a dramatic change in the transport mechanism, shifting for instance from a counter-exchange mechanism to a much less efficient uniport, as has been demonstrated for several mitochondrial substrate carriers [for a review, see Krämer and Palmieri (1992) and Palmieri et al. (1993b)].

In addition to Cys-184, the OGC contains two other cysteine residues located in the fifth transmembrane segment. The observed labeling of only Cys-184 by PM suggests that Cys-221 and Cys-224 either are linked by a disulfide bridge or else have a very low reactivity (due to a very high p*K* or their inaccessibility even to the hydrophobic PM). That these two residues are predicted to be on the same face of helix V (Runswick et al., 1990; Bisaccia et al., 1994) is consistent with the existence of a disulfide bridge between these two cysteines. Moreover, this hypothesis is supported by the finding that, under conditions in which Cys-184 is protected by FM, the OGC is labeled by PM after reduction of the reconstituted protein with DTE or 2-mercaptoethanol (Figure 6).

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