Abundant bacterial expression and reconstitution of an intrinsic membrane-transport protein from bovine mitochondria

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The oxoglutarate carrier, an intrinsic membrane-transport protein of the inner membranes of bovine-heart mitochondria, has been expressed at an abundant level in *Escherichia coli*. It accumulates in the bacterium as inclusion bodies, and none of the protein was detected in the bacterial inner membrane. The mitochondrial ADP/ATP carrier, a member of the same superfamily of transport proteins as the oxoglutarate carrier, has also been expressed in *E. coli*. However, the expression of the ADP/ATP carrier in bacteria retards their growth, and so the levels of expression that were attained were lower than those of the oxoglutarate carrier. The oxoglutarate carrier inclusion bodies have been disaggregated with the detergent *N*-dodecanoylsarcosine, and the protein has been incorporated into liposomes. In its ability to transport oxoglutarate and malate and other known substrates of the carrier in mitochondria, and in its inhibition characteristics by a wide range of non-competitive and competitive inhibitors, this reconstituted oxoglutarate carrier is similar to the natural protein in the inner membranes of mitochondria, and to the carrier that has been purified from mitochondria and reconstituted in liposomes. These experiments remove significant obstacles to crystallization trials and to site-directed mutagenesis of the oxoglutarate carrier.

INTRODUCTION

Many eukaryotic globular proteins have been expressed at high levels in bacteria, and this has often facilitated their structural analysis. In contrast, most attempts to express eukaryotic membrane proteins in bacteria have been unsuccessful, and this remains an impediment to establishing their three-dimensional structures. In all but one of the few examples where bacterial expression has been observed, the levels of expression of eukaryotic membrane proteins are far below those that have been achieved for globular proteins (Schertler, 1992). The exception is the membrane-bound bovine 17α-hydroxylase cytochrome *P*-450 from microsomes, where levels of expression of up to 16 mg/l of *Escherichia coli* culture were obtained (Barnes et al., 1991). More typical are the levels of expression of 30–400 adrenergic receptor molecules per bacterial cell (Strosberg, 1992). A number of suggestions have been advanced to account for these difficulties, including that the expressed proteins are toxic to the cell, that they are unstable and are degraded rapidly in the bacterium, and that the codon usage in the eukaryotic gene is incompatible with a high level of expression in *E. coli*.

We describe below a second example of the bacterial expression at a high level (10–15 mg/l) of a eukaryotic intrinsic membrane protein, namely the oxoglutarate-carrier protein that is naturally found in the inner membranes of mitochondria, and also the expression at somewhat lower levels of a second related mitochondrial membrane protein, the ADP/ATP or adenine-nucleotide carrier. The oxoglutarate carrier transports oxoglutarate into the mitochondrial matrix in an electroneutral exchange for malate or for other dicarboxylic acids (Palmieri et al., 1972), and has a central role in several metabolic processes, including gluconeogenesis from lactate, the malate–aspartate shuttle, the oxoglutarate–isocitrate shuttle, and nitrogen metabolism (Meijer and van Dam, 1974, 1981). The adenine-nucleotide carrier belongs to the same protein superfamily as the oxoglutarate carrier (Krämer and Palmieri, 1992; Walker, 1992), and exchanges ATP from the mitochondrial matrix for ADP from the cytoplasmic side of the membrane. In *E. coli*, the oxoglutarate carrier accumulates in the cytoplasm as insoluble inclusion bodies. This material has been solubilized in the presence of detergents and has been reconstituted into phospholipid vesicles. The transport properties of this reconstituted carrier are very similar to those of the natural carrier in mitochondrial membranes.

MATERIALS AND METHODS

Materials

Dowex AG1-X8 was purchased from Bio-Rad Laboratories SRL (I-20090 Segrate Milano, Italy), and Amberlite XAD-2 from Fluka (CH-9740 Buchs, Switzerland). 2-[5-^{14}C]Oxoglutarate was obtained from Amersham Radiochemicals (Amersham, U.K.), and turkey-egg-yolk L-α-phosphatidylcholine, Pipes, bathophenanthroline disulphonate and Triton X-114 from Sigma Chemical Co. Ltd. (Poole, Dorset BH17 7BR, U.K.). Pyridoxal 5′-phosphate was supplied by Boehringer Mannheim (20126 Milano, Italy).

Construction of the expression plasmids

The coding regions for the bovine oxoglutarate and ADP/ATP carriers were each amplified from total bovine-heart cDNA (10 ng) by 60 cycles of PCR. The forward and reverse oligonucleotide primers employed in these reactions corresponded to nucleotides 48–64 and 976–992, and nucleotides 12–28 and 888–908 respectively of the cDNAs for the oxoglutarate carrier (Runswick et al., 1990) and for the T1 isoform of the adenine-nucleotide carrier (Powell et al., 1989). A linker sequence attached to the forward primer contained *Eco*RI and *Nde*I restriction sites, and a linker in the reverse primer

Abbreviation used: sarkosyl, *N*-dodecanoylsarcosine.

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consisted of a stop codon and a *Hin*dIII site. The reaction products (10 μl) were fractionated on 1.2% agarose gels. The amplified fragments were purified by the Geneclean procedure (BIO101, La Jolla, CA, U.S.A.), cloned into M13mp9, and their sequences were verified by the dideoxy chain-termination method (Sanger et al., 1977; Biggin et al., 1983). Double-stranded fragments coding for the carriers, which had been prepared from replicative forms of M13 clones, were recloned into the expression vector pKN172 (Way et al., 1990). This vector is derived from pRK172 (McLeod et al., 1987) and is based on the pET vectors (Studier et al., 1990). It contains the bacteriophage T7 RNA polymerase gene under the control of the *lac* UV5 promoter, and is designed for use in *E. coli* BL21(DE3). DE3 is a λ lysogen that carriers *lac* I, the lac UV5 promoter, the beginning of the *lac* Z gene, and the gene for T7 polymerase. The production of the T7 RNA polymerase and the transcription of the target gene in the plasmid can be induced by the addition of isopropyl β-D-thiogalactopyranoside (Studier and Moffatt, 1986; McLeod et al., 1987). The plasmids were amplified in *E. coli* TG1, grown at 37 °C for 4.5–5 h. Colonies containing the coding sequences for the carriers were identified using PCR. Plasmid was prepared from these colonies and used to transform *E. coli* BL21(DE3).

Bacterial expression of the carrier proteins

A colony of recently transformed cells of *E. coli* BL21(DE3) was inoculated into growth medium (50 ml or 1 litre) containing ampicillin (100 μg/ml), and the culture was grown at 37 °C for 4–5 h, or until the optical density of the culture at 600 nm was 0.7–1.0. Then isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.4–0.5 mM to induce the expression of the carrier protein, and incubation was continued for a further 4.5 h. Control cultures containing the pKN172 vector only were grown at the same time. Both carriers accumulated in the respective bacteria as inclusion bodies. The cells were harvested by centrifugation, and either were used immediately for the preparation of inclusion bodies as follows, or were frozen.

Cells from a 1 litre culture were resuspended in TE buffer (20 ml), containing 10 mM Tris/HCl, pH 7.0, 0.1 mM EDTA and 1 mM dithioerythreitol, disrupted in a French press and centrifuged at 4 °C at 27000 *g* for 15 min. The pellet was resuspended in the same buffer (2 ml) and fractionated by centrifugation at 131000 *g* for 4.5 h at 4 °C, through a step gradient made 10 ml of 40%, 15 ml of 53% and 4 ml of 70% (w/v) solutions of sucrose dissolved in the buffer. The inclusion bodies both from the oxoglutarate carrier and from the ADP/ATP translocase formed a grey-gold band at the interface between the 53% and 70% sucrose layers. They were washed with buffer (20 ml) and centrifuged at 12000 *g* for 15 min at 4 °C. The pellet was resuspended in a further 1.5–2 ml of buffer.

Membranes were prepared from *E. coli* cells that had been ruptured by sonication. The broken cells were centrifuged at 27000 *g* for 15 min at 4 °C, and the resulting supernatant was centrifuged for a further 2 h at 70000 *g* at 4 °C. A suspension of the membranous sediment in TE buffer (1 ml) was applied to a discontinuous sucrose-density gradient made from 10 ml of 26%, 14 ml of 49% and 4 ml of 72% (w/v) solutions of sucrose dissolved in TE buffer, and centrifuged at 100000 *g* for 18 h at 4 °C (Janoff et al., 1979; Yatvin et al., 1986). The inner membranes sediment at the interface between the 26% and the 49% layers, and the outer membranes sediment at the interface between the 49% and the 72% layers.

Inclusion bodies, membrane fractions and samples taken from cultures at various points of growth were examined by PAGE in the presence of SDS on 17.5% gels (Laemmli, 1970). The proteins either were stained with Coomassie Blue dye or were transferred to poly(vinylidene difluoride) membranes, stained with Coomassie Blue dye, and their N-terminal sequences were determined using a modified Applied Biosystems 890B protein sequencer (Matsudaira, 1987; Fearnley et al., 1989). The total oxoglutarate carrier produced in a 1 litre bacterial culture was estimated by amino acid analysis of acid hydrolysates of purified, washed inclusion bodies with an Applied Biosystems 420A analyser.

Western-blot analysis of expressed proteins

After electrophoresis, samples of proteins from the inclusion bodies and in the membrane fractions were transferred to nitrocellulose sheets (Towbin et al., 1979). The sheets were then incubated for 2 h in the presence either of a rabbit anti-oxoglutarate carrier serum (donated by Dr. L. Capobianco, Bari, Italy) raised against the protein purified from bovine-heart mitochondria, or of a rabbit antiserum against bovine ADP/ATP translocase (a gift from Dr. G. Brandolin, Grenoble, France). The sheets were then incubated for 2 h with an anti-rabbit Ig antibody to which horseradish peroxidase had been attached (Amersham, U.K.). The binding of the second antibody was detected by the development of a brown colour after the addition of a solution (20 ml) made in phosphate buffer (0.01 M, pH 7.0) containing 0.14 M sodium chloride, 0.05% (w/v) 4-chloro-1-naphthol, 16% (v/v) methanol and 0.5% (w/v) BSA, followed by hydrogen peroxide (12 μl of 100 vol. reagent).

Solubilization and reconstitution of the expressed oxoglutarate carrier

Inclusion bodies (1% of those obtained from a 1 litre culture of *E. coli*) were solubilized in a buffer (60 μl) consisting of 1.67% (w/v) *N*-dodecanoylsarcosine (sarkosyl), 0.1 mM EDTA, 1 mM dithioerythreitol and 10 mM Tris/HCl, pH 7.0. The solution was cooled for 5 min on ice, diluted 2.8-fold with water (to give a final detergent concentration of 0.6%) and centrifuged at 12000 *g* for 10 min at 4 °C. The solubilized carrier in the supernatant was reconstituted into liposomes by hydrophobic chromatography on Amberlite beads (Indiveri et al., 1987a) as follows. To the solubilized inclusion bodies (10 μl, containing 8–12 μg of protein) were added 10% (w/v) Triton X-114 (100 μl), phospholipids (8.5 mg) in the form of sonicated liposomes (Bisaccia et al., 1985), 2-oxoglutarate and other substrates (see Table 2), and Pipes (pH 7.0, final concentration 10 mM). The mixture (final volume 700 μl) was recycled 13 times through an Amberlite column (3.2 cm × 0.5 cm internal diameter) in the presence of a buffer (10 mM Pipes, pH 7.0) containing the substrate at the same concentration as in the starting mixture. Except the passage of the mixture through Amberlite, which was carried out at room temperature, all of these operations were performed at 4 °C.

Measurements of transport activity

External substrate was removed by passage of the proteoliposomes (550 μl) through a column of Sephadex G-75 (15 cm × 0.7 cm internal diameter), equilibrated in a buffer containing 50 mM sodium chloride and 10 mM Pipes, pH 7.0. The eluted proteoliposomes (700 μl) were distributed in reaction vessels (150 μl), and used for transport measurements by the inhibitor-stop method (Palmieri and Klingenberg, 1979). Transport at 25 °C was started by the addition of 0.1 mM [^{14}C]oxoglutarate

(10 μl; see Tables 2 and 3 and Figure 4) or a range of concentrations of [^{14}C]oxoglutarate (see Figure 5), and stopped after the desired time interval, by the addition of a solution (10 μl) containing 480 mM pyridoxal 5'-phosphate and 160 mM bathophenanthroline (Passarella et al., 1973; Indiveri et al., 1987a). In control samples, the inhibitors were added together with the labelled substrate at the beginning of the time interval. External radioactivity was removed from each sample of proteoliposomes (150 μl) on an anion-exchange column (Dowex AG1-X8, acetate form; 5 cm × 0.4 cm internal diameter). The proteoliposomes were eluted with 50 mM sodium chloride (1.0 ml) into scintillation fluid (4 ml), and their radioactivities measured. The values from samples containing the carrier were corrected by subtracting the respective control values. The transport rates were calculated from measurements made within 20 s of substrate addition, which fall in the initial linear range of [^{14}C]oxoglutarate uptake into the proteoliposomes. The reconstituted protein was assayed by the inhibitor-stop procedure for its ability to catalyse various other exchange activities.

RESULTS

Expression of the mitochondrial carrier proteins

The growth rates of *E. coli* cultures were unaffected by the presence of the expression plasmid for the oxoglutarate carrier, whereas the expression plasmid containing the coding sequence for the ADP/ATP translocase slowed down the bacterial growth, even before the induction of expression of the protein. Therefore, in order to reach reasonable levels of expression of the ADP/ATP carrier, it was necessary to prolong the growth period of the culture until a sufficiently high cell density had been attained, before adding the inducer, isopropyl β-D-thiogalactopyranoside. The retardation of the growth of cultures containing the expression plasmid for the ADP/ATP carrier suggested that the protein was expressed at a basal level before the addition of the inducer.

The natural oxoglutarate carrier from bovine-heart mitochondria has an apparent molecular mass of about 31.5 kDa (Indiveri et al., 1987b), and analysis of the total protein from cells containing the oxoglutarate-carrier expression plasmid showed that the maximal accumulation of a protein of this apparent molecular mass was reached about 5 h after the addition of the inducer. This protein was concentrated by low-speed centrifugation of broken cells into an insoluble pellet. The material in the pellet gathered at the interface between the 53% and 70% sucrose layers in a discontinuous sucrose-density centrifugation. It consisted almost entirely of the 31.5 kDa protein (see Figure 1). It therefore appears that the 31.5 kDa protein had formed inclusion bodies in the bacterium. The N-terminal sequence of this protein was Ala-Ala-Thr-Ala-Ser-Pro-Gly-Ala-Ser-Gly-Met-Asp-Gly-Lys-Pro-Arg-Thr, which is identical to the sequence of amino acid residues 1–16 of the authentic bovine oxoglutarate carrier (Runswick et al., 1990). The natural carrier in mitochondria has a modified α-amino group, but this modification is not present in the bacterially synthesized protein. The identity of the 31.5 kDa protein was confirmed by its reaction with an antiserum raised against the carrier isolated from bovine heart mitochondria in an immunoblotting experiment (see Figure 2).

Immunoblotting experiments conducted on membranes from *E. coli* cells containing the expressed protein showed that they were devoid of the 31.5 kDa protein. Small amounts of the protein detected in the outer membrane fraction were attributed to residual contaminating inclusion bodies in that fraction. It was estimated by amino acid analysis of the purified inclusion bodies that a 1 litre culture of *E. coli* cells yielded about 15 mg of mitochondrial oxoglutarate-carrier protein. Yields of 10–15 mg/l of culture of the 31.5 kDa protein were obtained consistently from subsequent expression experiments.

In similar studies of the expression of the mitochondrial ADP/ATP translocase in *E. coli*, the levels of expression were considerably lower than those that were achieved with the oxoglutarate carrier. The natural ADP/ATP carrier has a molecular mass of 30 kDa, but a band with this apparent

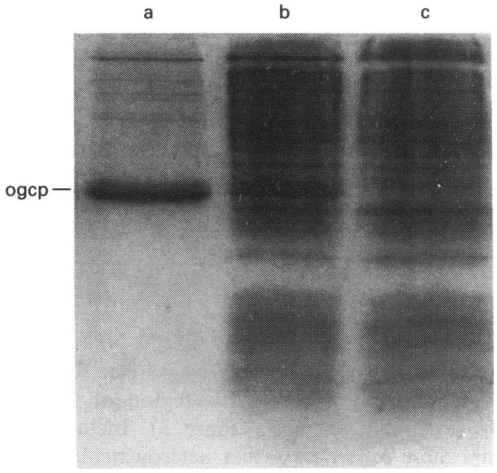

Figure 1 Expression of the bovine mitochondrial oxoglutarate-carrier protein in *E. coli*

Inclusion bodies (lane a) containing the oxoglutarate carrier (ogcp) were purified from *E. coli* cells (shown in lane b) at the end of the induction period. Lane c contains total proteins from a control bacterial culture containing the expression plasmid without an insert. Proteins were separated by denaturing PAGE and stained with Coomassie Blue dye. The oxoglutarate-carrier protein was identified by N-terminal sequence analysis.

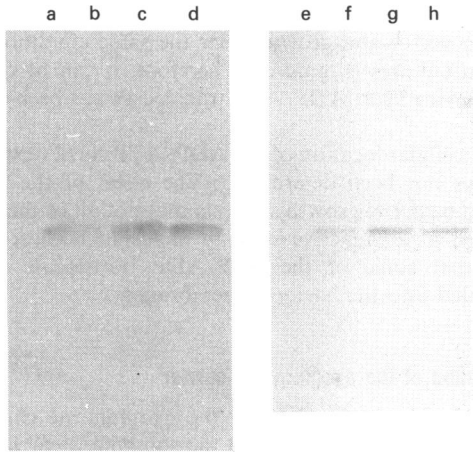

Figure 2 Immunoreactivity of the bovine-mitochondrial oxoglutarate- and adenine-nucleotide-carriers expressed in *E. coli*

The following samples were examined in Western blots using antisera against the oxoglutarate carrier (lanes a–d) or the ADP/ATP translocase (lanes e–h): lanes a and e, control *E. coli* cells containing the expression vector without an insert; lanes b and f, *E. coli* cells after the induction of expression of the oxoglutarate (b) and ADP/ATP (f) carriers; lanes c and g, inclusion bodies containing the oxoglutarate carrier (c) and the ADP/ATP carrier (g); lane d, oxoglutarate carrier and lane h, ADP/ATP carrier purified from bovine-heart mitochondria.

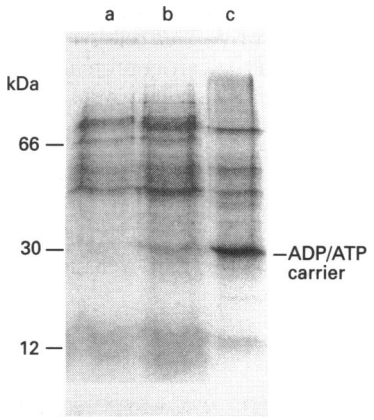

Figure 3 Expression of the bovine-heart ADP/ATP translocase in *E. coli*

Comparison by denaturing PAGE of the total bacterial protein from a control culture containing the expression plasmid without the insert (lane a), with a similar culture containing the expression plasmid for ADP/ATP translocase (lane b), 5 h after the induction of expression of the protein. Lane c, inclusion bodies prepared from the culture shown in lane b. The positions of molecular mass markers are shown at the left-hand side.

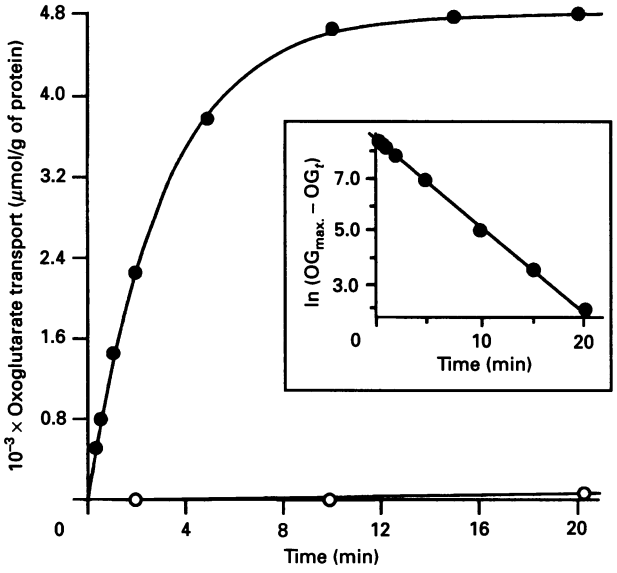

Figure 4 Uptake of external oxoglutarate by the bacterially expressed oxoglutarate carrier reconstituted into liposomes

[^{14}C]Oxoglutarate (0.1 mM) was added at time zero to liposomes reconstituted with the expressed oxoglutarate carrier and containing 20 mM oxoglutarate (●) or 20 mM sodium chloride (○). The inset is a plot of ln ($OG_{max.} - OG_t$) according to the relation ln ($OG_{max.} - OG_t$) = ln $OG_{max.} - kt$, where $OG_{max.}$ is the maximum oxoglutarate exchange/g of protein and OG_t is the oxoglutarate exchange at time t. The value of $OG_{max.}$ (4743 μmol/g of protein) was obtained by extrapolation to infinite time by non-linear regression analysis.

molecular mass could not be detected by gel electrophoresis of total cell protein, partly because this region of the gel was obscured by a cellular protein (Figure 3). Inclusion bodies prepared from these cells were rather heterogeneous in protein composition, but they did contain as a major constituent a protein with an apparent molecular mass of about 30 kDa. This protein had the N-terminal sequence Ser-Asp-Gln-Ala-Leu-Val, the same as residues 1–6 of the N-terminal sequence of the authentic T1 isoform of the ADP/ATP carrier from bovine heart (Powell et al., 1989), where the N-terminal serine is *N*-acetylated (Aquila et al, 1982). In immunoblotting experiments, the 30 kDa protein reacted with an antibody raised against the ADP/ATP carrier isolated from bovine-heart mitochondria, both in total cell protein from bacteria in which expression of the carrier had been induced, and in inclusion bodies prepared from those cells. No protein was detected by this means in bacteria lacking the expression vector and grown under the same conditions as the expression cultures (Figure 2). Therefore, it can be concluded that the bovine heart ADP/ATP translocase has been expressed in *E. coli*.

The subcellular location of the ADP/ATP carrier expressed in *E. coli* has not been determined. The effect of the expressed protein on bacterial growth suggests that not all of the carrier is sequestered in an inactive state in inclusion bodies, and it is possible that some of the ATP/ADP translocase has been incorporated into the bacterial membranes.

Reconstitution of the oxoglutarate carrier

The inclusion bodies containing the oxoglutarate carrier were disaggregated with sarkosyl, and the solubilized carrier protein was reconstituted into liposomes. The transport properties of the reconstituted carrier were studied by loading the liposomes with oxoglutarate during the incorporation of the protein, and by subsequently measuring the uptake of external [^{14}C]oxoglutarate by the proteoliposomes. The carrier catalysed a strict counter-exchange of [^{14}C]oxoglutarate for oxoglutarate with first-order kinetics (see Figure 4), isotopic equilibrium being approached exponentially (see the inset to Figure 4). The native oxoglutarate carrier has similar exchange kinetics, both in intact mitochondria (Palmieri et al., 1972) and after purification and reconstitution into liposomes (Indiveri et al., 1987b; see Table 1). In contrast, in the absence of internal substrate in the liposomes, or if the solubilized protein was boiled before the incorporation into liposomes, there was no uptake of the external substrate. No exchange activity was detected by the reconstitution of sarkosyl extracts of sonicated *E. coli* cells lacking the expression vector and grown under the same conditions as those used for the expression of the mitochondrial carrier.

Table 1 Comparison of the kinetic parameters of the reconstituted bacterially expressed oxoglutarate carrier with the oxoglutarate carrier from bovine-heart mitochondria

Parameter	Protein			References
	Bacterially expressed	Native in mitochondria	Native reconstituted	
K_m oxoglutarate/oxoglutarate exchange (mM)	0.20 ± 0.013	0.046	0.31 ± 0.08	a, b
$V_{max.}$ (μmol/min per mg)	2.96	0.043	8–11	a, b
K_i malate (mM)	0.7	0.12	0.27	a, c
First-order rate constant (min^{-1})	0.33	2.13	0.15	a, c

a, Palmieri et al. (1972); b, Indiveri et al. (1991); c, Indiveri et al. (1987b).

Kinetic properties of the reconstituted oxoglutarate-carrier protein

The dependence of the exchange rate in proteoliposomes on substrate concentration was studied by changing the concen-

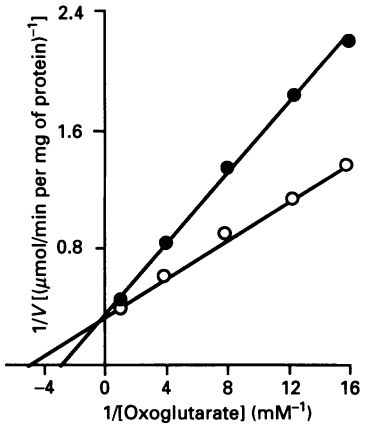

Figure 5 Dependence of the rate of oxoglutarate exchange in reconstituted liposomes on the external oxoglutarate concentration and competitive inhibition by L-malate

Liposomes reconstituted with the bacterially expressed oxoglutarate carrier were loaded with 20 mM oxoglutarate. [^{14}C]Oxoglutarate was added at various concentrations. Except in the controls, 0.6 mM L-malate was added simultaneously with the [^{14}C]oxoglutarate. Control (○); with L-malate (●). The exchange activity V is expressed in μmol/min per mg of protein.

Table 2 Dependence on the internal substrate of the oxoglutarate exchange by proteoliposomes containing bacterially expressed oxoglutarate carrier

Transport was started by the addition of 0.1 mM [^{14}C]2-oxoglutarate followed by incubation for 10 min.

Internal substrate (10 mM)	Oxoglutarate transport (μmol/10 min per g of protein)
None (Cl^- present)	20
Oxoglutarate (reconstituted with boiled protein)	16
Oxoglutarate	3666
L-Malate	3504
Succinate	1617
Oxaloacetate	1585
Malonate	1503
Maleate	1514
Fumarate	0
D-Malate	459
α-Ketoadipate	417
Oxomalonate	16
Pyruvate	28
Glutamate	10
Phosphate	15
Sulphate	18
Citrate	13
ADP	1

Table 3 Effect of inhibitors and externally added substrates on the oxoglutarate exchange by proteoliposomes containing reconstituted bacterially expressed oxoglutarate-carrier protein

Transport was started as described in Table 2. Thiol reagents were added 2 min before the labelled substrate, and other inhibitors and external anions were added together with the [^{14}C]oxoglutarate. The concentrations of the thiol reagents, pyridoxal 5′-phosphate, bathophenanthroline and carboxyatractyloside were 1, 10, 5 and 0.1 mM respectively; other inhibitors were used at a concentration of 2 mM. In experiments 1 and 2, respectively, the control values of uninhibited oxoglutarate exchange were 3441 and 3779 μmol/10 min per g protein.

Reagents	Inhibition (%)
Experiment 1	
Phthalonate	91
Butylmalonate	57
Phenylsuccinate	62
Mersalyl	80
p-Chloromercuriphenylsulphonate	55
N-Ethylmaleimide	14
Pyridoxal 5′-phosphate	95
Bathophenanthroline	96
Pyridoxal 5′-phosphate and bathophenanthroline	99
Carboxyatractyloside	10
1,2,3-Benzenetricarboxylate	17
Experiment 2	
Oxoglutarate	89
L-Malate	81
Succinate	64
Malonate	60
Maleate	65
D-Malate	18
Fumarate	7
Oxomalonate	8
2-Ketoadipate	13

tration of externally added [^{14}C]oxoglutarate at a constant internal concentration of 20 mM oxoglutarate. The K_m and $V_{max.}$ values for oxoglutarate exchange at 25 °C, calculated from a typical experiment (see Figure 5), were approx. 0.19 mM and 2974 μmol/min per g of protein respectively, and the average values from nine independent experiments were 0.2 ± 0.013 mM and 2960 ± 240 μmol/min per g of protein respectively. L-Malate is also a physiological substrate for the oxoglutarate carrier, and, as expected, inhibits oxoglutarate exchange in a competitive manner (Figure 5). The inhibition constant, K_i, for malate (0.68 mM) is close to the value that has been measured previously (Indiveri et al., 1991) and also close to the K_m for malate measured with the purified oxoglutarate carrier reconstituted into liposomes (Palmieri et al., 1972; Indiveri et al., 1987b). The K_m for oxoglutarate uptake is also close to the values measured for the native oxoglutarate carrier in mitochondria (Palmieri et al., 1972) and for the purified carrier reconstituted into liposomes (Indiveri et al., 1991), but the $V_{max.}$ value is somewhat lower than the value of 8–11 μmol/min per mg of protein that has been found for the purified oxoglutarate carrier (Indiveri et al., 1991). This is probably a consequence of protein impurities in the reconstituted oxoglutarate carrier from inclusion bodies. It should also be borne in mind that the proportion of reconstituted protein that is active is not known.

The specificity of [^{14}C]oxoglutarate exchange with respect to intraliposomal counteranions was investigated by pre-loading the proteoliposomes with a variety of substrates (summarized in Table 2). The highest activities were observed in the presence of internal oxoglutarate and L-malate. To a lesser extent, succinate, oxaloacetate, malonate and maleate also exchanged for external radiolabelled oxoglutarate, whereas D-malate and 2-ketoadipate were very poorly transported. No significant exchange was observed with internal fumarate, oxomalonate, pyruvate, glutamate, phosphate, sulphate, citrate or ADP. The residual

low activity in the presence of these substrates is approximately the same as the activity observed in the presence of sodium chloride. These transport characteristics are the same as those determined previously for the native oxoglutarate carrier in mitochondria (Palmieri et al., 1972) and for the native carrier after purification from mitochondria and reconstitution into liposomes (Bisaccia et al., 1985, 1988).

The effects of inhibitors on the reconstituted bacterially expressed oxoglutarate carrier (see Table 3) were again similar to their effects on the native oxoglutarate carrier, both in mitochondria (Palmieri et al., 1972) and after purification and reconstitution (Bisaccia et al., 1985; Indiveri et al., 1987a,b). For example, the substrate analogues phthalonate, butylmalonate and phenylsuccinate strongly inhibited the reconstituted transport activity, as did the thiol reagents mersalyl and *p*-chloromercuriphenylsulphonate, and the inhibitors pyridoxal 5′-phosphate and bathophenanthroline. In contrast, inhibitors of other mitochondrial transporters, such as *N*-ethylmaleimide (phosphate carrier), carboxyatractyloside (ADP/ATP carrier) and 1,2,3-benzenetricarboxylate (tricarboxylate carrier), had little or no effect on the reconstituted bacterially expressed protein. Similarly, the [^{14}C]oxoglutarate/oxoglutarate exchange was prevented by competitive inhibition after the external addition of the well-known substrates of the oxoglutarate carrier, namely oxoglutarate, L-malate, succinate, malonate and maleate, and it was unaffected by D-malate, fumarate, oxomalonate and 2-ketoadipate, and by substrates of other mitochondrial carriers such as phosphate, citrate and ADP. The reconstituted protein did not catalyse the exchange reactions of the adenine nucleotide, dicarboxylate and tricarboxylate carriers (ADP/ADP, malate/phosphate and citrate/citrate respectively).

DISCUSSION

The oxoglutarate and ADP/ATP carriers belong to a family of related mitochondrial transport proteins that so far have been found only in mitochondria (Krämer and Palmieri, 1992; Walker, 1992). Other members of the family are the phosphate carrier (Runswick et al., 1987) and the uncoupling protein from brown adipose tissue (Aquila et al., 1985). The common characteristic feature of established members of the family is that their polypeptide chains consist of three tandemly repeated related sequences of approx. 100 amino acids (Saraste and Walker, 1982). Each of these three repeated elements is probably folded into two transmembrane α-helices linked by an extensive polar region, forming a structure with six transmembrane α-helices. These characteristics have also been detected in the sequences of a number of proteins of unknown function, that are also presumed on this basis to be mitochondrial carrier proteins. It is probable that other carriers of unknown sequence (for example, the aspartate/glutamate, dicarboxylate, pyruvate and tricarboxylate carriers) also belong to the family.

The structural model of the carriers is supported by a range of biochemical data (Krämer and Palmieri, 1992; Walker, 1992), but, because no carrier has been crystallized, high-resolution structural information is lacking. The oxoglutarate carrier is a relatively rare constituent of the inner membranes of bovine-heart mitochondria, which has proved to be difficult to purify in sufficiently large amounts to permit crystallization trials to be undertaken. Therefore, the expression of this protein in abundant amounts in bacteria, and its reconstitution in an active form in liposomes, are significant steps towards overcoming the problem of the supply of protein for crystallization. They also provide an opportunity to study which amino acids are essential for the function of the carrier by site-directed mutagenesis.

The expression of the mitochondrial ADP/ATP carrier in bacteria is also a step towards this latter goal, although the essential reconstitution step has not yet been achieved. In contrast with the oxoglutarate carrier, the ADP/ATP carrier is a major constituent of the inner membranes of bovine-heart mitochondria, and this remains the richest source of material for structural analysis, providing that it can be induced to crystallize.

It is not yet known whether the mitochondrial phosphate carrier, the uncoupling protein from brown-fat mitochondria, or any of the members of the carrier family of known sequence but of unknown function, can be expressed in the vectors that we have used for the expression of the oxoglutarate and ADP/ATP carriers. Previous attempts to express the intact mitochondrial phosphate carrier in *E. coli* have not been successful, although fragments of the carrier lacking the C-terminal 64 or more amino acids were expressed to high levels by fusing them either to the α- or to the β-subunit of ATP synthase, together with the alkaline-phosphatase signal sequence (Ferreira and Pedersen, 1992). Bacterial expression of fragments of the uncoupling protein from brown-fat mitochondria was obtained by fusing them to the Mal E protein (Miroux et al., 1992). If the expression of the carriers that are of unknown function can be achieved, it would provide a number of potential routes to identify their transport specificities, either by the reconstitution of the carriers into liposomes and the determination of their transport properties, or by using the expressed proteins to raise antibodies to aid in the purification and biochemical characterization of these carriers from mitochondria.

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