

## Targeting and assembly of the oxoglutarate carrier: general principles for biogenesis of carrier proteins of the mitochondrial inner membrane

Annamaria PALMISANO<sup>\*1</sup>, Vincenzo ZARA<sup>\*†1</sup>, Angelika HÖNLINGER<sup>‡</sup>, Angelo VOZZA<sup>\*</sup>, Peter J. T. DEKKER<sup>‡</sup>, Nikolaus PFANNER<sup>‡</sup> and Ferdinando PALMIERI<sup>\*2</sup>

<sup>\*</sup>Dipartimento Farmaco-Biologico, Università di Bari, Via E. Orabona 4, I-70125 Bari, Italy, <sup>†</sup>Dipartimento di Biologia, Università di Lecce, I-73100 Lecce, Italy, and <sup>‡</sup>Institut für Biochemie und Molekularbiologie, Universität Freiburg, Hermann-Herder-Strasse 7, D-79104 Freiburg, Federal Republic of Germany

We have studied the targeting and assembly of the 2-oxoglutarate carrier (OGC), an integral inner-membrane protein of mitochondria. The precursor of OGC, synthesized without a cleavable presequence, is transported into mitochondria in an ATP- and membrane potential-dependent manner. Import of the mammalian OGC occurs efficiently into both mammalian and yeast mitochondria. Targeting of OGC reveals a clear dependence on the mitochondrial surface receptor Tom70 (the 70 kDa subunit of the translocase of the outer mitochondrial membrane), whereas a cleavable preprotein depends on Tom20 (the 20 kDa subunit),

supporting a model of specificity differences of the receptors and the existence of distinct targeting pathways to mitochondria. The assembly of minute amounts of OGC imported *in vitro* to the dimeric form can be monitored by blue native electrophoresis of digitonin-lysed mitochondria. The assembly of mammalian OGC and fungal ADP/ATP carrier occurs with high efficiency in both mammalian and yeast mitochondria. These findings indicate a dynamic behaviour of the carrier dimers in the mitochondrial inner membrane and suggest a high conservation of the assembly reactions from mammals to fungi.

### INTRODUCTION

The mitochondrial inner membrane has to transport a large variety of metabolites. Functional studies indicate the presence of at least 20 different transport systems [1,2]. Major representatives are the ADP/ATP carrier (AAC), the phosphate carrier, the 2-oxoglutarate carrier (OGC), the dicarboxylate carrier, the ornithine carrier and the uncoupling protein [3–7]. All these transporters have related primary structures and belong to the same carrier protein family [8,9]. Each member of this family so far characterized has a tripartite structure, made up (as a monomer) of three tandemly repeated homologous domains of approx. 100 amino acid residues in length. Each repetitive domain contains two hydrophobic stretches, which are thought to span the membrane as  $\alpha$ -helices [9,10]. From the position of the introns in the genes of several mitochondrial carriers it is assumed that these genes were derived from a common ancestor encoding the 100-residue domain [11,12]. A systematic analysis of the fully sequenced genome of the yeast *Saccharomyces cerevisiae* revealed the presence of 35 homologous genes for mitochondrial carrier proteins [6]. By using cross-linking reagents, evidence has been provided that the AAC, OGC and uncoupling protein exist as homodimers [13–16], although a tetrameric form of the AAC has also been inferred [17].

Whereas most mitochondrial proteins studied so far are synthesized at cytosolic polysomes with N-terminal presequences that are proteolytically removed after import into the organelle, the carrier proteins are typically synthesized as non-cleavable mature-sized proteins [18–20]. The carrier proteins thus form a special class of mitochondrial preproteins, the biogenesis of which cannot be deduced from the import pathways of cleavable preproteins. Interestingly, a few species-specific exceptions were

observed: the phosphate carrier of mammals [4,21] and the AAC of plants [22] were found to be synthesized with a cleavable presequence; the presequence of the mammalian phosphate carrier was shown, however, to be dispensable for targeting of the protein into mitochondria [23], supporting the model of targeting signals present in the mature part of the carrier proteins.

A characterization of the targeting pathways of carrier proteins into mitochondria has relied on very few examples, the AAC and phosphate carrier from fungal mitochondria. It was reported that those two carrier proteins preferentially used the import receptor Tom70 (the 70 kDa subunit of the translocase of outer mitochondrial membrane) at the mitochondrial outer membrane and only to a minor degree the receptor Tom20 (the 20 kDa subunit) [24–27]; Tom20 is mainly used by presequence-containing preproteins [27,28]. Alternatively to this model of two distinct targeting pathways into mitochondria that converge at the general import pore of the outer membrane [29], a model was proposed in which Tom70 and Tom20 function in a linear targeting chain to the general import pore. In this latter model, cytosolic cofactors (chaperones) determine whether a preprotein directly contacts Tom20 or whether it is bound to Tom70 and then transferred to Tom20 [30,31]. Binding studies with purified receptor domains showed the principal ability of both Tom70 and Tom20 to bind carrier proteins [32,33]; however, they could not assign the relevance of the receptors for preprotein targeting in the organelle. The insertion of carrier proteins into the inner membrane requires a membrane potential  $\Delta\psi$  and involves a machinery, Tim10–Tim12–Tim22 (the 10, 12 and 22 kDa subunits, respectively, of the translocase of the inner mitochondrial membrane), that is distinct from the general import machinery for presequence-containing preproteins [34–36].

Abbreviations used: AAC, ADP/ATP carrier;  $\Delta\psi$ , membrane potential; OGC, 2-oxoglutarate carrier; Tom70 and Tom20, 70 and 20 kDa subunits respectively of the translocase of the outer mitochondrial membrane.

<sup>1</sup> These authors contributed equally to this work.

<sup>2</sup> To whom correspondence should be addressed (e-mail fpalm@farmbiol.uniba.it).

In the present situation it is thus difficult to make a conclusion about general principles of the biogenesis of carrier proteins, in particular as the number of known examples is too limited and possible species-specific differences have not yet been studied. Moreover, only scarce information is available on the assembly reactions of carrier proteins to the putative dimeric forms in the inner membrane. For this report we used a carrier protein, the OGC, the biogenesis of which has not previously been studied, and performed a detailed analysis of its targeting mechanism. In addition we show how assembly to the dimeric forms can be monitored directly and demonstrate the specificity of assembly of AAC and OGC in both homologous and heterologous systems.

## EXPERIMENTAL

### Materials

The expression vector pGEM4 was purchased from Promega; [<sup>35</sup>S]methionine/[<sup>35</sup>S]cysteine labelling mix, rabbit reticulocyte lysate system and PVDF membranes (Hybond P) from Amersham International; BSA (fatty acid free), sodium succinate, valinomycin, oligomycin, PMSF, trypsin, soybean trypsin inhibitor, apyrase, digitonin, Bis-Tris, 6-aminohexanoic acid, Tricine, Coomassie Brilliant Blue G-250 and 3,3'-diaminobenzidine from Sigma; and proteinase K (20 units/mg), NADH and SP6 RNA polymerase from Boehringer Mannheim. All other reagents were of analytical grade.

### *S. cerevisiae* strains and growth media

The *S. cerevisiae* strains used in this study were YPH 499 (*MAT $\alpha$*  *ade2-101 his3- $\Delta$ 200 leu2- $\Delta$ 1 ura3-52 trp1- $\Delta$ 63 lys2-801*) as the control strain (wild type) and *tom70 $\Delta$*  (*MAT $\alpha$*  *ade2-101 his3- $\Delta$ 200 leu2- $\Delta$ 1 ura3-52 trp1- $\Delta$ 63 lys2-801 tom70::HIS3*) as the strain from which the gene *TOM70* was deleted [24,27]. For the isolation of mitochondria, both strains were grown in YP medium [1% (w/v) yeast extract/2% (w/v) bacto-peptone (pH 5.0)] containing 3% (v/v) glycerol and 2% (v/v) ethanol.

### Import of preproteins into isolated mitochondria

Mitochondria from rat liver and from *S. cerevisiae* were prepared by standard procedures. The cDNA encoding the bovine heart OGC [5] was subcloned into the expression vector pGEM4. The precursor of OGC was expressed by transcription and translation *in vitro* in rabbit reticulocyte lysates in the presence of [<sup>35</sup>S]methionine/[<sup>35</sup>S]cysteine. Rabbit reticulocyte lysate containing <sup>35</sup>S-labelled OGC, <sup>35</sup>S-labelled AAC or <sup>35</sup>S-labelled Su9-DHFR (a fusion protein between the presequence of F<sub>0</sub>-ATPase subunit 9 and dihydrofolate reductase) and mitochondria isolated from rat liver (100  $\mu$ g of protein) or from *S. cerevisiae* wild-type or *tom70 $\Delta$*  strains (25  $\mu$ g of protein) were incubated in BSA-containing buffer [3% (w/v) BSA/250 mM sucrose/80 mM KCl/5 mM MgCl<sub>2</sub>/10 mM Mops/KOH (pH 7.2)] in a final volume of 100  $\mu$ l for 30 min at 30 °C (rat liver mitochondria) or for 20 min at 25 °C (*S. cerevisiae* mitochondria) [37]. Sodium succinate (rat liver mitochondria; 10 mM final concentration) or NADH (with the use of the external NADH dehydrogenase of *S. cerevisiae* mitochondria; 2 mM final concentration) was added from a 50-fold-concentrated stock solution in water. Valinomycin and oligomycin (1  $\mu$ M and 20  $\mu$ M final concentrations respectively) were added from a 100-fold-concentrated stock solution in ethanol. The samples were made chemically similar by adding the same amount of reagent-free solvent to the control samples. For protease treatment the samples were cooled to 0 °C and incubated with trypsin (50  $\mu$ g/ml) for 15 min at 0 °C (rat liver mitochondria) or with proteinase K (250  $\mu$ g/ml) for 20 min at 0 °C (*S. cerevisiae*

mitochondria). Then a 30-fold excess of soybean trypsin inhibitor (rat liver mitochondria) or PMSF (*S. cerevisiae* mitochondria; 3 mM final concentration) was added to the samples. Rat liver and *S. cerevisiae* mitochondria were then reisolated by centrifugation and analysed by SDS/PAGE, fluorography and laser densitometry as previously described [21,23,26].

In some experiments *S. cerevisiae* wild-type mitochondria were preincubated for 35 min at 4 °C with monospecific immunoglobulins G (100  $\mu$ g/10  $\mu$ g of mitochondrial protein) directed against Tom20 or Tom70, essentially as previously described [37]. Control samples were preincubated with preimmune serum. The import of the precursors of OGC, AAC and Su9-DHFR into these antibody-pretreated yeast mitochondria was then performed as described above.

### Blue native gel electrophoresis

Rat liver mitochondria (100  $\mu$ g) or *S. cerevisiae* mitochondria (25  $\mu$ g) were lysed in 50  $\mu$ l of ice-cold digitonin buffer [1% digitonin/20 mM Tris/HCl (pH 7.4)/0.1 mM EDTA/50 mM NaCl/10% (w/v) glycerol/1 mM PMSF] or in 50  $\mu$ l of SDS buffer [2% (w/v) SDS/100 mM Bistris (pH 7.0)/500 mM 6-aminohexanoic acid] for 10 min at 0 °C. Insoluble material was removed by centrifugation for 30 min at 20000 g. The supernatant of each sample, supplemented with 2.5  $\mu$ l of sample buffer [5% Coomassie Brilliant Blue G-250/100 mM Bistris (pH 7.0)/500 mM 6-aminohexanoic acid], was analysed by blue native electrophoresis as described [35,38,39]. Proteins were separated on a 6–16.5% (w/v) polyacrylamide gradient gel (with 4% stacking gel) in 50 mM Bistris (pH 7.0)/66 mM 6-aminohexanoic acid. The run was performed for approx. 4 h at 500 V in a cooled (4 °C) gel chamber (SE600; Hoefer). After 1.5 h the cathode buffer [15 mM Bistris (pH 7.0)/50 mM Tricine/0.02% Coomassie Brilliant Blue G-250] was changed for the same buffer without the Coomassie dye. After the run, gels were dried and analysed by fluorography.

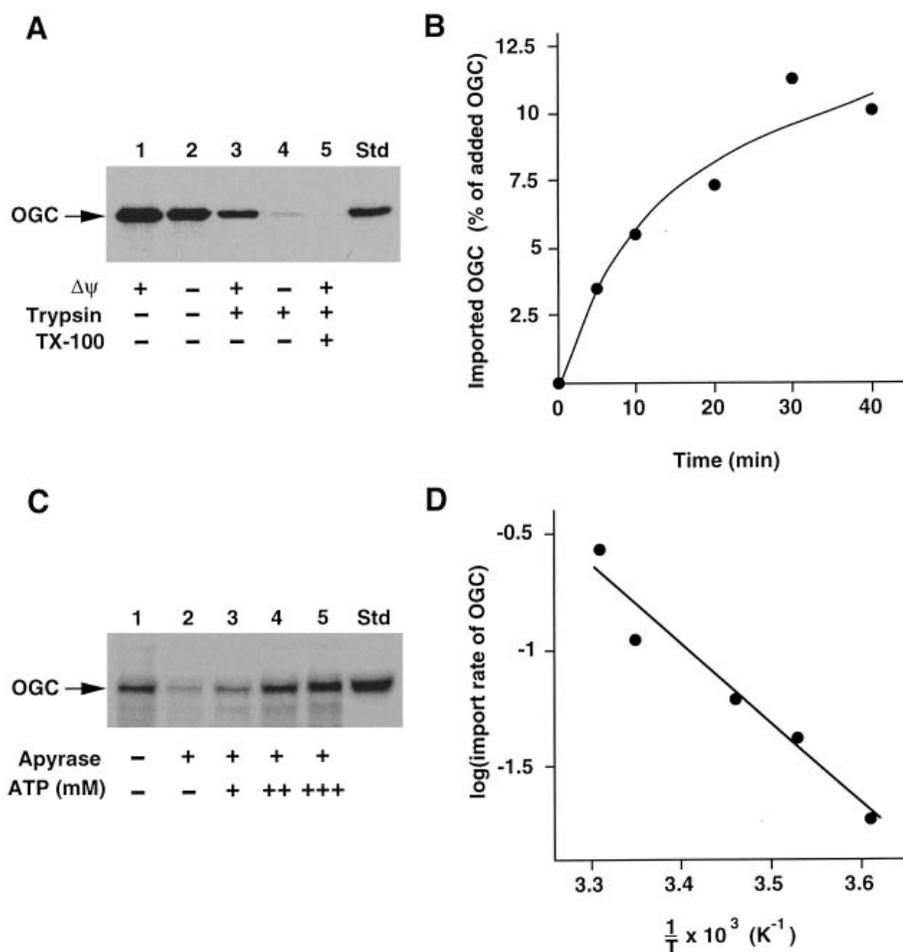
For Western blotting, the blue native gel was soaked in blot buffer [20 mM Tris/150 mM glycine/0.02% SDS/20% (v/v) methanol] for 10 min; the proteins were then transferred to PVDF membranes in the same buffer. Immunodecoration was performed by standard procedures and was revealed by 3,3'-diaminobenzidine as substrate for the peroxidase reaction.

## RESULTS AND DISCUSSION

### Import of mammalian OGC into rat liver mitochondria

On SDS/PAGE, the precursor of OGC expressed *in vitro* showed an apparent molecular mass of 31.5 kDa (Figure 1A, lane labelled Std), which corresponds to that of the endogenous OGC purified to homogeneity from bovine heart mitochondria by hydroxyapatite and celite chromatography [40,41]. Reticulocyte lysate containing the <sup>35</sup>S-labelled OGC was incubated with isolated rat liver mitochondria in the presence of a  $\Delta\psi$  across the inner membrane. The protein was transported to a location where it was protected against externally added trypsin (Figure 1A, lane 3), unless the mitochondria were disrupted by the addition of Triton X-100 (Figure 1A, lane 5). Dissipation of  $\Delta\psi$  with the potassium ionophore valinomycin (in the presence of K<sup>+</sup> ions in the import buffer) blocked the import of OGC (Figure 1A, lane 4). The gel mobility of the imported OGC was indistinguishable from that of the preprotein synthesized in reticulocyte lysate (Figure 1A, lanes 3 and Std), confirming that the OGC was synthesized without a cleavable presequence.

A time course revealed that the import of OGC into isolated



**Figure 1** Import of OGC into isolated rat liver mitochondria *in vitro*

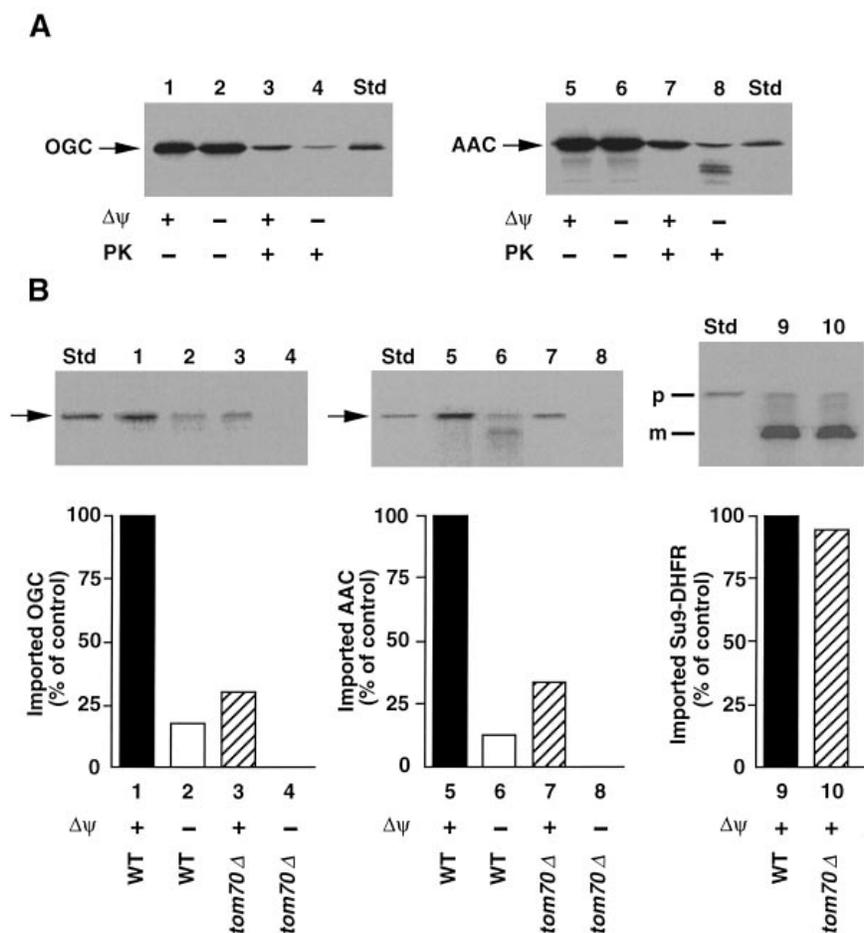
(A) Import requires a  $\Delta\psi$ . Rabbit reticulocyte lysate containing  $^{35}\text{S}$ -labelled OGC (5% by vol.) and isolated rat liver mitochondria (100  $\mu\text{g}$  of protein per sample) were incubated in the presence of 10 mM sodium succinate (lanes 1, 3 and 5) or 1  $\mu\text{M}$  valinomycin and 20  $\mu\text{M}$  oligomycin (lanes 2 and 4) in BSA-containing buffer in a final volume of 100  $\mu\text{l}$  for 30 min at 30  $^{\circ}\text{C}$ . Samples 3, 4 and 5 were treated with trypsin (50  $\mu\text{g}/\text{ml}$ ) for 15 min at 0  $^{\circ}\text{C}$  after the import; sample 5 was treated with 1% (w/v) Triton X-100 before the protease treatment. After treatment with protease, soybean trypsin inhibitor was added to a final concentration of 1.5 mg/ml to samples 3, 4 and 5. The mitochondria (samples 1–4) were then reisolated and analysed by SDS/PAGE and fluorography. Sample 5 was subjected to precipitation with trichloroacetic acid before SDS/PAGE. The lane labelled Std represents 10% of the radiolabelled OGC added to each sample. (B) Kinetics of transfer of OGC into isolated rat liver mitochondria. Import of  $^{35}\text{S}$ -labelled OGC into energized rat liver mitochondria was performed at 30  $^{\circ}\text{C}$  for the durations indicated, as described for (A). The amount of OGC transferred into a trypsin-resistant location was determined. The reisolated mitochondria were analysed by SDS/PAGE and fluorography. Imported OGC was quantified by laser densitometry. The total amount of  $^{35}\text{S}$ -labelled OGC added to the import reaction was taken as 100%. (C) Import of OGC requires ATP. Reticulocyte lysate, containing  $^{35}\text{S}$ -labelled OGC, and rat liver mitochondria were incubated with apyrase (10 units/ml; samples 2–5) for 30 min at 30  $^{\circ}\text{C}$  and for 15 min at 30  $^{\circ}\text{C}$  respectively. Reticulocyte lysate and rat liver mitochondria of sample 1 were incubated under the same conditions as described above with an apyrase preparation (10 units/ml) that had been heated to 95  $^{\circ}\text{C}$  for 10 min before use. After being cooled to 0  $^{\circ}\text{C}$ , samples were then added to the import mixture in a final volume of 100  $\mu\text{l}$  in the presence of sodium succinate 10 mM. Samples 3, 4 and 5 received 5 (+), 10 (++) or 15 (+++) mM ATP respectively. The import incubation was performed for 30 min at 30  $^{\circ}\text{C}$ . The lane labelled Std represents 10% of the radiolabelled OGC added to each sample. (D) Arrhenius plot of the temperature dependence of the import of OGC into mitochondria. Import of OGC into energized mitochondria was performed at the indicated temperatures for 15 min as described for (A). The rates of import were calculated as described for (B). An import rate of 1 represents 1% imported OGC/min.

mitochondria was linearly dependent on the time of incubation up to at least 5 min (Figure 1B). To determine whether ATP was needed as energy source for the import in addition to the requirement for a  $\Delta\psi$ , reticulocyte lysate and isolated rat liver mitochondria were depleted of ATP and ADP by a preincubation with apyrase [37]. The removal of ATP inhibited almost completely the import of OGC (Figure 1C, lane 2). Re-addition of increasing amounts of ATP fully restored the import (Figure 1C, lanes 4 and 5). Figure 1(D) shows the temperature dependence of OGC import evaluated by an Arrhenius plot. When the import incubation was performed for 15 min at different temperatures, a linear dependence between  $\log(\text{import rate})$  and  $1/T$  was

found. The calculated activation energy was 64.2 kJ/mol (15.7 kcal/mol).

#### Heterologous import and receptor dependence of mammalian OGC

We then analysed whether the mammalian OGC was also imported into fungal mitochondria isolated from *S. cerevisiae*. The OGC was indeed efficiently transported to a protease-resistant location of energized yeast mitochondria (Figure 2A, lane 3). A dissipation of the membrane potential strongly inhibited the import (Figure 2A, lane 4), as already found for the import into rat liver mitochondria (Figure 1A, lane 4). For



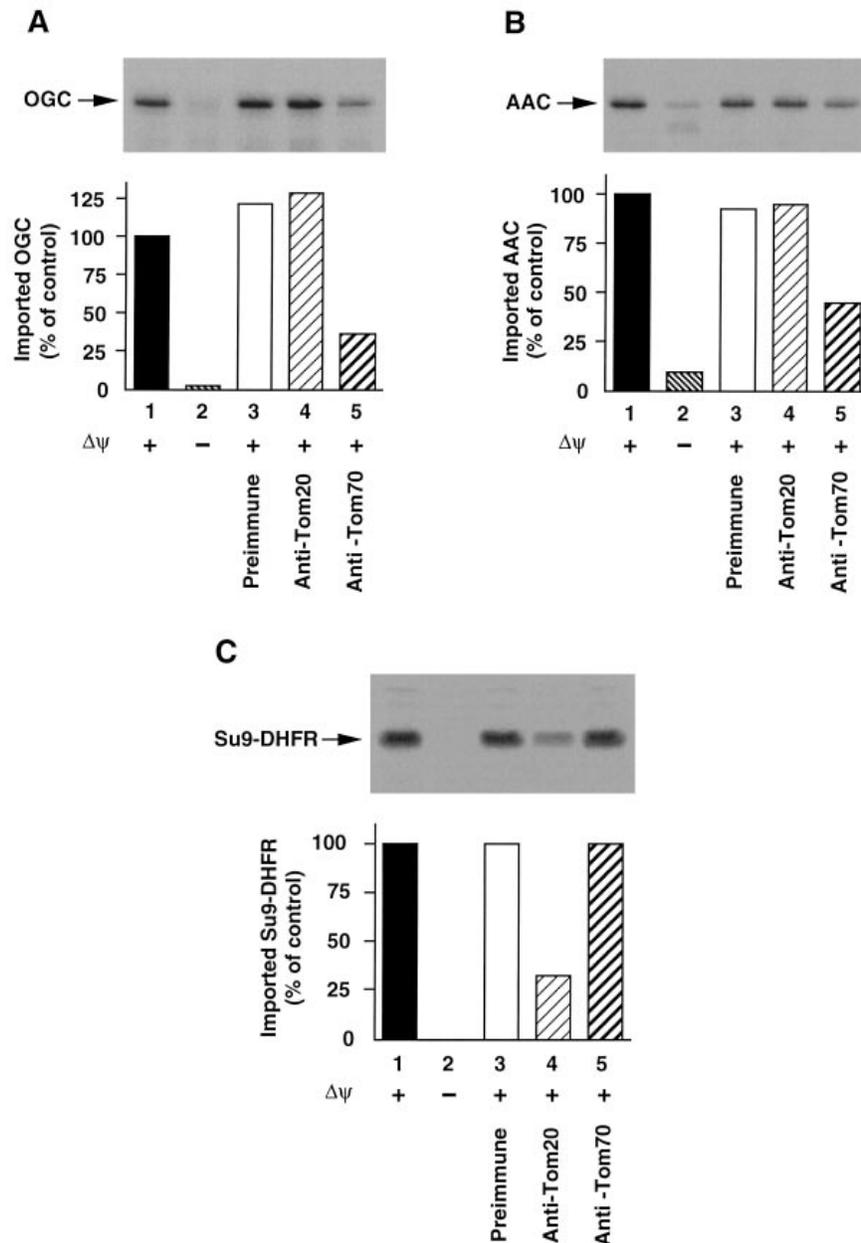
**Figure 2** Heterologous import of OGC

(A) Import of OGC and AAC into *S. cerevisiae* mitochondria. Reticulocyte lysate containing  $^{35}\text{S}$ -labelled bovine heart OGC (10% by vol.; lanes 1–4) or  $^{35}\text{S}$ -labelled *Neurospora crassa* AAC (10% by vol.; lanes 5–8) and isolated *S. cerevisiae* mitochondria (25  $\mu\text{g}$  of protein per sample) were incubated in the presence of 2 mM NADH (lanes 1, 3, 5 and 7) or 1  $\mu\text{M}$  valinomycin and 20  $\mu\text{M}$  oligomycin (lanes 2, 4, 6 and 8) in BSA-containing buffer in a final volume of 100  $\mu\text{l}$  for 20 min at 25  $^{\circ}\text{C}$ . Lanes 3, 4, 7 and 8 were treated with proteinase K (250  $\mu\text{g}/\text{ml}$ ; PK) for 20 min at 0  $^{\circ}\text{C}$  after the import. After the treatment with protease, PMSF was added to a final concentration of 3 mM. The reisolated mitochondria were analysed by SDS/PAGE and fluorography. The lane labelled Std represents 10% of the radiolabelled OGC or AAC added to each sample respectively. (B) Import of OGC, AAC and Su9-DHFR into *tom70Δ* mitochondria. Reticulocyte lysate containing  $^{35}\text{S}$ -labelled OGC (lanes 1–4),  $^{35}\text{S}$ -labelled AAC (lanes 5–8) or  $^{35}\text{S}$ -labelled Su9-DHFR (lanes 9 and 10) and isolated *S. cerevisiae* mitochondria from wild-type strain (WT) (25  $\mu\text{g}$  per sample; lanes 1, 2, 5, 6 and 9) or from *tom70Δ* strain (25  $\mu\text{g}$  per sample; lanes 3, 4, 7, 8 and 10) were incubated in the presence of 2 mM NADH (samples 1, 3, 5, 7, 9 and 10) or 1  $\mu\text{M}$  valinomycin and 20  $\mu\text{M}$  oligomycin (samples 2, 4, 6 and 8) in BSA-containing buffer in a final volume of 100  $\mu\text{l}$  for 20 min at 25  $^{\circ}\text{C}$ . All samples were treated with proteinase K (250  $\mu\text{g}/\text{ml}$ ) for 20 min at 0  $^{\circ}\text{C}$  after the import. The reisolated mitochondria were analysed by SDS/PAGE and fluorography. Imported proteins were quantified by laser densitometry. The import of the proteins into energized wild-type mitochondria was set to 100%. The lane labelled Std represents 5% of the radiolabelled OGC, AAC and Su9-DHFR added to each sample respectively. Abbreviations: p and m, precursor-sized and mature-sized forms of Su9-DHFR respectively.

comparison the import of fungal AAC into isolated yeast mitochondria is also shown (Figure 2A, lanes 5–8). Deletion of the gene *TOM70* from *S. cerevisiae* yields mitochondria that selectively lack the import receptor Tom70, whereas the import pathway via Tom20 and the  $\Delta\psi$ -dependent insertion of pre-proteins into the inner membrane are not impaired [24,25,27]; *tom70Δ* mitochondria are thus well suited to determine a dependence of import of OGC on the function of Tom70. We compared the import of OGC into wild-type and *tom70Δ* mitochondria (Figure 2B, lanes 1–4). With the mutant mitochondria, the import of the mammalian carrier was strongly decreased (Figure 2B, lane 3); the decrease in import was as strong as that observed for the import of fungal AAC (Figure 2B, lane 7). In contrast, the cleavable preprotein Su9-DHFR, a fusion protein between the presequence of  $F_0$ -ATPase subunit 9 and dihydrofolate reductase, was imported into wild-type and

*tom70Δ* mitochondria with comparable efficiencies (Figure 2B, lanes 9 and 10). We conclude that the receptor Tom70 is similarly required for the import of OGC and AAC.

We examined whether Tom20 was of equal importance for import of the carrier proteins. Mitochondria isolated from a yeast strain lacking the *TOM20* gene show a significant decrease in the membrane potential [42]. Therefore a decreased import into *tom20Δ* mitochondria might not be caused only by a lack of this receptor, but also by an impairment of translocation into the inner membrane. To circumvent this problem we used inhibitory antibodies as a means of distinguishing between the contributions of Tom70 and Tom20 in protein import. Immunoglobulins G specifically directed against either Tom70 or Tom20 were pre-bound to isolated wild-type mitochondria [37], followed by an incubation with  $^{35}\text{S}$ -labelled preproteins. As control, preimmune antibodies were used. The import of OGC was inhibited by anti-



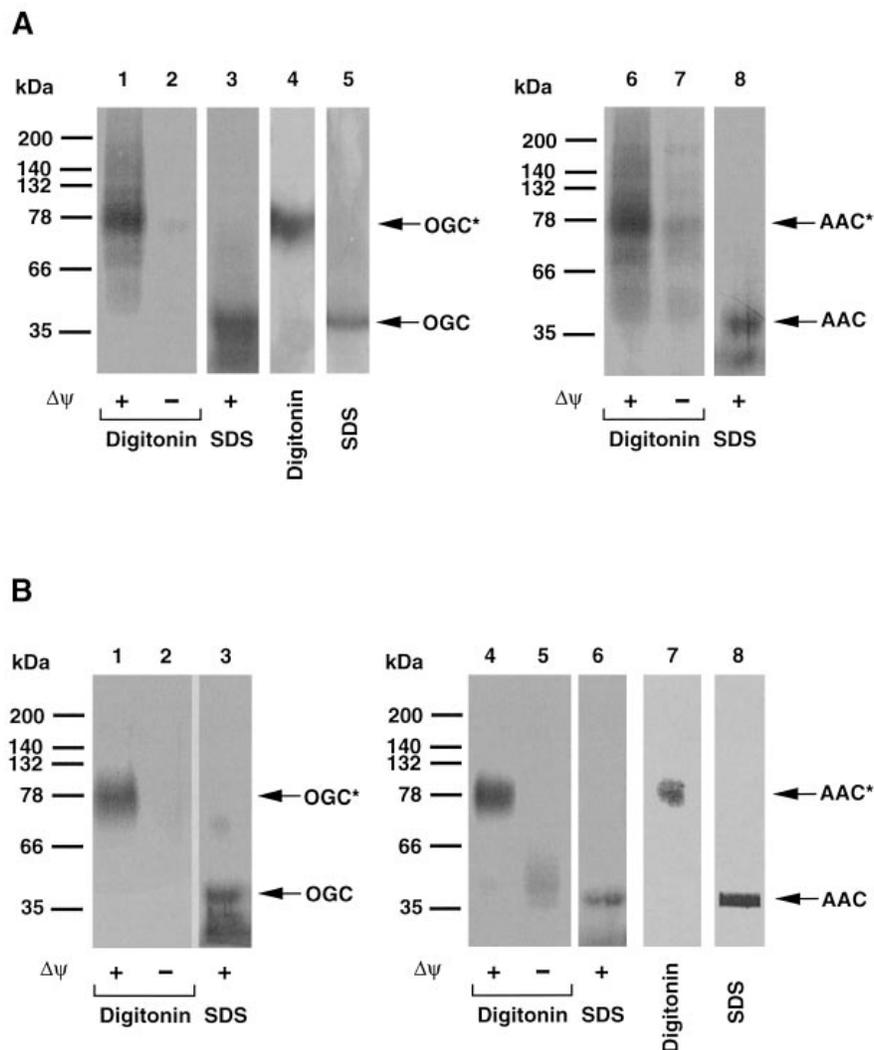
**Figure 3** Tom70 is required for the import of OGC and AAC into yeast mitochondria

Isolated *S. cerevisiae* mitochondria from the wild-type strain were preincubated with immunoglobulins G (100  $\mu$ g/10  $\mu$ g of mitochondrial protein) directed against Tom20 (sample 4), Tom70 (sample 5) or with preimmune serum (sample 3) for 35 min at 4 °C. The import of the precursors of OGC (A), AAC (B) and Su9-DHFR (C) into yeast mitochondria was performed as described in the legend to Figure 2(A). The mitochondria were then reisolated and analysed by SDS/PAGE and fluorography. Imported proteins were quantified by laser densitometry. The import of each protein into mitochondria not preincubated with antibodies (sample 1) was taken as 100%.

Tom70 only (Figure 3A, lane 5), not by anti-Tom20 (Figure 3A, lane 4). For comparison, the import of AAC revealed a similar preference for Tom70 (Figure 3B, lanes 4 and 5), whereas the import of Su9-DHFR was affected by the antibodies in a reverse manner, i.e. inhibition by anti-Tom20 (Figure 3C, lanes 4 and 5). We conclude that the receptor Tom70 of yeast mitochondria is required for the bulk of import of OGC and AAC, whereas Tom20 has a dominant role in the import of a presequence-carrying preprotein.

#### Assembly of endogenous and imported mammalian OGC

Many mitochondrial proteins do not function as monomers but have to assemble into oligomeric structures to become functional. A major limitation of import studies *in vitro* is the lack of suitable means of determining the assembly of the tiny amounts of radiolabelled preproteins. For studying hetero-oligomeric protein complexes, co-immunoprecipitations have been used although the efficiency is usually low [43,44]. For the carrier



**Figure 4** Analysis of OGC and AAC imported *in vitro* by blue native electrophoresis

(A) Import into rat liver mitochondria. The  $^{35}\text{S}$ -labelled precursors of OGC and AAC were incubated with isolated rat liver mitochondria (100  $\mu\text{g}$  protein per sample) in the presence (lanes 1, 3, 6 and 8) or in the absence (lanes 2 and 7) of a membrane potential for 30 min at 30  $^{\circ}\text{C}$ . All samples were then treated with trypsin (50  $\mu\text{g}/\text{ml}$ ) as described in the legend to Figure 1(A). Reisolated mitochondria were resuspended in 50  $\mu\text{l}$  of ice-cold digitonin buffer (samples 1, 2, 6 and 7) or in 50  $\mu\text{l}$  of SDS buffer (samples 3 and 8); insoluble material was removed by centrifugation for 30 min at 20 000  $g$ . The supernatant of each sample was supplemented with 2.5  $\mu\text{l}$  of sample buffer and analysed by blue native electrophoresis. Gels were dried and analysed by fluorography. Marker proteins were  $\beta$ -lactoglobulin B (35 kDa), albumin (monomeric and dimeric forms) (66 and 132 kDa), conalbumin (78 kDa), L-lactate dehydrogenase (140 kDa) and  $\beta$ -amylase (200 kDa); their migration positions are indicated at the left. Samples 4 and 5 represent endogenous OGC of rat liver mitochondria detected by Western blotting. Mitochondrial extracts from lysis with digitonin (sample 4) or SDS (sample 5) were separated by blue native electrophoresis, blotted to PVDF membrane and incubated with antibodies against OGC. (B) Import into yeast mitochondria. The  $^{35}\text{S}$ -labelled precursors of OGC and AAC were incubated with isolated *S. cerevisiae* mitochondria (25  $\mu\text{g}$  of protein per sample) in the presence (lanes 1, 3, 4 and 6) or in the absence (lanes 2 and 5) of a membrane potential for 20 min at 25  $^{\circ}\text{C}$ . All samples were then treated with proteinase K (250  $\mu\text{g}/\text{ml}$ ) as described in the legend to Figure 2(A). Reisolated mitochondria were resuspended in digitonin buffer (samples 1, 2, 4 and 5) or in SDS buffer (samples 3 and 6) and analysed by blue native electrophoresis and fluorography as described for (A). Samples 7 and 8 represent endogenous AAC of *S. cerevisiae* mitochondria detected by Western blotting and immunodetection. Abbreviations: OGC\* and OGC, dimeric and monomeric forms of OGC respectively; AAC\* and AAC, dimeric and monomeric forms of AAC respectively.

proteins that apparently assemble to homodimers, co-immunoprecipitations are not possible. For AAC and phosphate carrier, indirect assays for an assembly of subunits imported *in vitro* have been applied by following a scheme of partial purification of the functional form [21,45]. Here we employed the technique of blue native electrophoresis developed by Schagger et al. [38] after lysis of mitochondria in digitonin [35,39] to study the oligomeric state of OGC. By Western blotting we found that the endogenous OGC of rat liver mitochondria was quantitatively present in the dimeric form after lysis with the mild detergent digitonin (Figure 4A, lane 4), whereas after lysis with SDS only the monomeric

form of OGC was observed (Figure 4A, lane 5). OGC imported *in vitro* in the presence of a  $\Delta\psi$  assembled efficiently to the dimeric form (Figure 4A, lane 1); as expected, the dimeric form was stable in digitonin, but not in SDS (Figure 4A, lanes 1 and 3). Assembly was not observed when the precursor of OGC was incubated with mitochondria in the absence of a  $\Delta\psi$  (Figure 4A, lane 2). Blue native electrophoresis thus represents a quantitative method for determining the assembly of OGC imported *in vivo* and *in vitro*.

We examined whether the heterologous import reactions, fungal AAC into rat liver mitochondria and mammalian OGC

into yeast mitochondria, represented complete import reactions, i.e. whether the heterologous preproteins were assembled to dimeric forms. This was indeed so. AAC assembled to the dimeric form in rat liver mitochondria (Figure 4A, lane 6) and OGC assembled to the dimeric form in yeast mitochondria (Figure 4B, lane 1). It has to be emphasized that the heterologous assembly processes were of the same high efficiency as the homologous ones (Figure 4A, lane 1; the assembly of fungal AAC in yeast mitochondria is shown in lane 4 of Figure 4B). The lack of dimeric forms when the preproteins were incubated with isolated mitochondria in the absence of a  $\Delta\psi$  (Figure 4A, lane 7; Figure 4B, lane 2) and the dissociation of the dimers in SDS (Figure 4A, lane 8; Figure 4B, lane 3) confirm the specificity of the heterologous assembly reactions. An OGC of yeast mitochondria has not been identified at the molecular level but its existence has been inferred by metabolite transport studies [46,47]. The assembly reactions reported here support the presence of an OGC in yeast.

### Conclusions

This study has two major implications.

(1) Targeting of carrier proteins into mitochondria. Together with studies on fungal AAC and phosphate carrier [26], the findings reported here with mammalian OGC indicate the following general principle of carrier targeting. The carrier proteins preferentially use the receptor Tom70, whereas Tom20 has only a minor role. Because inactivation of Tom20 with an antibody strongly inhibits the import of a presequence-containing preprotein, but not that of the carrier proteins, a linear targeting pathway from Tom70 to Tom20 and then to the general import pore [30,31] is unlikely for the carrier proteins. Alternatively it is conceivable that Tom20 contains two distinct binding sites for preproteins [32,33] and that the antibody inhibits the major site for presequence-containing preproteins, but not a second site that might be used by carrier proteins. Although a clarification of this point awaits a detailed structure-function analysis of Tom20, it is clear that carrier proteins enter the mitochondrial targeting machinery at a different pathway from cleavable preproteins.

(2) Assembly of carrier proteins. Blue native electrophoresis represents an accurate and convenient technique for studying the assembly of carrier proteins to the dimeric forms both for carriers imported *in vivo* and for the small amounts of carriers imported *in vitro*. The assembly reactions are of a remarkably high efficiency in both the homologous and the heterologous systems. The concentration of preproteins synthesized *in vitro* is very low [48], and in comparison with the quantities of mitochondria used here it can be calculated that the chances are low that more than one preprotein is imported into a single mitochondrion. It is thus highly unlikely that the efficient formation of dimers occurs between two subunits imported *in vitro*. Moreover it can be excluded that carrier subunits synthesized *in vitro* assemble in the reticulocyte lysate because the  $\Delta\psi$ -dependent import into the mitochondrial inner membrane is a prerequisite for assembly ([21,45], and this study). Therefore carriers imported *in vitro* have to assemble with pre-existing subunits of the mitochondria, implying that pre-existing dimers dissociate. In our analysis at least 98% of carrier subunits imported *in vivo* or *in vitro* were found in the dimeric form. This suggests the presence of a small pool of non-assembled carrier subunits in mitochondria that is in rapid equilibrium with the large pool of assembled forms. We propose that the carrier dimers in the mitochondrial inner membrane show a dynamic behaviour with a cycling between assembled (dimeric) and non-assembled

(monomeric) forms. The equilibrium is strongly shifted to the dimeric form and thus newly imported subunits pass rapidly through the non-assembled pool to the dimeric form.

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