Biogenesis of yeast dicarboxylate carrier: the carrier signature facilitates translocation across the mitochondrial outer membrane

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Summary

A family of related carrier proteins mediates the exchange of metabolites across the mitochondrial inner membrane. The carrier signature Px[D/E]xx[K/R] is a highly conserved sequence motif in all members of this family. To determine its function in the biogenesis of carrier proteins, we used the dicarboxylate carrier (DIC) of yeast as a model protein. We found that the carrier signature was dispensable in binding of the newly synthesized protein to the import receptor Tom70, but that it was specifically required for efficient translocation across the mitochondrial outer membrane. To determine the relevance of individual amino acid residues of the carrier signature in the transport activity of the protein, we exchanged defined residues with alanine and reconstituted the mutant proteins in vitro. Substitution of the carrier signature in helix H1 reduced the transport activity for [^{33}P]-phosphate by approximately 90% and an additional substitution of the carrier signature in helix H5 blocked the transport activity completely. We conclude that the carrier signature of the dicarboxylate carrier is involved both in the biogenesis and in the transport activity of the functional protein.

Key words: Mitochondria, Metabolite carrier proteins, Carrier signature

Introduction

The exchange of metabolites across the mitochondrial inner membrane is mediated by a family of carrier proteins. All mitochondrial carrier proteins are membrane proteins of about 33 kDa and show remarkable structural similarities, suggesting that they have a common ancestor in evolution (Palmieri, 1994; Zara et al., 2003a; Kunji, 2004). The primary structure of carrier proteins shows three homologous modules, each containing two membrane-spanning helices, connected by a loop at the matrix side. The carrier signature Px[D/E]xx[K/R] is a conserved sequence motif found at the C-terminal end of the first helix of each of these modules, in proximity to the matrix-exposed loop (Nelson et al., 1998; Nury et al., 2006). The crystal structure of the ADP/ATP carrier (AAC) confirmed that the charged residues of the carrier signatures form salt bridges, connecting the odd-numbered helices H1, H3 and H5 of the protein to form a basket-like structure (Pebay-Peyroula et al., 2003). The prolines of the motif induce sharp kinks responsible for the closed form of the central cavity towards the exit at the matrix side.

A genetic study on the AAC of yeast demonstrated that neutralization of a single residue of the carrier signature causes a respiration defect of the cells (Nelson et al., 1998). However, the precise role of the carrier signature is still unclear. Initially it was speculated that the carrier signature might play a role in the transport activity of the carrier proteins (Nelson et al., 1998). This notion was recently supported by a study on the oxoglutarate carrier, showing that the carrier signature is essential for its function (Cappello et al., 2007). However, the carrier signature could additionally have a function in the biogenesis of carrier proteins. In contrast to other precursor proteins, newly synthesized carrier proteins identify their target organelle by binding to the import receptor TOM70 (a component of the translocase of the mitochondrial outer membrane, TOM) (Pfanner and Geissler, 2001; de Marcos-Lousa et al., 2006; Neupert and Herrmann, 2007). Subsequent translocation across the mitochondrial outer membrane is driven by a hexameric complex of small proteins, each of about 10 kDa, named Tim9 and Tim10, in the intermembrane space. [The complex subsequently cooperates with the translocase of the inner membrane, TIM (Sirrenberg et al., 1998; Koehler et al., 1998; Leuenberger et al., 1999; Endres et al., 1999; Luciano et al., 2001; Truscott et al., 2002; Vasiljev et al., 2004)]. It is conceivable that the carrier signature might be involved in specific binding of carrier proteins either to Tom70 or to the Tim9-Tim10 complex (Sirrenberg et al., 1998; Bauer et al., 2000), but experimental evidence is lacking. Moreover, a function of the carrier signature in the import pathway of the carrier proteins was questioned by studies on the biogenesis of yeast AAC, showing that interactions of preproteins both with Tom70 and with the small Tim proteins are mainly determined by hydrophobic interactions (Brix et al., 2000; Curran et al., 2002).

To determine the possible functions of the carrier signature in the biogenesis and in the function of a carrier protein, we investigated both aspects in detail, using the dicarboxylate carrier (DIC) of *Saccharomyces cerevisiae* as a model protein.

Table 1. The carrier signature in the three modules of different carrier proteins

Protein	CS1	CS2	CS3	Reference
AAC (*B. taurus*)	..VA**P**I**E**RV**K**LL...	VY**P**L**D**FA**R**TR...	SY**P**F**D**TV**R**RR..	(Aquila et al., 1982)
AAC (*N. crassa*)	..AA**P**I**E**RI**K**LL...	VYSL**D**YA**R**TR...	SY**P**L**D**TI**R**RR..	(Arends and Sebald, 1984)
CIC (*A. anguilla*)	..TF**P**T**E**YV**K**TQ...	VC**P**M**E**TV**K**VK...	NT**P**L**D**VI**K**TR..	(Zara et al., 2007)
CIC (*S. cerevisiae*)	..TY**P**F**E**FA**K**TR...	VT**P**F**E**AI**K**TA...	TM**P**L**D**TV**K**TR..	(Kaplan et al., 1995)
DIC (*R. norvegicus*)	..TH**P**L**D**LL**K**VH...	GT**P**A**D**LVNV**R**...	CQ**P**L**D**VL**K**TR..	(Fiermonte et al., 1998)
DIC (*S. cerevisiae*)	..TH**P**L**D**LA**K**VR...	GNFA**D**VVNI**R**...	CS**P**A**D**VM**K**TR..	(Palmieri et al., 1996)
Flx1p (*S. cerevisiae*)	..VH**P**L**D**LL**K**VR...	TN**P**IWVI**K**TR...	VY**P**FQLL**K**SN..	(Tzagoloff et al., 1996)
Ndt1p (*S. cerevisiae*)	..VC**P**L**D**VA**K**TR...	TN**P**IWVV**K**TR...	TY**P**H**E**IL**R**TR..	(Todisco et al., 2006)
OAC (*S. cerevisiae*)	..TN**P**I**E**LI**K**IR...	GS**P**LFLVK**R**TR...	MN**P**W**D**VILTR..	(Palmieri et al., 1999)
OGC (*B. taurus*)	..VQ**P**L**D**LV**K**NR...	GT**P**A**E**VALI**R**...	SM**P**V**D**IV**K**TR..	(Cappello et al., 2007)

ADP/ATP carrier (AAC) of *Bos taurus* (acc. no. P02722); AAC of *Neurospora crassa* (acc. no. P02723); citrate carrier (CIC) of *Anguilla anguilla* (acc. no. CAG30841); CIC of *Saccharomyces cerevisiae* (acc. no. P38152); dicarboxylate carrier (DIC) of *Rattus norvegicus* (acc. no. NP_596909); DIC of *S. cerevisiae* (acc. no. Q06143); Flx1p, FAD carrier of *S. cerevisiae* (acc. no. P40464); Ndt1p, NAD^+ carrier (acc. no. NP_012260) of *S. cerevisiae*; oxaloacetate carrier (OAC) of *S. cerevisiae* (acc. no. P32332); and oxoglutarate carrier (OGC) of *Bos taurus* (acc. no. NP_777096). The carrier signature motifs Px[D/E]xx[K/R] (bold) are separated from each other by about 100 residues, indicated by dots (…).

The DIC is a protein of 33 kDa, mediating the exchange of dicarboxylates such as malate and succinate with phosphate (Lancar-Benba et al., 1996; Palmieri et al., 1996; Kakhniashvili et al., 1997). The DIC was particularly suitable for this study because, within the three modules of the protein, a carrier signature is only contained in module I (helix 1) and module III (helix 5), facilitating a complete exchange of the corresponding residues (Table 1). We started the project by stepwise exchanging all triplets encoding residues of the carrier signature in helix 1 and helix 5 by site-directed mutagenesis.

Results

All residues of the DIC carrier signature in helix 1 (CS1; residues P33, D35 and K38) and helix 5 (CS2; residues P227,

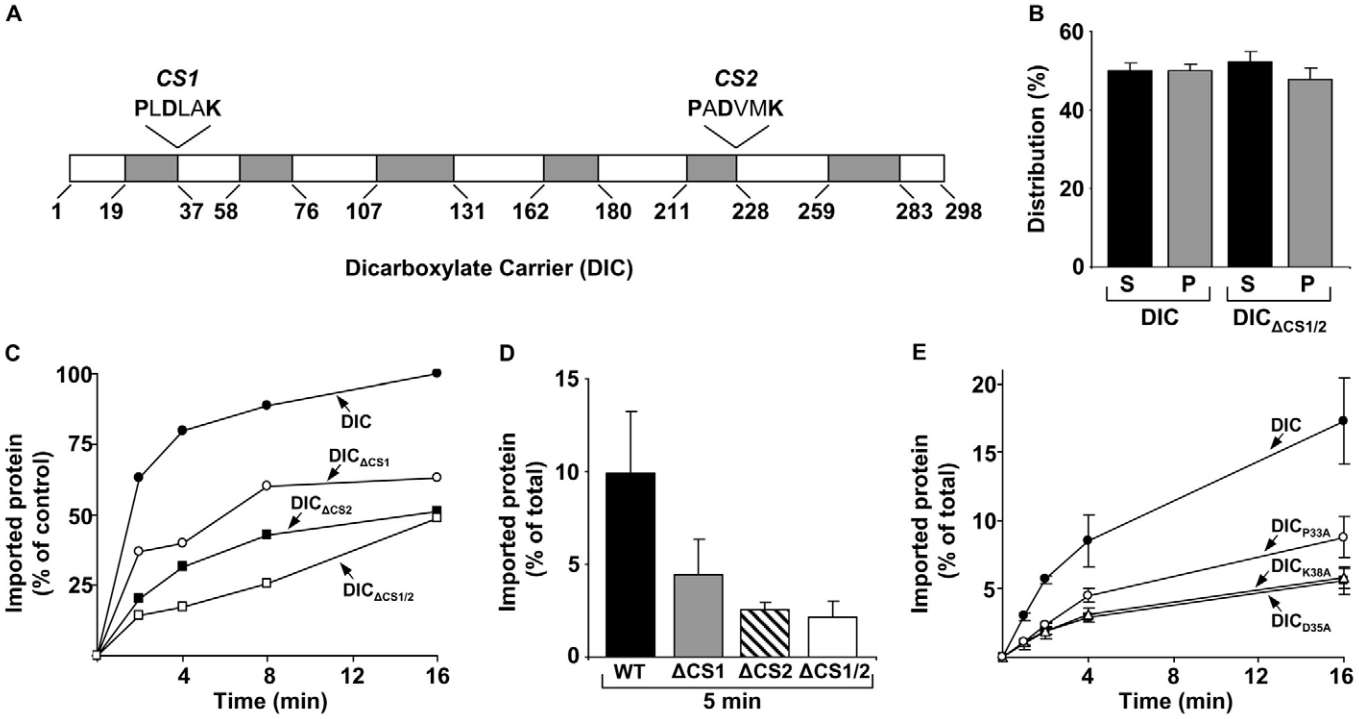

Fig. 1. Import of radiolabelled DIC into isolated mitochondria. (A) The DIC is a protein of 298 residues containing six putative membrane-spanning domains (grey segments). A carrier signature is found in two positions (CS1 and CS2). (B) Wild-type DIC and a mutant version lacking the carrier signatures (exchange of the corresponding residues with alanine, ΔCS1/2) were synthesized in reticulocyte lysate in the presence of ^{35}S-labelled methionine and centrifuged at 2°C for 45 minutes at 100,000 ***g***. The pellets (P) and samples of the supernatants (S) were analyzed by SDS-PAGE and fluorography to determine the distribution of the radiolabelled DIC or $DIC_{\Delta CS1/2}$, respectively. Four samples were tested in parallel to calculate the standard deviation. (C) Mitochondrial import of DIC and derivatives lacking carrier signature CS1, CS2 or both. The ^{35}S-labelled proteins were incubated with isolated yeast mitochondria at 25°C for different times, as indicated. The mitochondria were subsequently treated with proteinase K, re-isolated and the proteins were separated by SDS-PAGE. A PhosphorImager was used for quantification; the highest value was set to 100% (control). (D) The radiolabelled proteins were imported into mitochondria for 5 minutes and analyzed as in C. Standard samples of the reticulocyte lysates were included to allow the calculation of the import efficiency (n=3). (E) Import of DIC and derivatives containing the amino acid exchanges P33A, D35A or K38A. The radiolabelled proteins were imported into isolated mitochondria for different times and subsequently analyzed as in C. Bars represent standard deviation.

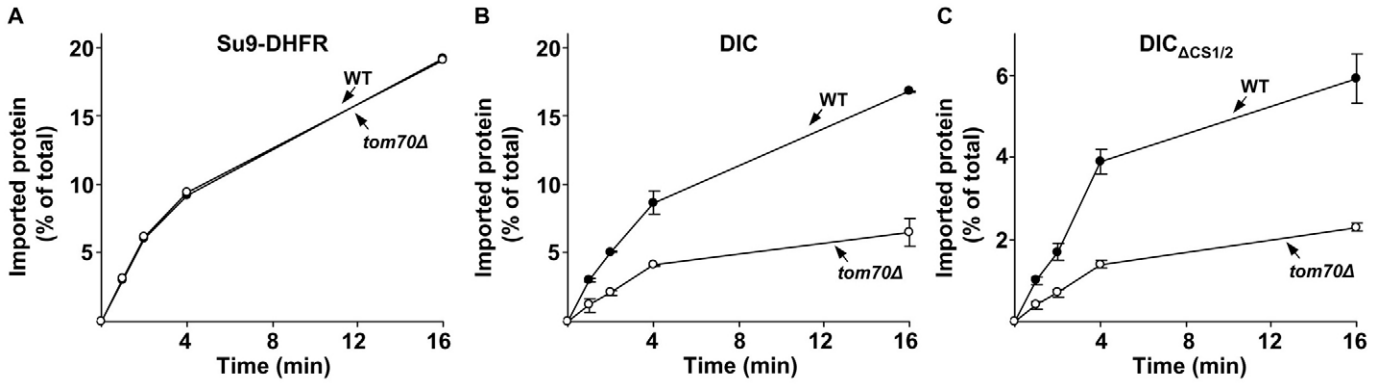

Fig. 2. Protein import into mitochondria lacking Tom70. (A) Import of Su9-DHFR. ^{35}S-labelled Su9-DHFR (comprising the first 69 residues of *Neurospora crassa* ATP synthase subunit 9 fused to mouse dihydrofolate reductase) was imported into yeast mitochondria isolated from a strain lacking the import receptor Tom70 (*Δtom70*) or from the corresponding wild-type strain (WT). For calculation of the import efficiency, the total amount of the radiolabelled preprotein in the samples was set to 100%. (B) Import of wild-type DIC into mitochondria from the same preparation as in A. (C) Import of $DIC_{\Delta CS1/2}$ into mitochondria from the same preparation as in A. Bars represent standard deviation.

D229 and K232; Fig. 1A) were exchanged with alanine using the QuikChange method (Papworth et al., 1996) for mutagenesis. The DNA fragments encoding the different constructs were cloned into a pGEM-4Z vector and used for coupled transcription/translation in reticulocyte lysate in the presence of ^{35}S-methionine. Because the exchange of a single amino acid in the citrate carrier (CIC) can significantly affect the solubility of the protein (Zara et al., 2003b; Zara et al., 2007), we tested the solubility of the DIC derivative lacking the carrier signature in helix 1 and helix 5 by centrifugation of the reticulocyte lysate at 100,000 ***g*** (Fig. 1B, $DIC_{\Delta CS1/2}$). Only about 50% of the radiolabelled protein stayed in the soluble fraction. However, the distribution between the soluble and pellet fraction was nearly identical to the authentic wild-type DIC (Fig. 1B, left columns). The carrier signature does therefore not seem to determine the solubility of the protein.

We then synthesized ^{35}S-labelled authentic DIC, derivatives lacking either the carrier signature in helix 1 (ΔCS1) or in helix 5 (ΔCS2), and the ΔCS1/2 construct, and imported these proteins into isolated yeast mitochondria. For this purpose, the reticulocyte lysates containing the radiolabelled proteins were incubated with the mitochondria at 25°C, and the mitochondria were subsequently treated with proteinase K. The mitochondria were re-isolated and analyzed for protease-protected radiolabelled protein (Fig. 1C). Following this procedure, wild-type DIC was found to be slowly but efficiently imported into the mitochondria. However, the rate of import was drastically reduced for the ΔCS1/2 construct. The constructs lacking only one of the two carrier signatures showed an intermediate behaviour. Repeating the assay several times, we found that import of the ΔCS2 construct was reduced more significantly than that of the ΔCS1 construct (Fig. 1D), indicating that the carrier signature in helix 5 is more relevant for import than the corresponding motif in helix 1.

To determine which of the three residues of a carrier signature is essential to allow a maximum rate of import, we exchanged the individual residues in the carrier signature in helix 1 with alanine and tested the constructs for import (Fig. 1E). The exchange of the proline in position 33 reduced the import rate significantly, but a stronger effect was observed with the exchange of the charged residues D35 or K38.

It is thus obvious that an intact system of carrier signatures is required to allow rapid import of DIC into mitochondria. To determine whether the carrier signatures are primarily required for efficient targeting and binding or more directly during translocation, we took advantage of previous observations showing that binding of carrier proteins to the mitochondrial outer surface is mainly mediated by the import receptor Tom70, and that yeast strains lacking Tom70 are viable and able to grow on non-fermentable carbon sources (Neupert and Herrmann, 2007). We isolated mitochondria from a *tom70Δ* deletion strain and from the corresponding wild-type strain and used these mitochondria in the import assay. As reported previously for other presequence-targeted proteins (Neupert and Herrman, 2007), import of the model protein Su9-DHFR was not affected by the loss of Tom70 (Fig. 2A). As expected, import of wild-type DIC was clearly reduced (Fig. 2B). Strikingly, import of $DIC_{\Delta CS1/2}$ was again much less efficient than the original DIC, but the dependence on Tom70 was similar for both proteins (Fig. 2B versus Fig. 2C). The interactions of DIC with Tom70 seemed to be independent of the carrier signature. This conclusion was confirmed by the observation that the effects of the amino acid exchanges were also retained in the import of the ΔCS1/2 construct into protease-pre-treated mitochondria (data not shown).

The experiments show that the carrier signatures play an important role in the import of DIC into mitochondria. Their effect is independent of the binding of DIC to the import receptor Tom70, indicating that the intact carrier signatures are not essential in targeting but are primarily required in translocation across the mitochondrial outer membrane. In this function, the carrier signature CS2 in helix 5 is more relevant than the carrier signature CS1 in helix 1, and the charged residues are more relevant than the proline residues.

A complete loss of the carrier signatures CS1 and CS2 produces a delay in import; however, a significant fraction of the ΔCS1/2 construct was still transported across the mitochondrial outer membrane (Fig. 1C). Is the carrier signature involved in subsequent insertion into the mitochondrial inner membrane? To investigate the fate of the imported DIC inside the mitochondria, we opened the outer

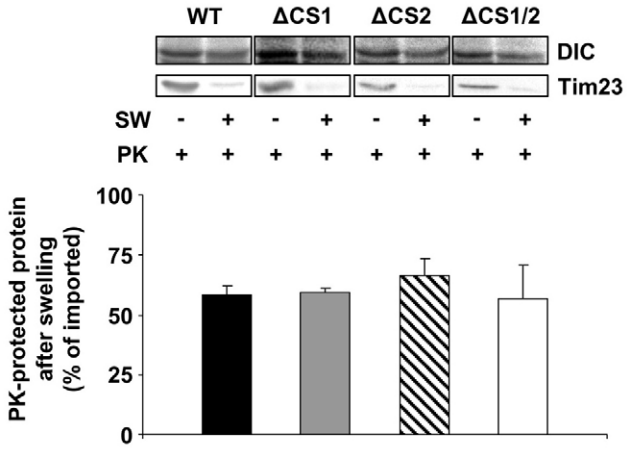

Fig. 3. Accessibility of DIC to proteases after import into mitochondria. ^{35}S-labelled wild-type DIC and the mutant versions ΔCS1, ΔCS2 and ΔCS1/2 were synthesized in reticulocyte lysate and incubated with isolated yeast mitochondria for 5 minutes at 25°C. The mitochondria were then cooled to 0°C and re-isolated by centrifugation. The mitochondria were then resuspended either in 1 mM EDTA, 10 mM MOPS/KOH, pH 7.2 to allow swelling (+SW) of the mitochondria and rupture of the outer membrane, or in 250 mM sucrose, 1 mM EDTA, 10 mM MOPS/KOH, pH 7.2 to keep the mitochondria intact (–SW). All samples were treated with 250 μg/ml proteinase K for 20 minutes at 0°C. Mitochondria and mitoplasts were re-isolated, the proteins were separated by SDS-PAGE, blotted on nitrocellulose and the radiolabelled proteins were visualized using a PhosphorImager (upper panel). For quantification of each construct, the amount of ^{35}S-labelled protein in the –SW sample was set to 100%. The nitrocellulose was eventually immuno-decorated with polyclonal antibodies directed against the hydrophilic N-terminus of the inner-membrane protein Tim23. PK, proteinase K. Bars represent standard deviation.

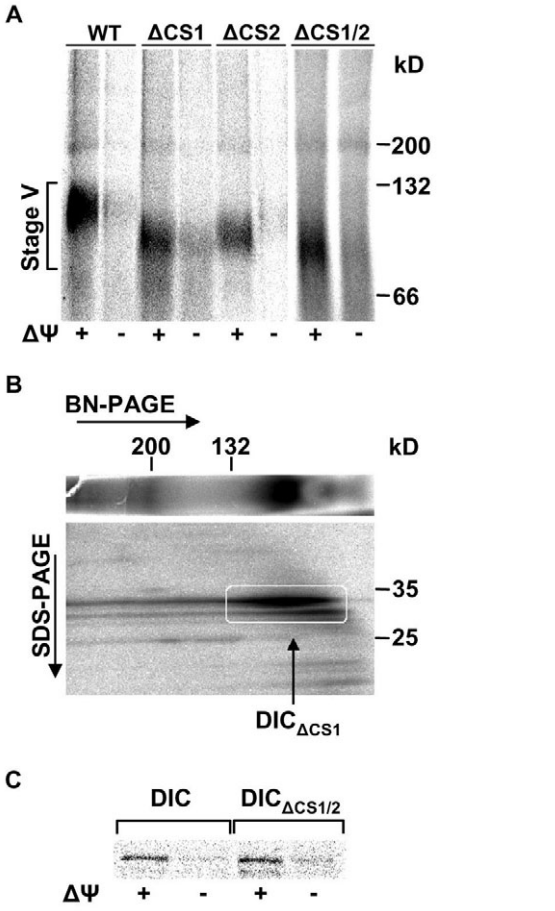

Fig. 4. Maturation of DIC in the mitochondrial inner membrane. (A) Analysis by BN-PAGE. ^{35}S-labelled DIC and the derivatives ΔCS1, ΔCS2 and ΔCS1/2 were imported into isolated yeast mitochondria for 20 minutes at 25°C, the mitochondria were treated with proteinase K, re-isolated, dissolved in the presence of 1% digitonin, and separated by BN-PAGE. The radiolabelled proteins were visualized using a PhosphorImager. Some samples contained valinomycin to dissipate the mitochondrial membrane potential (–ΔΨ). As indicated, mature carrier proteins (import stage V) were usually detected by this method at a range corresponding to 80-120 kDa. (B) 2D analysis of $DIC_{\Delta CS1}$. A stripe from a BN-PAGE as shown in A was excised and layered on top of an SDS-PAGE for separation of the proteins in a second dimension. The radiolabelled proteins were subsequently visualized using a PhosphorImager. Upper panel, stripe from a $DIC_{\Delta CS1}$ BN-PAGE sample run in parallel; lower panel, SDS-PAGE. (C) Affinity of DIC for hydroxyapatite. Radiolabelled DIC and $DIC_{\Delta CS1/2}$ were imported into mitochondria for 15 minutes at 25°C. As indicated, parallel samples contained valinomycin to dissipate the membrane potential (–ΔΨ). The mitochondria were then treated with proteinase K, re-isolated and solubilized in the presence of 2.5% Triton X-100. Following a clarifying spin for 5 minutes at 16,000 ***g***, the supernatants were passed through small columns containing hydroxyapatite. The proteins of the eluates were collected by precipitation with trichloro-acetic acid and analyzed by SDS-PAGE and fluorography.

membrane by osmotic swelling of the mitochondria and tested accessibility for externally added proteinase K (Fig. 3). Rupture of the outer membrane was confirmed by the complete degradation of the soluble domain of the inner-membrane protein Tim23. We found that, after import for 5 minutes at 25°C, the authentic DIC as well as the constructs ΔCS1, ΔCS2 and ΔCS1/2 were partially protected against the protease, but a considerable fraction of each was quickly degraded. Testing the constructs in parallel under identical conditions, we found that the ratio of protease-protected versus protease-accessible protein was similar for all four proteins (Fig. 3, lower panel). In some experiments, we observed a slightly reduced protease-protection of the ΔCS1/2 protein; however, the effect was much less pronounced than the delay in import as shown in Fig. 1C. The integration of the DIC into the mitochondrial inner membrane seemed to be essentially independent of the carrier signature. In agreement with this conclusion, we found that the fraction of DIC that was resistant to extraction by carbonate at pH 11.5 was similar for wild-type DIC and for ΔCS1/2 DIC (data not shown).

Eventually, we tested whether the carrier signature was required for the maturation of DIC in the inner membrane. For this purpose we imported different radiolabelled DIC constructs into mitochondria, solubilized the imported proteins in the presence of 1% digitonin and separated the proteins by blue native electrophoresis (BN-PAGE; Fig. 4A). Similar to other mitochondrial carrier proteins (Ryan et al., 1999; Wiedemann et al., 2001; Zara et al., 2007), wild-type DIC showed an apparent molecular mass of approximately 90 kDa,

possibly representing homodimers. Formation of the complex was dependent on the mitochondrial membrane potential (Fig. 4A, lanes $+\Delta\Psi$ versus $-\Delta\Psi$). Similar protein complexes were also observed with the DIC derivatives lacking one or both carrier signatures. However, the derivatives showed a slightly higher mobility, probably resulting from a difference in the conformation. The identity of the radiolabelled proteins was confirmed by separating the polypeptides of a lane from the blue native gel by SDS-PAGE (as shown for $DIC_{\Delta CS1}$, Fig. 4B).

An independent assay for the maturation of carrier proteins is based on the different affinities of native and non-native carriers to hydroxyapatite (Klingenberg et al., 1995). We imported radiolabelled wild-type DIC and $DIC_{\Delta CS1/2}$ into mitochondria, solubilized the membranes in the presence of 2.5% Triton X-100, passed the lysates through small columns containing hydroxyapatite, and analyzed the radiolabelled proteins by SDS-PAGE and fluorography (Fig. 4C). In this assay, $DIC_{\Delta CS1/2}$ showed the same behaviour as wild-type DIC. Maturation of both proteins required the mitochondrial membrane potential. The result shows again that the carrier signatures 1 and 2 are not essential in the maturation of the DIC in the mitochondrial inner membrane.

The data shown in Figs 1-4 demonstrate a role of the carrier signature in the import of newly synthesized DIC into mitochondria. To investigate the possible function of the carrier signature in the transport activity of the mature protein in the inner membrane, we expressed wild-type DIC and three mutant DIC in *Escherichia coli*, isolated the proteins and reconstituted them into liposomes (Palmieri et al., 1995). We then determined the transport activity by testing for the uptake of externally added [^{33}P]-phosphate in exchange for non-labelled phosphate. The liposomes were incubated with the radiolabelled phosphate for 10 minutes at 20°C. The results are summarized in Fig. 5. Within 10 minutes, wild-type DIC mediated an uptake of about 1 μmole [^{33}P]-phosphate per mg protein. Relative to this value, we detected an activity of about 50% for DIC_{P33A}, 10% for $DIC_{\Delta CS1}$ and 0.8% for $DIC_{\Delta CS1/2}$. Hence, the mutant $DIC_{\Delta CS1/2}$ that we found to be imported at a reduced rate (Fig. 1C) and to mature in the inner membrane with good efficiency (Figs 3 and 4) was nearly devoid of any transport activity.

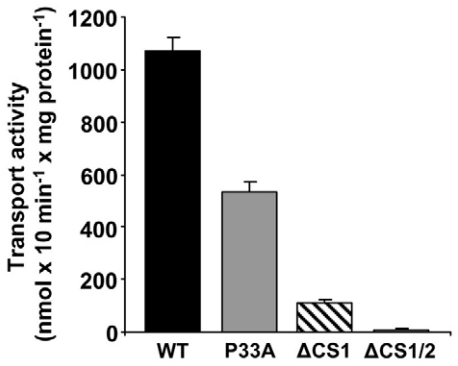

Fig. 5. Transport activity of DIC after reconstitution in liposomes. The sarkosyl-solubilized proteins were reconstituted into liposomes in the presence of 20 mM potassium phosphate, pH 7.2. The external substrate was removed from proteoliposomes on Sephadex G-75 columns. The transport was started by adding 0.1 mM [^{33}P]-phosphate and terminated after 10 minutes. Finally, the external substrate was removed on Sephadex G-75 columns and the accumulation of labelled substrate in the proteoliposomes was measured. The values reported in the figure represent the means ± s.d. (n=4). WT, wild type.

Discussion

Because the carrier signature Px[D/E]xx[K/R] is conserved in all carrier proteins, these residues seem to participate in important functions. Investigating the yeast dicarboxylate carrier, DIC, we found that the carrier signature is in fact essential in the function of the protein as a mediator of substrate transport across the mitochondrial inner membrane. An exchange of a single residue of the carrier signature with alanine (P33 in helix 1) reduced the transport activity of the purified and reconstituted protein by about 50%; an exchange of the complete P-D-K motif in helix 1 reduced the activity by about 90%. An exchange of all the residues of the carrier signature in the entire protein blocked the transport activity nearly completely. Similar observations were recently published with regard to the bovine oxoglutarate carrier (Cappello et al., 2007). Characterizing the consequences of several point mutations, it turned out that most of the residues that were crucial for function belonged to the carrier-signature motif. An essential function of the carrier signature in the transport of metabolites has already been proposed in the genetic study on the AAC that was carried out by Nelson et al. (Nelson et al., 1998). The combined data on the yeast oxoglutarate carrier (Cappello et al., 2007) and on the yeast dicarboxylate carrier (this study) suggest that the carrier signature is indeed an intrinsic element in the transport mechanism of carrier proteins.

It is remarkable that this conserved sequence motif is involved in interactions with substrates despite each member of the carrier family recognizing a different set of metabolites (Palmieri, 1994). Although it is obvious that the carrier signature cannot determine the specific selection of substrates, it is more likely that the carrier signature is a conserved part of the machinery that drives the translocation of the substrates. Data on the AAC indicate that the transport mechanism relies on kink and tilt modifications of the six transmembrane helices of the protein. These are accompanied by substantial rearrangements of the matrix-exposed loops of the protein (Nury et al., 2006). The scaffold of carrier proteins is similar in all members of the family, suggesting that they share a common transport mechanism (Nury et al., 2006). It is therefore tempting to speculate that the residues of the carrier signature participate in an essential switch between different states of the structure, probably due to their vicinity to the mobile loops at the matrix side of the protein.

The crystal structure of the bovine AAC shows not only the basket-like structure formed by the charged residues of the carrier signature but also the function of the proline residues within the odd-numbered helices in closing the inner cavity (Pebay-Peyroula et al., 2003). It was suggested that an exchange of these should have a significant effect on the overall structure in any carrier protein (Nury et al., 2006). In agreement with this prediction, we found a limited but clearly increased mobility of $DIC_{\Delta CS1}$, $DIC_{\Delta CS2}$ and $DIC_{\Delta CS1/2}$ relative to wild-type DIC in BN-PAGE. Relative to each other, the three mutant proteins showed only minor differences. In its native structure, wild-type DIC seemed to adopt a tight structure,

whereas the loss of a carrier signature causes a more relaxed conformation.

A role of the carrier signature in the catalytic mechanism does not exclude an additional role in the biogenesis of the proteins. This notion was suggested several years ago by studies on the function of the Tim9-Tim10 complex, a chaperone-like protein complex in the mitochondrial intermembrane space (Sirrenberg et al., 1998; Bauer et al., 2000). The authors showed that carrier proteins in transit across the mitochondrial outer membrane bind directly to this complex, and it was proposed that charged residues within the complex play a role in the specificity of substrate recognition. However, subsequent studies revealed that Tim9 and Tim10 can accept substrates that do not contain a carrier signature (Davis et al., 2000; Vasiljev et al., 2004), and two detailed investigations on the basis of a peptide-scanning approach showed that the affinity of carrier proteins to the complex of the small Tim proteins is mainly dependent on the hydrophobic segments of the transmembrane helices (Curran et al., 2002; Vasiljev et al., 2004). Our data on the biogenesis of the DIC confirm that a carrier protein that does not contain any residue of the carrier signature can still be imported and even assemble in the inner membrane. The carrier signature is obviously not essential in the biogenesis of the protein.

However, there is evidence that the carrier signature might at least support the interactions of carrier proteins with the small Tim proteins during translocation in the intact mitochondria: (1) the complete loss of the carrier signature in the DIC caused a reduction of the import efficiency by about 75%; (2) we found that this effect is independent from receptor sites at the mitochondrial outer surface; and (3) it is known from previous studies that the small Tim proteins drive the translocation of carrier proteins across the outer membrane space (Sirrenberg et al., 1998; Koehler et al., 1998; Leuenberger et al., 1999; Endres et al., 1999; Luciano et al., 2001; Truscott et al., 2002). In summary, the affinity of carrier proteins to the small Tim proteins appears to depend on hydrophobic interactions, but the kinetics of transport seem to depend on an intact carrier signature.

The purified Tim9-Tim10 complex shows a general chaperone activity for different substrates (Vial et al., 2002). However, the carrier proteins that are transferred from the Tom complex to the Tim9-Tim10 complex adopt a specific conformation (Endres et al., 1999; Wiedemann et al., 2001; Vasiljev et al., 2004). Interestingly, the pattern of loops and helices that is formed by the mature carrier proteins in the inner membrane seems to have already been preformed in binding to the Tim9-Tim10 complex (Endres et al., 1999). The transfer from the Tom complex to the Tim9-Tim10 complex is directly related to the process of translocation across the outer membrane, i.e. to the step in the biogenesis of the DIC, that shows a clear dependence on the intact carrier signatures. The carrier signatures seem to facilitate the structural rearrangements that are required in this context. It is remarkable that just the loss of a single residue of the carrier signature, P33 in helix 1 of DIC, is sufficient to cause a significant reduction in the rate of translocation (Fig. 1C). In contrast to matrix-targeted precursor proteins, which completely unfold to pass the translocation machinery (Rassow et al., 1990; Huang et al., 1999; Neupert and Herrman, 2007), the mechanism that drives the import of carrier proteins seems to be adjusted to specific protein conformations (de Marcos-Lousa et al., 2006).

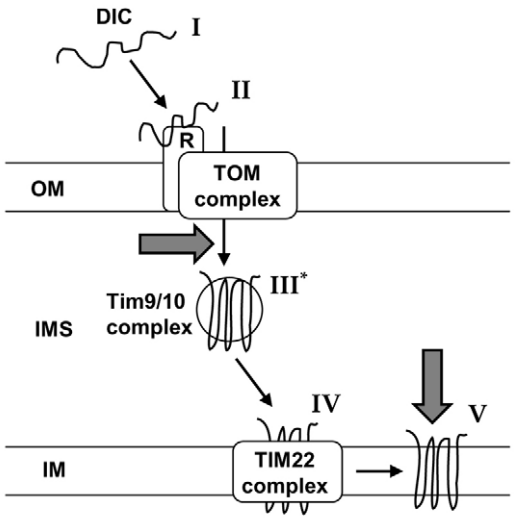

Fig. 6. Stages of DIC import into mitochondria. (I) Newly synthesized DIC is bound to chaperone proteins in the cytosol; (II) DIC then becomes bound to the import receptor Tom70; (III*) DIC is bound the Tim9-Tim10 complex in the intermembrane space [as defined by Zara et al. (Zara et al., 2001)]; (IV) insertion into the inner-membrane Tim22 complex; V, mature DIC. The carrier signature facilitates the transition from stage II to stage III* and is essential in the transport activity of the mature protein (large arrows). IM, inner membrane; IMS, intermembrane space; OM, outer membrane; R, receptor.

We cannot exclude some additional role of the carrier signature in the insertion of the DIC into the mitochondrial inner membrane. However, this effect would be limited to a kinetic effect that is not resolved within the time-frame of our experiments. Whereas the carboxy-terminal third of the DIC is crucial for productive association with the inner membrane TIM complex (Brandner et al., 2005), the carrier signature is not essential.

The biogenesis of carrier proteins is traditionally discussed in five stages (Pfanner and Neupert, 1987; Rehling et al., 2004). In this study, we followed the DIC in these stages from synthesis to its function in the inner membrane (Fig. 6). We studied the possibility of a role of the carrier-signature motif in keeping the newly synthesized protein in a soluble state (stage I), in interacting with the import receptor Tom70 (stage II), in the translocation into the intermembrane space [stage III* (Zara et al., 2001)], in insertion (stage IV) and in maturation in the inner membrane (stage V). We found only two steps that were significantly affected by the carrier signature: the carrier signature substantially facilitated the translocation across the outer membrane, and it was essential in the activity of the mature protein. It will now be interesting to determine the role of the carrier signature in these two steps in more detail.

Materials and Methods

Preproteins were synthesized in rabbit reticulocyte lysate (TNT coupled reticulocyte lysate system, Promega) in the presence of ^{35}S-methionine. For synthesis of the yeast DIC, a DNA fragment encoding the protein (GenBank acc. no. U79459) was cloned into the vector pGEM4. The construct Su9-DHFR was described previously

(Zara et al., 2005). Mitochondria were isolated from yeast, used for import of radiolabelled proteins and analyzed by BN-PAGE and SDS-PAGE following standard procedures as published previously (Zara et al., 2005; Zara et al., 2007; Papatheodorou et al., 2007). For the import experiments involving *Δtom70* mitochondria, mutant and wild-type mitochondria were isolated in parallel, using genetically equivalent strains (Moczko et al., 1994) and identical growth conditions. Import experiments that only required a wild-type strain were carried out using mitochondria of the strain YPH499 (Sikorski and Hieter, 1989). Some samples ($-\Delta\psi$) received 1 μM valinomycin to dissipate the membrane potential. To open the mitochondrial outer membrane, isolated mitochondria (30 μg of protein) were suspended in 500 μl 1 mM EDTA, 10 mM MOPS/KOH, pH 7.2 and incubated at 0°C for 20 minutes. Proteinase K (PK) was used at a concentration of 250 μg/ml for 10 minutes at 0°C. To detect Tim23, we used a polyclonal rabbit antiserum directed against the hydrophilic N-terminus of the protein. The DIC constructs were expressed in *E. coli* as described previously (Palmieri et al., 1996). For this purpose, the corresponding DNA fragments were cloned into the pET28a vector and transformed into the strain C41 (DE3). For reconstitution of the purified DIC in liposomes, we followed the protocol of Palmieri et al. (Palmieri et al., 1995). The protein content of the liposomes was determined as published by Capobianco et al. (Capobianco et al., 1996).

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