Positively charged residues, the helical conformation and the structural flexibility of the leader sequence of pALDH are important for recognition by hTom20

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Abstract Tom20, a mitochondrial outer membrane receptor necessary for protein translocation, was found to interact specifically with mitochondrial preproteins. The interaction of proteins containing an N-terminal matrix targeting signal was enhanced in an hydrophobic environment and the dependence of this interaction on the alpha helical conformation of the presequence was postulated. In order to test this hypothesis and to gain insights about the features of a matrix targeting signal necessary to be recognized by the receptor machinery including Tom20, the interaction of pALDH and signal sequence mutants to Tom20 in the absence and presence of a hydrophobic environment was investigated. Here we present evidence to show that in a hydrophobic environment the interaction between Tom20 and the leader sequence is strongly dependent on the positive charges within the signal sequence as well as on the flexibility of this signal.

Key words: Mitochondrial outer membrane; Protein translocation Tom20; pALDH

1. Introduction

Preprotein translocation into organelles is a highly regulated process [1,2]. One mechanism of regulation and sorting involves the specific interaction of the presequence with receptor proteins located for example on the mitochondrial surface [3,4]. In general, the receptor complex of yeast or *N. crassa* contains four proteins involved in early recognition. These are Tom70, Tom37, Tom22 and Tom20. In mammals only a homologue of Tom20, a 34 kDa protein (Tom34) and Metaxin were identified as receptor proteins [5]; other proteins linked to the mitochondrial import machinery are still under investigation [3,6].

The targeting signal for import into the matrix of the mitochondria was defined as a positively charged amphiphilic region either at the N- or C-terminus of the protein that can adobe a helical conformation in a hydrophobic environment [6]. However, it remained unclear which features of the leader sequence are important for the interaction between receptor and precursor. Furthermore the influence of the mature part on the interaction of the preproteins with the receptor complex remains to be investigated. The investigation of N-terminal targeting signals had revealed that preproteins are recognized by Tom20 through an electrostatic interaction [7–10]. However, the conformational change of the signal in a hydrophobic environment [11,12] strengthens this interaction and therefore a lipid contact prior to binding could be proposed [13]. Previous publications have demonstrated that the deletion of the linker region of a typical N-terminal targeting signal influences its ability to bind to the receptor complex and to be imported [14,15]. Now we were interested in determining if the specific position of basic amino acids or the overall electrostatic character within the presequence is important for recognition of the presequence by hTom20. Here we have compared the interaction of the presequence of pALDH and mutants of it to hTom20 in solution and on the lipid surface. We can demonstrate that the mature part of pALDH contributes to the interaction between Tom20 and the preprotein, however, the major contact is through the presequence as can be shown by competitive binding with an other presequence. For the interaction between the signal region and Tom20 the positive charged amino acids of the signal are important as well as is the conformational change that occurs in a hydrophobic environment.

2. Materials and methods

2.1. Material

Lipids from Avanti, Triton X-100 from Sigma and Glutathione Sepharose 4B from Pharmacia were used. All standard procedures were performed as described in previous articles [15,16]. All data were analyzed using Sigma Plot (Jandel).

2.2. Constructs

All pALDH constructs (see Fig. 1) were previously generated [14,15]. Generation of GST-Δ30hTom20 is described in [8] and of GST-CΔ30hTom20C100S in [17].

2.3. Binding assay

Binding of pALDH to hTom20 was performed as described [16] and binding to LUVs was described in [18]. In brief, the lipid composition (PC:PE:PI:PS 55:28:13:3-Tom20) or (PC:PE:PS:PI:PE-bmps 54.5:27.5:13:3:1+Tom20) was used [19] to prepare 100 nm LUVs [20] in Buffer A (170 mM sucrose, 20 mM Tris pH 7.0 and 2 mM $CaCl_2$). LUVs were incubated for 12 h with CΔ30hTom20C100S (in a ratio of Tom20:PE-bmps 10:1; coupling efficiency, 85–95%) followed by two 30 min incubations in medium lacking sucrose (using a ratio of loaded: not loaded vesicle of 1:10) in Buffer B (100 mM NaCl, 20 mM Tris and 2 mM $CaCl_2$) for 30 min in order to remove unbound Tom20. Liposomes were separated by 60 min centrifugation at $50\,000 \times g$ and then resuspended. Coupling efficiency was tested by Western blotting.

Lipid at a concentration of 0.02 mM was incubated with preprotein for 10 min at 23°C, diluted 20 times. The free preprotein was removed by centrifugation at $100\,000 \times g$ for 10 min. Bound preprotein was quantified as described for the binding assay [16].

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3. Results and discussion

3.1. The leader peptide of pALDH interacts with Tom20 like a matrix targeting signal

In order to analyze how Tom20 recognizes pALDH the interaction of the protein itself and the leader sequence fused to dihydrofolate reductase (pA-DHFR) with Tom20 were separately investigated. As shown in Fig. 2, Tom20 recognized both proteins (lane 3) in a salt dependent (lane 4) manner. In the absence of detergent it was necessary to add 750 mM NaCl to totally disrupt the interaction between Tom20 and pALDH and 400 mM NaCl for pA-DHFR (data not shown). The interaction could not be disturbed by the presence of detergent (lane 5), as typically found in the interaction between Tom20 and other N-terminal targeting signals [8]. In contrast, the interaction between Tom20 and pALDH increased in the presence of detergent, but not as much as was the interaction between Tom20 and pA-DHFR. This finding suggests the effect of the detergent on the signal region, similar as found for the interaction between Tom20 and the matrix targeting signal containing preprotein pODHFR [8]. Interestingly, in the presence of detergent the binding of both proteins was disrupted in the presence of just 300 mM NaCl (not shown). Furthermore, a point-mutation in the 'Glutamine face' [8] did not alter the binding affinity (data not shown), but the deletion of the first 30 amino acids of the cytosolic domain of Tom20 did (data not shown). The interaction between Tom20 and pALDH and pA-DHFR can be disrupted by the presence of the matrix targeting signal of preornithyl carbamyl transferase (pOCT) (lane 6) suggesting similar binding sites on Tom20 for both proteins. Further, the interaction appears to be to the signal sequence and a disruption of this interaction only results in a low affinity interaction between mALDH and Tom20 (not shown). We propose, based upon the data obtained that a direct electrostatic interaction of the signal of pALDH to Tom20 occurs that depends strongly on the conformation of the leader sequence in the hydrophobic environment. This binding system can further be used to investigate which properties of the precursor present in pALDH are necessary for the recognition by hTom20 since the result suggest no influence of the mALDH domain on interaction. This was also found during in vivo import [21].

3.2. Recognition of pALDH by Tom20 is dependent on the net charge and the conformation

It has been shown that the in vitro import of pALDH requires only one positively charged amino acid within the first part of the leader sequence and is relatively independent of the charges in the second domain of the leader sequence [14]. Our goal here was to identify which properties of the signal were important for import and which for recognition by the receptor complex, especially of Tom20. As seen in Table 1 the increase of secondary structure results in an increase of import suggesting that the helical conformation is important for insertion into the pore. This effect can only be overcome by deletion of all positive charges in the N-terminus of the leader sequence (R3Q/R10Q). Deletions of the charges in the C-terminal part of the signal or introduction of negative charges into the N-terminal part did not decrease import.

In order to test if the charge dependency for import was the same for binding the interaction of all mutants with GST-Δ30hTom20 was investigated (Table 1). In contrast to the results found for import of those proteins, the interaction of the preprotein in the absence of detergent was strongly dependent on the presence of the positive charges in both, N- and C-terminal region of the presequence (compare binding of wild-type (wt) to binding of R3Q, S7E/R14E, R3Q/R10Q and R3Q/R10Q/ΔRGP). Insertion of negative charges had only a minor effect on the binding of the precursor (wt vs. S7E) as was found during the import of these preproteins into isolated mitochondria [14].

In the presence of detergent, where a change of the conformation of the leader sequence can be expected to occur, as it did in the pOCT signal [10], or in a lipid environment [15], binding of wt-pALDH to hTom20 was increased two-fold compared to binding in the absence of detergent (Fig. 2 and Table 1). Furthermore, the binding was now not only dependent on the presence of positive charges, but the interaction also was disrupted by the presence of negative charges within the presequence (compare wt vs. R3Q and S7E in Fig. 1B and Table 1). This might be due to tertiary-structure stabilization of the presequence as a result of salt bridge formation between the negative charge and a positive charge of the second helix of the signal sequence as was proposed [14]. The decrease in the interaction of Tom20 with the preproteins containing the linker deletion might be explained either as a result of the

Table 1
Structural properties and binding activity of pALDH and mutants

1. Mutation	2. Mutation	3. Mutation	Import	Structure pH5.2	Binding T20−Tx	Binding T20+Tx	Binding Lipid	Binding Lipid+T20
–	–	–	100%	21%	100%	100%(×2)	100%	100%(×4)
R3Q	–	–	116%	n.d.	66%	47%	123%	55%
S7E	–	–	84%	42%	87%	25%	75%	43%
ΔRGP	–	–	141%	55%	55%	38%	113%	56%
S7E	R11E	–	23%	n.d.	52%	18%	60%	29%
S7E	R14E	–	254%	52%	38%	12%	71%	15%
R3Q	R10Q	–	6%	33%	30%	14%	85%	40%
R3Q	R10Q	ΔRGP	58%	n.d.	15%	6%	93%	36%

The import efficiency into mitochondria of pALDH and mutants is presented in column 4 (taken from [14]). The structure of the targeting signal was determined using CD-spectroscopy and the α-helical content in lipid-like environment (40% TFE) is given (column 5, taken from [14] and [15]). The interaction of the pALDH mutants with GST-Δ30hTom20 (T20) in the presence (+Tx) or absence (−Tx) of 0.05% Triton X-100 was determined and compared with wt-pALDH (column 6 and 7 in % wt binding). The value in brackets shows the increase of wt-pALDH binding to hTom20 in the presence of Triton compared to the binding in the absence of Triton. Column 8 and 9 gives the values for pALDH binding to liposomes comprised by the lipid composition of the outer mitochondrial membrane [19] in the absence or presence (+T20) of coupled Tom20. The number in brackets (column 9, row wt-pALDH) indicates the increase of binding of wt-pALDH to liposomes containing hTom20 compared to liposomes without Tom20. (n.d., not determined).

deletion of one positive charge since the decrease of interaction is as strong as found for R3Q, or as a result of the loss of flexibility, since deletion of the linker region results in a continuous helix and therefore forms a less flexible tertiary structure.

3.3. Binding and recognition of pALDH on a lipid surface

In order to confirm the results found with the GST-fusion protein binding assay in the presence of detergent and to determine how the affinity of the signal sequence for the lipid surface influences the binding ability of this sequence to Tom20, the binding of the preproteins to protein free liposomes and liposomes containing Tom20 was investigated (Table 1). To study the interaction of the signal sequence of pALDH with Tom20, this receptor was crosslinked to the liposome surface as previously described [18]. The lipids used for preparation of the liposomes represented the composition of the outer membrane of rat mitochondria [18,19].

The introduction of negative charges in the leader sequence resulted in a loss of binding to the negatively charged surface whereas deletion of positive charges did not produce such an effect. This finding leads us to suggest that a hydrophobic interaction rather than electrostatic interaction between pALDH and lipid surface occurs. Consistent with that is the finding that the increase of the α-helical content results in a stabilization of the binding with the surface. This could be a result of the hydrophobic amino acids being located on one side in a helical conformation increasing the hydrophobic interaction. The drastic increase of binding by the R3Q-mutation over that of the native precursor might be due to an insertion of the signal into the lipid bilayer by decreasing the interaction with the negatively charged headgroups or the increase of the polarity of the leader sequence. This finding suggests that the dependency of import on the hydrophobicity of the N-terminus of the signal [22] (Table 1) is not due to the interaction with the receptor components since the interaction of this mutant with the lipid surface containing Tom20 is not as strong as the interaction of the wt pALDH with this liposomes). Possibly it is due to the interaction with the first binding site of the trans side of the outer membrane composed by Tom40 [23].

The presence of Tom20 increased the binding of native pALDH to liposomes four-fold. Interestingly, the interaction of R3Q, S7E/R11E, ΔRGP, R3Q/R10Q and R3Q/R10Q/ΔRGP to the lipid surface was only slightly increased by the presence of Tom20 indicating that the affinity of those constructs to the lipid surface is higher or in the same range as the affinity to hTom20. In the case of the S7E/R14E-mutant the binding to the lipid surface was decreased, suggesting that the affinity for the lipid surface is higher then the affinity to hTom20. Therefore, it becomes clear that the decrease of the charges does not affect the binding to the lipid surface, whereas introduction of negatively charges does. This is consistent with the idea that the leader sequence undergoes a structural change on the lipid surface and the negatively charged amino acids are located on the site not facing the membrane surface. The latter is consistent with a previous report showing from NMR exchange data that the hydrophobic residues are more buried than are the hydrophilic ones on the leader [24]. Therefore, the charges are then in conflict with the recognition site of Tom20, since Tom20 contains negatively charged residues in its binding site in order to interact with the positively charged signal peptide. In addition, this might reflect the results found in vivo, where only the S7E mutant (from the mutants tested in here) was imported with high efficiency compared to wt pALDH [21]. Interestingly, deletion of positively charges or the linker region does not have such a strong effect on the interaction with Tom20 coupled to the

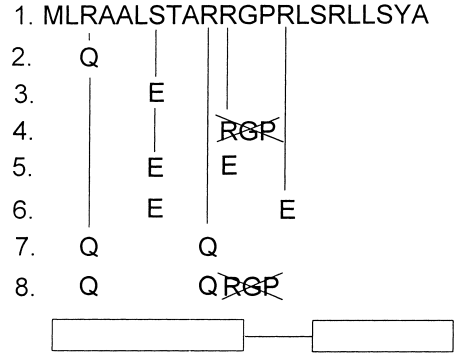

Fig. 1. Mutants of pALDH. Mutants of the leader sequence of pALDH used in the binding studies.

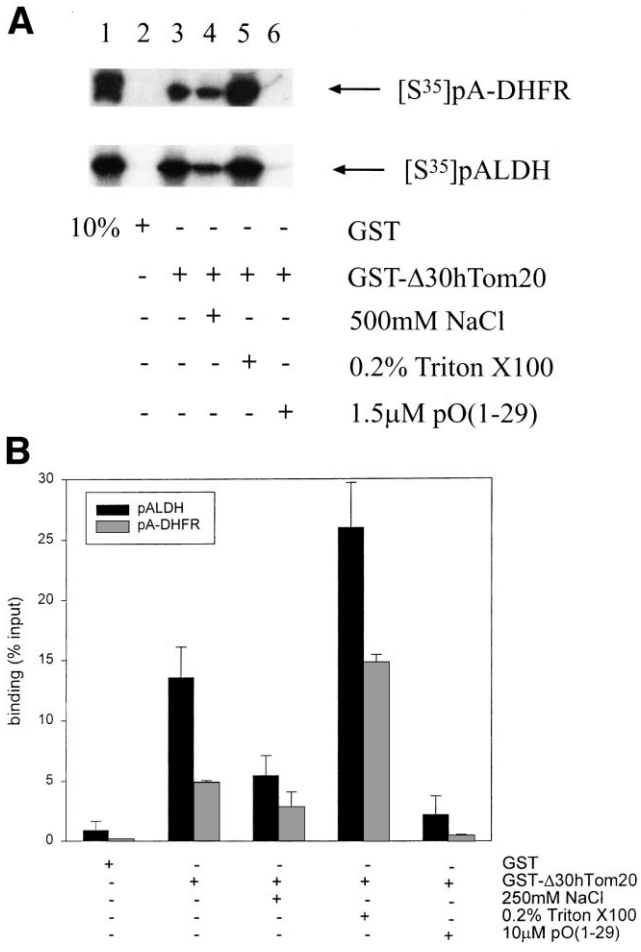

Fig. 2. pALDH binds to hTom20 like a matrix targeting signal containing protein. [S35]pALDH was incubated with GST (lane 2), GST-Δ30hTom20 (lane 3), GST-Δ30hTom20 Q100A (lane 4), GST-Δ60hTom20 (lane 5) and GST-Δ30hTom20 in the presence of the 10 μM matrix targeting signal peptide pO(1–27) (lane 6). In (A) a representative experiment is shown and in (B) the average of six independent experiments is presented as histogram.

liposomes as seen for the binding with Tom20 in detergent. That might be a result of the ability of these preproteins to interact very efficiently with the membrane surface again suggesting a hydrophobic interaction between preprotein and membrane surface. The recognition of preproteins by Tom20 in detergent as well as on the membrane surface requires the positive charges, can be disrupted by introduction of negative charges into the leader sequence and is also dependent on the flexibility and the helical conformation of the signal.

3.4. Conclusions

In this study we have shown that the signal sequence of pALDH interacts with Tom20 in a manner as did pOCT and it occupies the same binding site on Tom20. The results strengthen the notion that the interaction is an electrostatic and requires the presence of the positively charged amino acids. The leader, though, can tolerate negativity to a certain extent in the presence of charged residues in the sequence. The second important feature of the interaction between receptor and signal is the structural reformation of the signal in hydrophobic environment. Our data, however, suggest that for recognition by Tom20 the flexibility of the tertiary structure of the signal might play an important role. This was not found when these same modified precursors were studied in an import system.

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