Influence of the Mature Portion of a Precursor Protein on the Mitochondrial Signal Sequence*

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Most mitochondrial proteins are synthesized with an N-terminal signal sequence that targets these proteins to various compartments within the mitochondria. Signal sequences have been shown to be functional by fusing them to a nonmitochondrial passenger protein and observing import. In many cases, a signal sequence has been fused to passenger proteins, such as dihydrofolate reductase, and import occurred. There are, though, several unexplained instances in which a signal sequence was attached to a passenger protein and import was not observed. In this study, the N-terminal 23 residues of the matrix enzyme rhodanese could import several passenger proteins but were unable to import the mature form of mitochondrial aldehyde dehydrogenase (mALDH). However, if these same 23 residues were fused to the middle portion of mALDH, import was recovered, suggesting that the rhodanese signal sequence and N terminus of mALDH were incompatible for import. Circular dichroism data indicated that a peptide corresponding to the region of fusion between rhodanese and mALDH had less structure than corresponding peptides from imported fusion proteins, suggesting that mALDH may alter the helix in the rhodanese signal sequence, thus preventing import.

The majority of mitochondrial proteins are encoded in the nucleus and translated in the cytoplasm with an N-terminal signal sequence necessary for mitochondrial import (1, 2). It is believed that the signal sequence binds to various cytosolic factors that maintain the precursors in a loosely folded conformation necessary for translocation through the mitochondrial outer and inner membranes (3–5). Following import into the mitochondrial processing peptidase (6, 7), the signal sequence is removed, suggesting that the rhodanese signal sequence and N terminus of mALDH were incompatible for import. Circular dichroism data indicated that a peptide corresponding to the region of fusion between rhodanese and mALDH had less structure than corresponding peptides from imported fusion proteins, suggesting that mALDH may alter the helix in the rhodanese signal sequence, thus preventing import.

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*The abbreviations used are: DHFR, dihydrofolate reductase; ALDH, aldehyde dehydrogenase; mALDH, mature ALDH; PCR, polymerase chain reaction; AS, ALDH signal sequence; Rhod, rhodanese; TFE, trifluoroethanol.

EXPERIMENTAL PROCEDURES

Construction of Chimeric Proteins—The various constructs used in these studies are illustrated in Fig. 1. cDNA encoding rhodanese had been cloned into the plasmid pT7-7 as described previously (23). δ51-Rhod and δ27-Rhod were constructed by amplifying residues 52 to the stop codon of rhodanese or residues 28 to the stop codon of rhodanese with flanking 5′-NdeI and 3′-BamHI sites using the polymerase chain reaction (PCR) and rhodanese as a template. The oligonucleotides used for δ51-Rhod were 5′-TTTCATATGCGGTCCTGCGCTGCCTTCC-3′ and 5′-TTTGGATCCGCTAGCTGCCCTCCACTCTTC-3′. The oligonucleotides used for δ27-Rhod were 5′-TTTATATGACGCCCTCGCTCCT-3′ and 5′-TTTGGATCCGCTAGCTGCCCTCCACTCTTC-3′. The resulting PCR products were digested with NdeI and BamHI and cloned into pT7-7. 55 Rhod/mALDH was constructed by amplifying the entire pT7-7 vector and cDNA encoding the N-terminal
Circular Dichroism—Circular dichroism spectra were obtained on a Jasco J-600 spectropolarimeter (28). The samples were typically scanned from 350 to 290 nm at 25°C with a path length of 0.1 cm. The buffer was 50 mM potassium phosphate, pH 5.2. For spectra that included TFE, the buffer was mixed with TFE in a 4:1 proportion (v/v) to give a 20% TFE solution. Baseline spectra for each solvent were obtained prior to those for the peptide spectra. Peptide concentrations were determined from the UV absorbance of the tryptophan residue and for CD spectra, they were in a range of 20–25 μM. Helical content was estimated using a non-linear least squares fit of the experimental spectra to standard values which were derived from a data base of protein CD spectra (29).

**RESULTS**

The N Terminus of Rhodanese Contains Information Necessary for Import—Rhodanese is a mitochondrial matrix enzyme that possesses a noncleaved signal sequence (23, 32). The crystal structure of rhodanese revealed that residues 11–22 form an {alpha}-helix (33), and two-dimensional NMR showed that residues 4–21 in a peptide corresponding to the N-terminal 23 residues of rhodanese can form an {alpha}-helix in a micellar environment (28). To determine if these N-terminal residues were sufficient for import and that the targeting information did not lie in other regions of the protein, the N-terminal 51 and 27 residues of rhodanese were removed (referred to as Δ51-Rhod and Δ27-Rhod, respectively), and import of these truncated proteins was examined. As predicted, deletion of this N-terminal region from rhodanese abolished import in both cases (data not shown).

To determine if the N-terminal 23 residues of rhodanese fusion protein still did not occur. Mitochondria were isolated from rat liver and imported into isolated rat liver mitochondria (55 Rhod/mALDH, 26 Rhod/mALDH, and 23 Rhod/mALDH in Fig. 1). The remaining 350 residues of mALDH were used in Fig. 2). Surprisingly, 23 Rhod/mALDH and 26 Rhod/mALDH were imported, it was expected that these residues existed that the initial 22 residues of rhodanese were fused to the mature form of the mitochondrial rat liver ALDH (referred to as 55 Rhod/mALDH, 26 Rhod/mALDH, and 23 Rhod/mALDH in Fig. 1, respectively), or the 23 N-terminal residues of rhodanese were fused to DHFR (referred to as 23 Rhod/mALDH in Fig. 1). After incubation with isolated rat liver mitochondria, 55 Rhod/mALDH and 23 Rhod/mALDH were poorly imported. Full-length rat liver rhodanese has 297 amino acids, whereas ALDH has 500 amino acids. The possibility existed that the initial 23 or 26 residues of rhodanese were incapable of importing a protein the size of ALDH. Therefore, approximately 150 C-terminal residues were truncated 26 Rhod/mALDH (referred to as 26 Rhod/mALDH in Fig. 1). The remaining 350 residues of mALDH approximate the size of rhodanese. However, import of this truncated ALDH fusion protein still did not occur.

Since structural data from crystallography and two-dimensional NMR indicated an {alpha}-helix existed through the initial 22 residues of rhodanese, it was expected that these residues contained information necessary for import to occur (Fig. 2). Thus, it appeared that fusion of the N-terminal 23 residues of rhodanese to mALDH was not sufficient for import, whereas ALDH has 500 amino acids. The possibility existed that the initial 22 residues of rhodanese were incapable of importing a protein the size of ALDH. Therefore, approximately 150 C-terminal residues were truncated 26 Rhod/mALDH (referred to as 26 Rhod/mALDH in Fig. 1). After incubation with isolated rat liver mitochondria, 55 Rhod/mALDH and 23 Rhod/mALDH were poorly imported. Full-length rat liver rhodanese has 297 amino acids, whereas ALDH has 500 amino acids. The possibility existed that the initial 23 or 26 residues of rhodanese were incapable of importing a protein the size of ALDH. Therefore, approximately 150 C-terminal residues were truncated 26 Rhod/mALDH (referred to as 26 Rhod/mALDH in Fig. 1). The remaining 350 residues of mALDH approximate the size of rhodanese. However, import of this truncated ALDH fusion protein still did not occur.

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Since structural data from crystallography and two-dimensional NMR indicated an {alpha}-helix existed through the initial 22 residues of rhodanese and since Δ24–56-Rhod and 23 Rhod/ DHFR were imported, it was expected that these residues should be sufficient to import mALDH. In fact, it was possible to fuse as few as 17 N-terminal residues of rhodanese to DHFR and observe import (Fig. 2). Thus, it appeared that fusion of the N-terminal 23 residues of rhodanese to mALDH was not sufficient for import, whereas ALDH has 500 amino acids. The possibility existed that the initial 22 residues of rhodanese were incapable of importing a protein the size of ALDH. Therefore, approximately 150 C-terminal residues were truncated 26 Rhod/mALDH (referred to as 26 Rhod/mALDH in Fig. 1). The remaining 350 residues of mALDH approximate the size of rhodanese. However, import of this truncated ALDH fusion protein still did not occur.
rhodanese to mALDH made the signal sequence incompatible with import function.

To test whether the N terminus of mALDH adversely affected the rhodanese signal sequence in 23 Rhod/mALDH, this signal sequence was fused instead, for the convenience of creating a new restriction site, to residue 117 of mALDH (referred to as Δ24–56-Rhod).

Urea-denatured native pALDH was imported, while 23 Rhod/mALDH was not imported (data not shown). Therefore, as the average of four different experiments ± 1 S.D. from the mean value.

### Table I

| Protein          | Import |
|------------------|--------|
| Rhodanese        | 100 ± 28 |
| Δ24–56 Rhod      | 29 ± 15 |
| 55 Rhod/mALDH    | 36 ± 9 |
| 26 Rhod/mALDH    | <1     |
| 26 Rhod/350 ALDH | <1     |
| 23 Rhod/mALDH    | <1     |
| 23 Rhod/DHFR     | 54 ± 19 |
| 23 Rhod/117 ALDH | 15 ± 5 |
| 23 Rhod/9AS/mALDH| 58 ± 21 |
| 17 Rhod/DHFR     | 16 ± 6 |
| pALDH            | 51 ± 13 |

[FIG. 2. The import of the fusion proteins into rat liver mitochondria](#).

Each of the fusion proteins was translated in rabbit reticulocyte lysate (Tx) and incubated with isolated rat liver mitochondria for 30 min at 30°C. Half of the reaction was treated with proteinase K (+PK) and half of the reaction was left untreated (−PK). Imported protein is represented by the protein remaining after the mitochondria is treated with proteinase K, which destroys translated protein outside the mitochondria. Proteins were analyzed using SDS-polyacrylamide gel electrophoresis and phosphoimaging.

**Fig. 1.** Fusion proteins consisting of the rhodanese signal sequence attached to different passenger proteins. The N-terminal signal sequence and C-terminal passenger protein are represented by boxes. Light gray refers to rhodanese, black refers to mALDH, and dark gray refers to DHFR. Regions where amino acids are deleted are represented by heavy lines. The 9 C-terminal residues of the ALDH signal sequence of 23 Rhod/9AS/mALDH are represented by a dashed line. Various lengths (55, 26, 23 or 17 N-terminal residues) of the rhodanese signal sequence were fused to either mALDH or DHFR. Additionally, residues 24–56 of native rhodanese were deleted (referred to as Δ24–56-Rhod).

This, 8 M urea was added to 23 Rhod/mALDH and precursor ALDH after translation in rabbit reticulocyte lysate in an attempt to unfold these proteins. Subsequently, the urea-denatured protein was diluted into mitochondrial import reactions. Urea-denatured native pALDH was imported, while 23 Rhod/mALDH was still not imported (data not shown). Therefore, misfolding did not appear to be the cause for the lack of import of 23 Rhod/mALDH.

The Region of Fusion between Rhodanese and mALDH Posesses Less Structure than Other Regions of Fusion—Since 23 Rhod/DHFR, 23 Rhod/117 ALDH and Δ24–56-Rhod were imported, whereas 23 Rhod/mALDH was not imported, it seemed possible that mALDH affected the structure of the rhodanese signal sequence. To test for the influence of the first few residues of mALDH on the secondary structure of the signal sequence, CD was performed on three different 22-residue peptides. The peptides represented the following: Rhod-mALDH, spanning residues 12–33 from the fusion of rhodanese with mALDH; Rhod-DHFR, containing the region spanning residues 12–33 from the fusion of rhodanese with
Deletion of residues from the N-terminal region of mitochondrial aspartate aminotransferase eliminated the interaction of this protein with hsp 70, demonstrating the importance of the mature protein for interactions with anti-folding proteins (35).

The characteristics of amino acids within the mature region of a protein may affect the ability of a precursor to interact with import receptors and the membrane. Although positive charges are important in the signal sequence, positive charges in the mature region of mitochondrial proteins may also be important for import. Such charged residues may potentially interact with import receptors or negatively charged regions of the mitochondrial membranes. When a pairwise comparison of homologous cytosolic and mitochondrial proteins was performed, it was found that in 75% of the cases the pI of the mature form of the mitochondrial protein was higher than that of the corresponding cytosolic protein (36). It was argued that a more acidic mature protein required a more basic signal sequence (36).

The calculated pI, which approximates the value expected for the unfolded state of a protein, was determined for several proteins used in this study. It is interesting to note that the calculated pI of mALDH is 5.77, whereas the calculated pI of rhodanese is 7.82. The signal sequence of precursor pALDH has five positive charges, but the initial 23 residues of rhodanese only have three positive charges. The mature form of ALDH may require a more basic signal sequence for import to occur. If the mature protein folds too rapidly, import may be hampered. This appears to be a likely possibility since it was found that point mutations, which partially unfolded the passenger of a fusion protein consisting of the first 16 residues of yeast cytochrome oxidase subunit IV attached to DHFR, increased import efficiency. It was shown that an increased import rate correlated with destabilization of DHFR (34).

Additionally, the mature region of a precursor protein may play a role in binding to heat shock proteins that maintain the protein in a translocation-competent state. For example, both cytosolic and mitochondrial forms of aspartate aminotransferase exist. Since only the mitochondrial form interacted with hsp 70, which maintains proteins in their unfolded state, the cytosolic form folded much more rapidly than did the mitochondrial form in rabbit reticulocyte lysate. Deletion of residues from the N-terminus of the mature form of mitochondrial aspartate aminotransferase eliminated the interaction of this protein with hsp 70, demonstrating the importance of the mature protein for interactions with anti-folding proteins (35).

The hydrophobicity of a passenger protein may also influence import. For instance, if the signal sequence of the ATPase subunit 9 was fused to bI4 RNA maturase, import occurred.

**DISCUSSION**

It is well established that N-terminal regions of precursor proteins carry information essential for mitochondrial targeting since hybrid proteins, which each consist of a fusion of a signal sequence and passenger protein of 23 Rhod/mALDH and 23 Rhod/DHFR. The amino acid sequences of peptides corresponding to the region of fusion between rhodanese and mALDH or between rhodanese and DHFR were used for CD and are shown. Additionally, as a control, a peptide corresponding to residues 12-33 of rhodanese was also used for CD. Even though rhodanese is not processed, the region of the protein following the 23 amino acid N-terminal signal sequence is designated as the “mature” protein.

**Fig. 3.** Peptides corresponding to the region of fusion of the signal sequence and passenger proteins of 23 Rhod/mALDH and 23 Rhod/DHFR. The amino acid sequences of peptides corresponding to the region of fusion between rhodanese and mALDH or between rhodanese and DHFR were used for CD and are shown. Additionally, as a control, a peptide corresponding to residues 12-33 of rhodanese was also used for CD. Even though rhodanese is not processed, the region of the protein following the 23 amino acid N-terminal signal sequence is designated as the “mature” protein.

**Fig. 4.** Circular dichroism spectra of peptides. CD spectra with mean residue ellipticity (MRE) of peptides corresponding to the natural rhodanese sequence (Rhod-Rhod) and the rhodanese-DHFR (Rhod-mALDH) and rhodanese-DHFR (Rhod-DHFR) fusion sites are shown. These spectra show that, under identical conditions, the Rhod-ALDH peptide displayed a significantly lower tendency than did the other two peptides to form secondary structure. Spectra were obtained in solutions that contained 20% (v/v) TFE and 50 mmoles phosphate buffer, pH 5.2, at 25°C.

**Table 1.** Import of Fusion Proteins

| Protein                | MRE (deg cm²/mole) | Wavelength (nm) |
|------------------------|--------------------|-----------------|
| Rhod-DHFR              | -15000             | 190             |
| Rhod-AlDH              | -10000             | 190             |
| Rhod-Rhod              | -5000              | 190             |

**Table 2.** Import of Fusion Proteins

| Protein                | MRE (deg cm²/mole) | Wavelength (nm) |
|------------------------|--------------------|-----------------|
| Rhod-DHFR              | -15000             | 190             |
| Rhod-AlDH              | -10000             | 190             |
| Rhod-Rhod              | -5000              | 190             |

**Fig. 2.** Spectra of peptides. Spectra of peptides were obtained in aqueous buffer and in 20% (v/v) TFE. Spectra are shown for the mature forms of mitochondrial proteins and their corresponding cytosolic proteins. The spectra show that, under identical conditions, the Rhod-ALDH peptide displayed a significantly lower tendency than did the other two peptides to form secondary structure. Spectra were obtained in solutions that contained 20% (v/v) TFE and 50 mmoles phosphate buffer, pH 5.2, at 25°C.
However, if hydrophobic stretches of amino acids derived from apocytochrome c were included between the signal sequence and maturase, import was drastically reduced. This reduction in import correlated with the number of hydrophobic stretches included in the fusions (38).

We and others have demonstrated that the mature protein and signal peptide must be compatible for efficient import to occur. Fusion of the 23 and 26 N-terminal residues of rhodanese did not allow for import of mALDH. However, the same 23 residues could direct a variety of other passenger proteins to the mitochondria, including mALDH if the N terminus of mALDH was deleted or if residues of the ALDH signal sequence were included in the fusion. It appeared that the N terminus of mALDH and the rhodanese signal sequence were incompatible for import.

It is possible that if 23 Rhod/mALDH misfolded, the signal sequence would not be available for mitochondrial targeting. Alternatively, the mature protein may have altered the structure of the signal sequence. The latter situation seems likely in our case since CD data indicated that the area of fusion between rhodanese and DHFR or the comparable region in native rhodanese. A stable N-terminal helix is essential for import (17, 39), and the loss of a stable helix in the nonimported fusion proteins may have affected their import competency.

Several unexplained instances exist in which fusion of a signal sequence to a passenger protein did not result in mitochondrial import. It is possible that the structure of the signal sequence was adversely affected by the mature protein in these cases. It may prove interesting to determine the structure of the region of fusion between the signal sequence and passenger protein of these nonimported proteins to determine if the signal sequence is altered by the passenger protein.

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