The loss in hydrophobic surface area resulting from a Leu to Val mutation at the N-terminus of the aldehyde dehydrogenase presequence prevents import of the protein into mitochondria

PHILIP K. HAMMEN, THOMAS S. HEARD, MARY WALTNER, AND HENRY WEINER

Department of Biochemistry, Purdue University, West Lafayette, Indiana 47907-1153

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Abstract

An apparent conservative mutation, Leu to Val, at the second residue of the rat liver mitochondrial aldehyde dehydrogenase (ALDH) presequence resulted in a precursor protein that was not imported into mitochondria. Additional mutants were made to substitute various amino acids with nonpolar side chains for Leu2. The Ile, Phe, and Trp mutants were imported to an extent similar to that of the native precursor, but the Ala mutant was imported only about one-fourth as well. It was shown that the N-terminal methionine was removed from the L2V mutant in a reaction catalyzed by methionine aminopeptidase. The N-terminal methionine of native pALDH and the other mutant presequences was blocked, presumably by acetylation. Because of the difference in co-translational modification, the L2V mutant sustained a significant loss in the available hydrophobic surface of the presequence. Import competence was restored to the L2V mutant when it was translated using a system that did not remove Met1. The removal of an Arg-Gly-Pro helix linker segment (residues 11–14) from the L2V mutant, which shifted three leucine residues toward the N-terminus, also restored import competence. These results lead to the conclusion that a minimum amount of hydrophobic surface area near the N-termini of mitochondrial presequences is an essential property to determine their ability to be imported. As a result, both electrostatic and hydrophobic components must be considered when trying to understand the interactions between precursor proteins and proteins of the mitochondrial import apparatus.

Keywords: acid-chain hypothesis; amino-terminal processing; hydrophobic surface area; mitochondrial protein import; presequence

To be translocated into mitochondria, nuclear-encoded proteins must possess an N-terminal sequence that targets the protein to the organelle. Most matrix space proteins are synthesized as precursor proteins from which the targeting sequence is cleaved by a protease after import. The lack of sequence homology among mitochondrial presequences has been documented (von Heijne et al., 1989). Consequently, critical residues that could be studied to determine if they are responsible for presequence function cannot be identified *a priori*. Despite the great diversity in presequence primary structure, precursor proteins appear to interact with a series

of membrane-bound proteins at the mitochondrial surface (Lithgow et al., 1995). This raises the fundamental biochemical question of how such a diverse population of mitochondrial presequences can function in a specific process such as mitochondrial protein import.

Recently, an “acid-chain” hypothesis has been proposed to explain the translocation of proteins across the outer mitochondrial membrane (Schatz, 1997). The hypothesis states that translocation is driven by electrostatic interactions between basic presequences and acidic regions of TOM proteins. Precursor proteins could be transferred along a series of outer and inner membrane proteins that have progressively increasing affinities for basic ligands. Evidence in support of the “acid-chain” hypothesis has been published recently (Komiya et al., 1998). The results imply that basic residues in the presequences play a critical role in the binding to acidic surfaces of the receptor proteins. Recently, it has been shown that as many as 15 acidic residues from the cytosolic domain of TOM22 could be changed without seriously disabling import (Nargang et al., 1998). Therefore, a complete understanding of the

Reprint requests to: Dr. Henry Weiner, Department of Biochemistry, Purdue University, West Lafayette, Indiana 47907-1153; e-mail: Weiner@biochem.purdue.edu.

Abbreviations: TOM, mitochondrial outer membrane translocator protein; COX IV, yeast cytochrome oxidase subunit IV; GFP, green fluorescent protein; DHFR, dihydrofolate reductase; pALDH, precursor aldehyde dehydrogenase; pALDH(-RGP), precursor aldehyde dehydrogenase lacking the R-G-P segment from its presequence; OTC, ornithine transcarbamoylase; MDH, malate dehydrogenase; MAP, methionine aminopeptidase.

interactions between presequences and mitochondrial proteins continues to be elusive.

The basic nature of mitochondrial presequences is due to Arg residues that are three times more abundant in presequences than in proteins, in general (Hammen et al., 1996). Arginine residues have been shown to be essential for an import competent presequence in OTC (Horwich et al., 1986), MDH (Chu et al., 1987a), F₁-ATPase β (Bedwell et al., 1989), and ALDH precursors (Hammen et al., 1996). Subsequently, it was shown that acidic residues could be incorporated into the ALDH presequence without changing import competence (Heard & Weiner, 1998). Presequence conformation also appears to be important. It was concluded that the ability to form a stable helix near the N-terminus was necessary for a functional ALDH presequence (Wang & Weiner, 1993). These observations led to the hypothesis that basic residues alone do not account for the import-related interactions between presequences and mitochondrial proteins.

Mitochondrial presequences are naturally amphiphilic, requiring both hydrophobic and hydrophilic residues for proper import function. The artificial sequence, SynB2, which contained five Arg and only one Leu did not function as a presequence (Roise et al., 1988). Other studies have suggested the importance of hydrophobic properties, or residues, in presequences (Chu et al., 1987b; Bedwell et al., 1989). The OTC presequence contains four Leu residues among the first nine (Leu2, Leu5, Leu8, and Leu9). Two studies showed that the replacement of three of these residues by Ala resulted in a substantial reduction in the amount of protein imported (Kraus et al., 1988; Skerjanc et al., 1988). The similar observations were interpreted differently, one concluding that import competence was lost because helix stability was disrupted (Kraus et al., 1988) while the other concluded that hydrophobicity was lacking (Skerjanc et al., 1988).

In the process of studying the import of pALDH, we prepared the L2V mutant. This conservative mutation was designed to reduce the helix propensity of the N-terminal part of the presequence while having a minimal effect on the amphiphilicity of the peptide. Somewhat to our surprise, we found that the L2V mutation resulted in the complete loss of import. This dramatic effect seemed unusual in light of the conservative nature of the mutation. Here we report the results of the investigation into the loss of import competence of the L2V mutant.

Results

When Leu2 of the ALDH presequence was changed to Val, import of pALDH into mitochondria was no longer observed (Fig. 1). The ALDH presequence was then attached to DHFR and was imported into rat liver mitochondria. When the L2V presequence was attached to DHFR, this passenger protein also was not imported. The conservative L2V mutation, which apparently resulted in the small compositional change of one carbon atom one residue from the N-terminus, resulted in a complete loss of import competence. To confirm that the import behavior in the cell-free system was indicative of import *in vivo*, presequences fused to GFP were incubated with cultured HeLa cells as described elsewhere (Ni et al., 1999). When the ALDH presequence was fused to GFP, fluorescence was detected only in mitochondria. In contrast, when the L2V presequence was attached to GFP, the fluorescence was found in a diffuse pattern throughout the cytoplasm as well as in mitochondria. Therefore, import of the L2V mutant was impaired in comparison with the fusion protein containing native pALDH.

Fig. 1. Mitochondrial import of precursor proteins. Image of a 12.5% polyacrylamide SDS-PAGE gels comparing import between precursor proteins containing the native ALDH presequence and the L2V mutant. The precursor proteins that appear on the gel are as follows: Lanes 1–3, native pALDH; Lanes 4–6, pALDH(-RGP); Lanes 7–9, L2V; Lanes 10–12, L2V(-RGP). The lanes for each precursor are (from left to right): translated protein, translated protein incubated with isolated mitochondria, and translated protein incubated with isolated mitochondria and treated with proteinase K. For the L2V mutant, no protein fragment was observed following treatment with proteinase K (Lane 9), showing that import did not occur. However, in the case of L2V(-RGP), a protein fragment can be seen in Lane 12, demonstrating that removal of the R-G-P segment allows a presequence containing Val2 to import ALDH.

To determine the requirements for the second residue of the presequence, a series of mutant precursor proteins were investigated including Ala, Ile, Phe, and Trp mutants. Import of the L2I, L2F, and L2W precursors could be observed by visual detection as well as with the more sensitive phosphorimaging system (Fig. 2). For L2A, evidence of imported protein could be detected only by the phosphorimaging system on the polyacrylamide gels used to analyze import of this mutant. In the past, we and others have found that about 10% of the precursor protein used in an *in vitro* import assay can be found inside of mitochondria (Jarvis et al., 1995; Hammen et al., 1996). Quantitative analysis of polyacrylamide gels containing the L2V mutant showed no evidence that it was imported, even after exposure of the gel for extended periods of time. We have determined the lower limit of detection to be 0.2–0.5% of the total amount of precursor protein used in the assay. Therefore, the maximum import of the L2V mutant relative to that of pALDH (arbitrarily set to 100%) was 5% (Table 1). The L2A mutant was imported to an extent that was about 25% of the pALDH import. The quantitative analysis of L2I, L2F, and L2W revealed that, on average, these mutants were imported ca. 70–80% as well as native pALDH. Therefore, we concluded that import of the L2A mutant was impaired, but to a lesser extent than the L2V mutant. Uncertainties in the results (Table 1) did not allow us to conclude that import was slightly impaired for the L2I, L2F, and L2W mutants. It could only be stated that these mutants were imported about as well as the native precursor.

Fig. 2. Import of Leu2 mutants. Image of a 12.5% polyacrylamide SDS-PAGE gels comparing import between precursor proteins containing the native ALDH presequence and the L2V mutant. The precursor proteins that appear on the gel are as follows: Lanes 1–3, L2F; Lanes 4–6, L2W; Lanes 7–9, native pALDH; Lanes 10–12, L2A, and Lanes 13–15, L2I. As in Figure 1, the lanes for each precursor are (from left to right): translated protein, translated protein incubated with isolated mitochondria, and translated protein incubated with isolated mitochondria and treated with proteinase K.

Table 1. Mitochondrial import of L2X mutant presequences

Mutant	Presequence/passenger	% Import ^a	n
pALDH	MLRAALSTARRGPRLSRL- ALDH	100 ± 16	4
L2V	MVRAALSTARRGPRLSRL- ALDH	nd ^b	5
pALDH(-RGP)	MLRAALSTAR---RLSRL- ALDH	167 ± 32	3
L2V(-RGP)	MVRAALSTAR---RLSRL- ALDH	52 ± 19	4
pALDH-DHFR	MLRAALSTARRGPRLSRL- DHFR	30 ± 16	3
L2V-DHFR	MVRAALSTARRGPRLSRL- DHFR	nd ^b	3
L2A	MARAALSTARRGPRLSRL- ALDH	26 ± 19	3
L2I	MIRAALSTARRGPRLSRL- ALDH	78 ± 21	4
L2F	MFRAALSTARRGPRLSRL- ALDH	70 ± 15	5
L2W	MWRAALSTARRGPRLSRL- ALDH	79 ± 23	4

^aPercent import is reported relative to pALDH, which was arbitrarily given the value of 100%. The uncertainties reported are the standard error of measurement for the number of measurements (*n*) shown in Column 4.

^bNone detected. No evidence of import was observed on the gel. The maximum possible import was 2–5%, based on the background counts measured in the phosphor-imaging analysis. Therefore, less than ca. 5% could be imported without detection.

Residues adjacent to the N-terminal Met are known to influence the action of MAP and *N*^α-acetyltransferase, two eukaryote enzymes that perform co-translational modifications of polypeptides (Arfin & Bradshaw, 1988). Proteins translated in cell-free systems have been shown to undergo similar modifications (Boissel et al., 1988). To properly compare the L2X sequences, it was necessary to know the nature of their N-termini under the experimental conditions. The concentration of proteins translated in rabbit reticulocyte lysate is not sufficient to purify and analyze by normal N-terminal sequencing techniques. Instead, the proteins were translated with ³⁵S-Met and ³H-Leu as external sources of these two amino acids. The proteins were electro-transferred from SDS-PAGE gels onto PVDF membranes and subjected to N-terminal sequencing. Eluted fractions were collected for each sequencing cycle and counted in a liquid scintillation counter to determine whether or not radionuclides were present. If Met1 were retained after translation, N-terminal sequencing would give ³⁵S in the fraction collected from the first sequencing cycle and ³H in the sixth fraction. If Met1 were removed, ³H would be found in the fifth fraction. If, however, the N-termini were blocked, disrupting the sequencing reactions, no radioactivity would be found. Radioactivity observed in the first nine fractions is plotted in Figure 3A. Scintillation counting of the L2V sequinator fractions revealed a ³H level significantly above background only in the fifth fraction. This result indicated that the N-terminal Met was removed from the L2V mutant after translation without subsequent acetylation. Neither ³⁵S nor ³H were detected in the sequinator fractions from pALDH and the other L2X mutants. For these proteins, the initial counts loaded into the sequinator were the same order of magnitude as those loaded in the case of L2V. These results suggested that native pALDH and the other L2X mutants were blocked at the N-terminus. It is presumed that they were all blocked due to acetylation. As a positive control, rat rhodanese was translated and subjected to sequencing. The rhodanese sequence contains valine as the second residue along with the Met1, Leu6, and Leu10, which should be labeled. When sequencing was performed, ³⁵S was not detected in the first fraction, but ³H was found in the fifth and ninth fractions. Therefore, Met1 was removed from

Fig. 3. Radioactivity detected in the microsequencing of translated pALDH and L2X mutants. **A:** Plots of cpm vs. sequinator cycle number for pALDH [³⁵S (Δ) and ³H (▽)] and L2V [³⁵S (□) and ³H (○)]. The only meaningful peak is due to ³H in cycle 5 of the L2V data. The rise above the background level of ³⁵S in the L2V samples remains unexplained at the present time. **B:** Plots of cpm vs. sequinator cycle number for L2V translated with the S30 *E. coli* extract [³⁵S (■); ³H (●)]. High counts of ³⁵S in cycle 1 indicate the presence of Met1, which is confirmed by the ³H peak in cycle 6, due to Leu6.

rhodanese without the subsequent blocking of the N-terminus. This result confirmed that proteins containing Val as the second residue were modified in a way that was consistent with a previous report (Boissel et al., 1988).

It was not known whether Met1 was removed from proteins translated in the rabbit reticulocyte lysate system before or after the N-terminus was blocked. To distinguish between acetylated Met1 and acetylated X at the N-terminus of the proteins, ³⁵S/³H ratios were determined from liquid scintillation counting of the precursor proteins after electro-transfer to PVDF membranes. A ³⁵S/³H ratio greater than that measured for the L2V mutant would suggest that Met1 was still present and had been acetylated after translation. A ³⁵S/³H ratio equivalent to that measured for L2V would indicate that Met1 had been removed before acetylation. Native pALDH and the L2I, L2F, and L2W mutants were found to have a ³⁵S/³H ratio greater than L2V (Table 2). Acetylation of Met1 was expected for all but the L2A mutant (Yamada & Bradshaw, 1991), which should have had acetylated Ala at its N-terminus. The ³⁵S/³H values with L2A gave equivocal results. From these data it could not be determined if Met1 was removed.

To more thoroughly identify the N-terminal modifications as the reason that L2V was not imported, it was desirable to find condi-

Table 2. Mean values of the ratios ^{35}S (cpm)/ ^3H (cpm), observed in in vitro translated pALDH and its Leu2 mutants following transfer to PVDF membranes

Sequence	$^{35}\text{S}/^3\text{H}$	n^a	σ_{n-1}^b
ALDH	1.21	4	0.08
L2A	0.82	4	0.35
L2V	0.71	4	0.14
L2I	1.00	4	0.08
L2F	1.02	4	0.10
L2W	1.02	2	—

^aNumber of measurements^bStandard deviation of the sample.

tions under which L2V could be imported. Three ways were found to restore import competence to the L2V sequence. First, L2V was produced with an in vitro translation system that employed an *Escherichia coli* extract. Protein translated with this system was imported at a level near to native pALDH from the reticulocyte lysate system. When the protein was sequenced for detection of radioactivity, ^{35}S was found in the first fraction and ^3H was found in the sixth fraction (Fig. 3B). This pattern is consistent with an unblocked Met at the N-terminus. The second way that import was restored to L2V was by inhibition of MAP. Fumagillin, a commercially available MAP inhibitor (Sin et al., 1997), was added to translation reactions at 10 nM and 1 μM concentrations. At either concentration, import of L2V was observed. These results demonstrated that the presence of Met1 was important for import of L2V. Finally, import was restored following a deletion mutation in the presequence. We have shown previously that the deletion of the three residue linker from the ALDH presequence led to an approximately 50% increase in import efficiency in vitro (Hammen et al., 1996). When this linker was removed from the nonimported L2V presequence, import competence was partially restored (Table 1). The L2V(-RGP) precursor was imported with about one-half of the efficiency of pALDH, and only about one-third as well as previously reported for pALDH(-RGP). This result revealed that Val could be tolerated in the second position of the presequence under conditions in which Met1 was removed. Therefore, it was not Met1, but a property associated with Met1 that was necessary for import. The comparison to ALDH(-RGP) showed that while some import function was regained by deletion of the linker, the presence of Val2 alone was sufficient to impair import.

Discussion

Despite many years of work, the precise features required for functional mitochondrial presequences remain poorly understood. One of the common features among presequences is their amphiphilicity. To be amphiphilic a presequence must contain both hydrophobic and hydrophilic residues. The L2V mutation, which began this study, was intended to provide a minimal alteration of the hydrophobic character of the presequence. Although the side chains of Leu and Val are similar, the L2V mutation resulted in the complete loss of import competence. We have shown that residues other than Leu can be well tolerated at this position of the presequence. Consistent with previously published work with the reticulocyte lysate system (Boissel et al., 1988), we found that, upon

translation, Met1 was removed from L2V. In contrast, Met1 appeared to be retained and acetylated in other Leu2 mutants that were studied. The presumed conservative L2V mutation resulted in a more dramatic change to the N-terminus of the precursor protein than simply the loss of one carbon atom. The resulting N-terminal modification had the effect of significantly reducing the hydrophobic surface area of the presequence. The following discussion will contend that the amount of available hydrophobic surface area is a critical property of functional mitochondrial presequences.

The Leu2 mutants that were prepared comprise a series in which the hydrophobicity and side-chain size vary from Ala to Trp. The effect of the mutations on hydrophobic character was estimated from the results of molecular surface calculations. Peptide models were constructed taking into account the N-terminal modifications observed for each sequence and surface area calculations were carried out for fully extended structures. In this way, the total available surface area was assessed independent of presequence conformation. Despite the differences at the N-termini, hydrophilic surface areas did not change dramatically. The results of these calculations appear in Table 3 and are plotted vs. percent import in Figure 4, from which it can be seen that import competence correlates with hydrophobic surface area ($r = 0.904$).

To assess the generality of the conclusions derived from the ALDH presequence, surface area calculations were also performed on the OTC presequence and its previously studied mutants (Kraus et al., 1988; Skerjanc et al., 1988). The OTC presequence contains four Leu residues near the N-terminus. Import studies showed that

Table 3. Accessible hydrophobic and hydrophilic surface areas along with helical hydrophobic moments calculated for residues 1–11 of mitochondrial presequences

Sequence	Hydrophobic surface area ($\text{\AA}^2$) ^a	Hydrophilic surface area ($\text{\AA}^2$)	μ_{H} ^b
ALDH	1,190	652	4.9
L2A	1,143	650	4.7
L2V	1,008	647	4.9
L2I	1,210	639	5.0
L2F	1,255	629	4.9
L2W	1,260	641	4.7
ALDH(-RGP)	1,188	631	4.9
L2V(-RGP)	1,105	622	5.2
Rhodanese	1,197	495	3.7
F ₁ -ATPase β	1,199	543	5.8
Cpn-10	1,345	438	3.1
COX-IV	1,299	573	3.8
Thiolase	1,315	343	2.4
MDH	1,129	417	3.1
Isopropylmalate synthase	1,187	517	2.2
OTC	1,285	558	2.8
OTC L(8,9)A	1,198	567	2.4
OTC L(5,8,9)A	1,157	566	2.2
OTC L(5,8,9)G	1,064	611	2.0
OTC L(5,8,9)G;S3F	1,167	566	1.1

^aSurface areas calculated by rolling a 1.4 $\text{\AA}$ radius probe over the molecular surface. All structures were in an extended conformation. Construction of the structures is described in Materials and methods.

^bHelical hydrophobic moment calculated for the first 11 residues, using the method and parameters described in Eisenberg et al. (1982).

Fig. 4. Plots of hydrophobic surface area vs. percent import of pALDH and L2X mutants. Values for percent import (Table 1) of pALDH, Leu2 mutants and linker-deleted mutants are plotted against the calculated accessible hydrophobic surface area (Table 3). Linear regression analysis was performed, taking the import error bars into account. The solid regression line is shown ($r = 0.904$).

multiple Leu to Ala or Leu to Gly substitutions led to a loss in import competence. The double mutant, L(8,9)A, was imported nearly as well as native OTC. Native OTC had a greater amount and the double mutant had nearly the same amount of hydrophobic surface area as native pALDH (Table 3). The triple mutant, L(5,8,9)A, was imported poorly and contained approximately the same hydrophobic surface area as the poorly imported L2A mutant of pALDH. Another triple mutant, L(5,8,9)G, which was not imported, contained less hydrophobic surface area than L(5,8,9)A. Import competence was restored to L(5,8,9)G by making an additional S3F mutation. The addition of the Phe to the presequence had the effect of increasing the hydrophobic surface area by approximately $100 \text{ \AA}^2$. Consistent with the ALDH data, import of the presequences from the OTC studies appeared to correlate very well with the accessible hydrophobic surface area.

An alternative role of a hydrophobic residue, such as Leu2 in pALDH, could be to provide sufficient hydrophobic moment to make the presequence import competent. In the case of the F_1 -ATPase β from *Saccharomyces cerevisiae*, the competence of mutant presequences for import correlated to their helical hydrophobic moment (Bedwell et al., 1989). In that study, many of the randomly generated mutants differed significantly from the native sequence in charge and hydrophobicity. Therefore, many of the mutations may have resulted in the change of more than one property that could be important for import. For the present study, the hydrophobic moment of the Leu2 mutants of pALDH varied only slightly (Table 3), showing that changes in import behavior were essentially independent of the hydrophobic moment. The OTC presequence and the Leu to Ala/Gly mutants possessed helical hydrophobic moments that decreased as Leu residues were replaced (Table 3). However, the helical hydrophobic moment of the S3F mutant, which restored import, was substantially lower than that of the other mutants. The change in helical hydrophobic moment was in the opposite direction of what would be expected if it were a critical property for the restoration of import competence.

It has been demonstrated that the loss of Met1 was responsible for the nonimport of L2V. Although these N-terminal modifications have been known for 10 years (Arfin & Bradshaw, 1988), their importance has not been addressed previously in studies of mitochondrial protein import. Some proteins are imported despite having Val2. Examples of these proteins include rat rhodanese and the F_1 -ATPase β subunit and isopropylmalate synthase from *S. cerevisiae*. Hydrophobic surface area calculations were carried out for each of these sequences (Table 3). Despite the absence of Met1, each Val2 sequence had available hydrophobic surface area comparable to that of the native ALDH presequence, which included acetylated Met1. It appears that each of these sequences contain sufficient hydrophobic surface area to compensate for the loss of Met1. Hydrophobic surface area calculations were carried out for four other frequently studied presequences to demonstrate the generality of the need for a minimum amount of hydrophobic surface (Table 3). We have shown that the mutant presequence L2V(-RGP) was imported. Restoration of import competence upon the removal of the R-G-P linker has previously been explained by an increase in length and stability of an α -helical conformation (Thornton et al., 1993). However, the removal of the linker also resulted in the shift of Leu15, Leu18, and Leu19 toward the N-terminus. This shift had the effect of increasing the available hydrophobic surface area of the N-terminal portion of the presequence and provides an alternative explanation of the restoration of import competence to L2V(-RGP).

During the course of import into mitochondria, precursors have been shown to interact with cytosolic proteins such as Hsp70 and mitochondria stimulating factor (MSF) (Komiya et al., 1996), with TOM proteins (Bolliger et al., 1995; Haucke et al., 1995; Nargang et al., 1995), and with the translocation proteins that form the general import pore (GIP) (Alconada et al., 1995). The results reported here suggest that due to insufficient hydrophobic surface area the L2V and L2A mutants of pALDH could have an altered ability to interact with one or more of these proteins. From a thermodynamic perspective, hydrophobic surface area removed from contact with water can substantially effect binding equilibria. Based on the hydrophobic effect (Tanford, 1980) and solvent transfer data, the free energy associated with burying hydrophobic surface has been estimated as $46\text{--}47 \text{ cal mol}^{-1} \text{ \AA}^{-2}$ (Sharp et al., 1991). Using the values of Table 3 to compare pALDH and L2V, the difference in the free energy of an interaction with a protein surface could be as large as 10 kcal/mol. A similar magnitude would result from the comparison of native OTC and the nonimported L(5,8,9)G mutant. These free energy changes could significantly shift binding equilibria away from an import competent condition.

The "acid-chain" hypothesis proposes that precursor proteins are transferred along a series of outer membrane proteins that have increasing affinities for basic ligands (Schatz, 1997). It was shown that SynB2, a peptide corresponding to a nonimported basic sequence, did not inhibit binding to the TOM proteins while a peptide corresponding to a functional presequence did inhibit binding (Komiya et al., 1998). We calculated the hydrophobic surface area of the first 11 residues of SynB2 (Roise et al., 1988). The SynB2 sequence possessed $996 \text{ \AA}^2$ of hydrophobic surface area, a quantity much smaller than those calculated for ALDH, OTC, and the other natural presequences considered in this study. This is consistent with the argument that hydrophobic surface area is an important contributing factor to the import process.

The intent of this research was to investigate the effect of a hydrophobic residue near the N-terminus of a mitochondrial pre-

sequence on the import of the precursor protein. It was found that the co-translational modification of the N-terminal methionine effected the L2V mutant differently than native pALDH and the other mutant presequences. This difference led to a significant loss in the available hydrophobic surface of the presequence. The amount of hydrophobic surface area correlated with the import competence of the various presequences. This correlation leads to the conclusion that a minimum amount of hydrophobic surface area in mitochondrial presequences is an essential property to determine their ability to be imported. As a result, both electrostatic and hydrophobic components must be considered when trying to understand the interactions between precursor proteins and TOM proteins or other proteins of the mitochondrial import apparatus.

Materials and methods

Site-directed mutagenesis

pALDH was cloned into the *EcoRI* site of pGEM-3z (Promega, Madison, Wisconsin) as described previously (Wang et al., 1989). Subsequently, cDNA encoding pALDH was amplified using the polymerase chain reaction (PCR) with a flanking 5' *NdeI* and a 3' *BamHI* site and was cloned into the plasmid pT7-7. Leu2 was changed to a Val using PCR and a 5' primer, which changed position 2 from CTG (a Leu codon) to GTG (a Val codon). The L2V cDNA was subcloned into the *XbaI* site of pGEM-7z (Promega). The RGP linker was removed from the L2V mutant in the pGEM-7z vector by using PCR and a 5' primer, which deleted the nucleotides encoding these three residues.

To prepare the L2A, L2I, L2F, and L2W mutants, cDNA encoding pALDH (pGEM-3z) was amplified using PCR and subcloned into the plasmid pBLUESCRIPT II SK (Promega). A 5' *HindIII* site was incorporated and a 3' *SacI* site at the end of the coding region was utilized. The CTG codon of Leu was replaced by GCG for Ala, ATC for Ile, TTC for Phe, and TGG for Trp.

DHFR had been cloned into the plasmid pSP64 (Promega). The cDNA encoding DHFR was amplified using PCR with a flanking 5' *BglII* site and a 3' *PstI* site, and was then cloned into pGEM-3z containing pALDH. The resulting construct encoded the presequence of ALDH along with the first 22 residues of mature ALDH, followed by DHFR. In a similar fashion, DHFR was amplified by PCR from the pALDH-DHFR construct, this time with a 3' *BamHI* site. It was then cloned into pGEM-7z containing L2V-pALDH.

In vitro import of precursor proteins into isolated mitochondria

Mitochondria were isolated from male Harlan Sprague Dawley Wistar rat livers and used for import assays as described previously (Wang et al., 1989; Pak & Weiner, 1990). Proteins, radiolabeled with [³⁵S] methionine, were made using the TNT T7 Quick coupled transcription translation system (Promega). For translation of L2V with *E. coli* extract, the S30 translation system (Promega) was used. The L2V construct was cloned into a pT7-7 vector. Translation reactions were carried out for 1.5 h at 37°C.

To quantify import, the polyacrylamide gel band intensities were determined using a Bio-Rad Phosphor Imaging system. The import efficiency was obtained from the ratio of the amount of imported protein to the amount of translated protein added to the assay. The

import experiments were performed on mitochondria isolated from different rats, and the results were averaged.

Fumagillin inhibition of MAP

Fumagillin (1 mg) was dissolved in ethanol. This solution was diluted with ethanol to give 0.5 and 50 μ M solutions. Translation reactions were carried out as previously described, except that 1 μ L of fumagillin solution was added to give final concentrations of 10 nM and 1 μ M.

N-terminal modifications of precursors

Proteins were produced using a modification of the TNT coupled transcription translation system from Promega. An amino acid mixture was prepared that had no Met or Leu. These amino acids were supplied separately with the [³⁵S] label in Met and the [³H] label in Leu. The translation reactions were carried out for 2 h at 30°C. The mixture was subjected to SDS-PAGE using a 7.5% acrylamide gel at 125 V. One microliter of bovine hemoglobin (10 mg/mL) was added to each sample before loading onto the gel. Proteins were electro-transferred to PVDF membranes over a 2 h period at 90 V (ca. 220 mA) using a Bio-Rad mini-protein transfer apparatus. Radiolabeled protein bands were located on the membrane after overnight exposure to X-ray film. These regions were cut from the membrane. Extraneous radioactivity was removed from the membranes by washing five times with water. The PVDF sections containing the proteins were sequenced using a Procise 491 Gas-Phase Sequencer in the Laboratory for Macromolecular Structure in the Purdue University Biochemistry Department. Fractions collected from each sequencing cycle were saved and dissolved in 500 μ L of n-butyl chloride and 3 mL of Ecolume (ICN) biodegradable scintillation fluid. Samples were counted using a Beckman (Costa Mesa, California) LS 1801 liquid scintillation counter, which was set to count windows with energies of 0–400 (all ³H and 70% of ³⁵S) and 0–1,000 (all ³H and ³⁵S). Proportions of the isotopes were determined using algebra. ³⁵S/³H ratios were obtained by counting the PVDF fragments, which were treated with 500 μ L of methanol before the addition of scintillation fluid.

Oligonucleotide synthesis and purification

All oligonucleotides were synthesized at the Laboratory for Macromolecular Structure in the Purdue University Biochemistry Department. The 5' and 3' oligos were purified with the use of NENSORB PREP columns (Dupont, Boston, Massachusetts).

Molecular modeling

pALDH and pOTC peptide structures were generated and calculations were carried out using QUANTA 4.1 on a Silicon Graphics Indigo² workstation. Structures were constructed using extended dihedral angles ($\phi = \psi = 180^\circ$). Energy minimization (200 steps of steepest descents) was carried out on the initial structures to eliminate high-energy steric interactions among side chains. Solvent accessible surface areas were calculated using the algorithm contained in QUANTA based on previous publications (Richards, 1974), with a probe radius of 1.4 Å.

Miscellaneous

DNA sequence analysis was performed according to Sanger et al. (1977). The cDNA for DHFR was a gift from Dr. Gordon

Shore. Proteinase K and pyruvate kinase were purchased from Boehringer Mannheim. Reagents for PCR were obtained from Perkin-Elmer Cetus. Fumagillin was purchased from Sigma. SDS-PAGE was performed according to published procedures (Laemmli, 1970).

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