A Regional Net Charge and Structural Compensation Model to Explain How Negatively Charged Amino Acids Can Be Accepted within a Mitochondrial Leader Sequence

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Mitochondrial leader sequences have been found to be statistically enriched for positively charged residues, with only a few known leader sequences possessing negatively charged residues. Mutational studies that have introduced negatively charged residues into various leader sequences have shown a general, but not absolute, trend toward reduced import. The leader sequence of rat liver aldehyde dehydrogenase has been previously determined by NMR to form a helix-linker-helix structure. A negative charge introduced into this leader did not prevent import, provided that a net positive charge remained in the N-helical segment. When the net charge of the N-terminal helical segment was reduced to zero, import could be recovered by removing the linker, which resulted in a longer, more stable leader. This structural recovery of import was effective enough to compensate for a net charge of zero within the first 10 residues, even when a glutamate is the first charged side chain presented in the sequence.

The majority of mitochondrial proteins are encoded from nuclear DNA and synthesized in the cytosol. These nascent proteins possess nonidentical N-terminal amino acid sequences that contain sufficient information to direct the protein from the cytosol to the mitochondria. Although mitochondrial leaders do not share any primary sequence identity, they are statistically rich in positively charged amino acid residues and display the ability to form an amphiphilic secondary structure (1, 2). Mitochondrial receptors, which recognize and interact with these leader sequences, possess regions in their primary sequence that are enriched with negatively charged residues (3). Although the structures of the various import receptor components are unknown, it is likely that certain receptor components bind the leader sequences amphiphilically (4, 5). The enrichment of negative charges within segments of the mitochondrial receptors implies an electrostatic complementation to the positive charges of the leader sequence.

From a statistical basis, negatively charged amino acid residues are infrequently found in leader sequences (1). However, a number of leader sequences that have been investigated, such as isopropyl malate synthase (6, 7), 10-kDa chaperonin (8), and rhodanese (9), possess a minimum of 1 negatively charged residue. Mutational studies have demonstrated both a tolerance and an intolerance to the introduction of negative charges within leader sequences (10–13). The statistical fact that naturally occurring leader sequences are biased to be rich in positive charges, coupled with conflicting import results from the mutagenic introduction of negatively charged residues, raises questions as to how negative charges could be tolerated.

The leader sequence for the pALDH1 has been studied extensively both structurally and in two separate systematic mutational investigations (14–16). As shown in Fig. 1, the leader is comprised of 19 amino acids that can be induced into a helix-linker-helix amphiphilic structure in the presence of trifluoroethanol or detergent micelles. Deletion of the linker results in a nonprocessible leader that is more stable and longer in helical content (17). Removal of the linker results in a leader capable of importing aldehyde dehydrogenase to greater levels than that of the native sequence (15). The enhanced stability of the linker-deleted leader has been successfully used to study structural aspects as a compensating factor for the loss of positive charges through arginine to glutamine mutational substitutions.

The leader sequence of pALDH contains five arginines with two arginines in each helix and one in the linker segment. It was found from a mutational study that numerous arginine to glutamine substitutions could be made with no apparent effect on import (15). Although each helical segment contains an equal number of positive charges, the N-terminal helical segment has been shown to provide the majority of the necessary charge for efficiently targeting the leader to the matrix. If both of the N-helical segment arginines were substituted with glutamine residues, the ability to import was essentially eliminated. This result implies that the net positive charge in the N-helical segment is more important than the total positive charge throughout the leader. In contrast, the linker-deleted structure could import a passenger protein even if both of the N-terminal arginine residues (Arg9 and Arg10) were mutated to glutamines. The linker-deleted leader demonstrated that a longer, more stable amphiphilic structure compensates for the absence of the N-terminal positive charges. Based on the positive charge versus structural compensation model developed from our previous studies, glutamic and aspartic acid residues were systematically introduced into the pALDH leader to ascertain how negative charges may be tolerated.

MATERIALS AND METHODS

Site-directed Mutagenesis—The plasmid containing rat liver mitochondrial pALDH, which was previously cloned into pGEM-3Z (Promega; Ref. 18), was digested with BglII and HindIII to remove the segment coding from amino acid residue 23 to the end the mature protein. Mouse dihydrofolate reductase cloned into SP64 was amplified

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† The abbreviations used are: pALDH, precursor aldehyde dehydrogenase; pALDH-RGP, linker-deleted aldehyde dehydrogenase leader; TFE, trifluoroethanol.
**Negative Charge Acceptance in Mitochondrial Leader Sequences**

**Fig. 1.** Helical wheel projections of pALDH and pALDH-RGP based on two-dimensional NMR structures. A, the helix-linker-helix structure of the native pALDH leader illustrates the number of charges to each helical segment and the linker. The native leader carries a gross positive side chain charge of +5. B, the pALDH-RGP leader sequence, which has lost one positively charged residue and two helix-disrupting amino acids. The resulting sequence forms a continuous helix that surpasses the ability of the native sequence to be induced into a secondary structure by TFE or detergent micelles.

by a polymerase chain reaction utilizing a 5' primer with a BglII overhang and a 3' primer with a HindIII overhang. The BglII- and HindIII-digested polymerase chain reaction fragment was ligated to the abovementioned vector, resulting in a plasmid that codes for the leader sequence of pALDH, the first 22 amino acids of the mature segment of pALDH, followed by dihydrofolate reductase. Subsequent point mutations within the leader sequence were performed by polymerase chain reaction with 5' primers encoding a SphI overhang and the desired codon change and a 3' primer with a BglII site. Oligonucleotides were obtained and purified as described previously (15). All mutational constructs were verified by sequencing.

In Vitro Import—Mitochondria were isolated from male Harlan Sprague-Dawley Wistar rat livers as described previously (15). Radio-labeled proteins were synthesized using the TNT Quick Coupled transcription and translation system (Promega). Quantification of import was performed using the band intensities from SDS-polyacrylamide gel electrophoresis gels with a Bio-Rad phosphor imaging system. The level of import was defined as the ratio of the total counts of the protease-protected bands:that of the total counts provided in the assay. The import values obtained from the mutant leaders were normalized to the values obtained from the native leader in the same experiment. The averages shown in Table I reflect a minimum of three separate import experiments.

Circular Dichroism—Circular dichroism was performed on a Jasco J-600 Spectroplanimeter. Peptides were obtained, purified, and analyzed for mass and amino acid composition as described previously (15). The experiments were performed at a peptide concentration of 20 μM at 25 °C in 50 mM phosphate buffer, pH 5.2 or pH 7.2. The percentage of TFE was based on volume. Three baseline determinations were obtained and averaged at the given percentage of TFE. Three measurements with the peptide under the same percentage of TFE were obtained and averaged. A value of ~35,600 was used to estimate the fraction of peptide in a helical conformation at [θ]_222 (15).

**RESULTS**

Neutral and Negatively Charged Amino Acid Mutations in the Precursor Aldehyde Dehydrogenase Sequence—The pALDH leader sequence is structurally comprised of two amphiphilic helical segments joined by a flexible, three-amino acid linker segment (Fig. 1). Each helical segment, referred to as either the N-terminal or the C-terminal segment, contains two arginines, whereas the linker contains one arginine. It was our aim to determine the effect on import by the introduction of negative charges, particularly in the N-terminal helical segment, because this helix segment is thought to be responsible for the positive charge required for import (14, 15). We chose Ser7 as the residue to begin the site-directed mutagenic introduction of a negative charge. A mutagenic replacement of this Ser with Glu or Asp would retain the amphiphilicity required for mitochondrial leader sequences, as illustrated in Fig. 1 (4). As a control for replacing Ser7 with the charged residues, both Gln and Asn were substituted at the same position. As shown in Fig. 2 and summarized in Table I, the S7N and S7Q mutants were capable of targeting the dihydrofolate reductase passenger protein to the matrix to the same extent as the native leader. The S7E mutant was imported to levels that averaged up to 84% of the native leader, indicating a tolerance to a negative charge. The S7D mutant was only capable of importing to about 30% of the native levels.

Previously, we showed that only one arginine was required in the N-terminal domain of the pALDH leader sequence, because both the R3Q and R10Q mutant leaders were imported as well as the native sequence (15). The double mutant R3Q(R10Q) was impaired at import, demonstrating a necessity for at least one positive charge in the first 10 amino acids. If the positive charges of the arginines could interact with the negative charge through ion pairing, this interaction could shield and reduce the effect of the introduced negative charge. To test this hypothesis, two mutants were made in which the flanking arginines were substituted with glutamine, a neutral, helix-promoting residue. As shown in Fig. 2, the R3Q(S7E) and R10Q(S7E) mutants were imported less than the S7E mutant, indicating that both arginines were required to achieve native import levels when a negatively charged amino acid was present at position 7.

In the R3Q(S7E) and R10Q(S7E) mutants, the total charge of the leader has been reduced from +5 to +3, whereas in the N-terminal helical segment, the charge was reduced from +2 to 0. The N-helical segment of the impaired R3Q(R10Q) leader also possessed a net charge of 0. If a net charge within the N-terminal helical segment is more important than the total charge of the leader, then we could potentially influence this net charge through a S7E(R11E) mutant. Although, this additional glutamic acid is not part of the N-terminal helical segment, its presence could cancel the effect of the adjacent positive charge of Arg10. As shown in Fig. 2, the S7E(R11E) mutant was impaired at import. The import results with the mutants of S7E, R3Q(S7E), and R10Q(S7E), as well as the R3Q(R10Q) previously studied, converge to a model that supports a regional net charge effect in the leader sequence of pALDH rather than the total number of positive charges in the entire...
leader, as has been previously implied with the ornithine transcarbamylase leader (19). However, S7E(R11E) does not fully support the model of a regional N-terminal net charge because the total charge of the leader may have been reduced too much.

To attempt to resolve whether S7E(R11E) was impairing import by influencing either the total charge of the leader or the N-helical segment's net charge, we introduced an additional negative charge into the C-helical segment of the S7E mutant. The two arginines in the C helix of the native leader could be separately mutated to glutamines without any effect on import, but the R14Q(R17Q) double mutant was imported to 50% efficiency (15). We chose Arg14 to preserve the processing site motif and thus made a S7E(R14E) leader. If the total charge of the leader were more important than the net charge of the C-helical segment, then we would expect import levels similar to that of R14Q(R17Q). As shown in Fig. 2, neither of the expected results was observed. The S7E(R14E) mutant was found to be more efficient at importing compared with the native leader. In the S7E(R14E) mutant, the overall net charge of the native leader sequence has been reduced, as in the S7E(R11E) mutant, to $\frac{1}{2}$, yet import was increased. What may have compensated for the C-terminal net charge deficiency was a network of potential ion pairs between the Glu and Arg residues that could form in both helical segments as well as between them. There are six potential ion pairs in the S7E(R14E) leader, and this ion pairing may have improved the stability of the leader, especially through the linker segment. If the potential increase in structural stability were the factor in compensating for net positive charge reduction in the C-helical segment, structural stability may also compensate for the loss of positive charge in the N-terminal helical segment by removing the linker.

Removing the linker segment in the leader sequence of pALDH has been shown to result in increased stability and helical length (17). Additionally, it has been shown to compensate for the loss of positive charges in the N-helical segment, as with the R3Q(R10Q) mutant (15). Removing the linker from the R3Q(S7E) and R10Q(S7E) double mutants restored import, as shown in Fig. 2. Although both mutants were imported, the efficiencies were dissimilar. The R10Q(S7E) leader restored import to levels equivalent to that of the linker-deleted leader, whereas the R3Q(S7E) leader was imported to native levels. What may have been a contributing factor for the reduced import in the R3Q(S7E)-RGP leader compared with that of R10Q(S7E)-RGP was that the first N-terminal side chain bearing a charge was negative. However, improving the apparent stability and increasing the helical length of the leader can compensate for an N-terminal positive charge deficiency.

![Fig. 2. SDS-polyacrylamide gel electrophoresis of the mitochondrial import experiments of the native and mutant precursor proteins.](image)

**TABLE 1**

| Leader Charge | N-helix Charge | Standard Deviation |
|---------------|----------------|-------------------|
| S7E(R11E)     | $\frac{1}{2}$ | $\frac{1}{2}$ |
| S7E(R14E)     | $\frac{1}{2}$ | $\frac{1}{2}$ |

*Import is reported on a relative scale to the native leader sequence. Import values for R3Q, R10Q, R3Q(R10Q), R14Q, R14Q(R17Q), pALDH-RGP, R3Q(R10Q)-RGP, R11Q-RGP, and R11Q(R14Q)-RGP are from a previous study (15).*

*The charge on the N-helix is either 1 or 0, depending upon whether or not residue 11 in the linker is considered part of the N-helix.*
The ability of S7E(R14E) to form such a stable structure is a likely deleted peptide. Analogous to the linker-deleted peptide, the TFE and pH 7.2, it was nearly identical to that of the linker-deleted peptide. At 40% TFE and pH 5.2, the ability to form a helix was approaching that of the linker-deleted peptide. At 40% TFE and pH 5.2, the population of peptide in a helical conformation was 27%. At pH 7.2, the population of helical peptide was approximately 2-fold higher in helical population than at pH 5.2. In contrast to the S7D peptide, the S7E peptide was capable of being induced into a helix at 20 and 40% TFE was surprisingly independent of the pH value.

As summarized in Table II, the ability of the S7D to be induced into a helical conformation at 20 and 40% TFE was comparable to that of the native peptide at pH 5.2. At pH 7.2, the S7D peptide displayed a greater fraction of peptide in a helical conformation compared with that of the native leader at both 20 and 40% TFE. In contrast to the S7D peptide, the S7E peptide was approximately 2-fold higher in helical population compared with that of the native leader at both 20 and 40% TFE and was surprisingly independent of the pH value.

The R3Q(S7E) peptide, in contrast to S7E, displayed a pH dependence on helical conformation and was more readily induced into a helix at 20 and 40% TFE, pH 7.2. As with the S7D and S7E peptides at 20% TFE, pH 7.2, the S7E and R3Q(S7E) peptides were indistinguishable at 20% TFE, pH 5.2. Although there is a greater ability to form a helical structure in R3Q(S7E) compared with the S7E peptide, neither peptide was as capable of being induced into a helix as well as the linker-deleted leader. The circular dichroism results with peptides S7D, S7E, and R3Q(S7E) show that their ability to form a helix has not been compromised. Thus, the reason for the reduced import of S7D and R3Q(S7E) is most likely to be charge related and not structurally related.

S7E(R14E) displayed greater levels of import, surpassing that of the linker-deleted leader. The peptide S7E(R14E) displayed a pH dependence on being induced into a helix. At 20% TFE and pH 5.2, the population of peptide in a helical conformation was essentially identical to that of S7E and R3Q(S7E). However, at 40% TFE and pH 5.2, the ability to form a helix was approaching that of the linker-deleted peptide. At 40% TFE and pH 7.2, it was nearly identical to that of the linker-deleted peptide. Analogous to the linker-deleted peptide, the ability of S7E(R14E) to form such a stable structure is a likely explanation for the enhanced import.

**DISCUSSION**

The mitochondrial import receptor complexes of translocator of the outer membrane and translocator of the inner membrane from yeast have been extensively studied, and the functional roles of each complex have been partially uncovered (3). The translocator of the outer membrane complex is thought to provide regions of net negative charge on both sides of the outer membrane, which may serve to electrostatically guide the leader to the intermembrane space. The translocator of the inner membrane complex, which is also thought to contain acidic patches for binding leaders, differs from the translocator of the outer membrane complex with respect to the transfer mechanism of a leader across the inner membrane. Translocation from the translocator of the inner membrane complex to the matrix requires a Δψ (negative inside) chemical potential across the inner membrane, used apparently for the electrophoretic migration of the leader (20, 21). Utilizing the helix-promoting glutamic acid residue while maintaining amphiphilicity in all of the constructs, we demonstrated a tolerance to a statistically uncommon acidic residue in a mitochondria leader sequence.

The leader sequences of the mutants S7E and S7E(R14E) revealed a remarkable tolerance to the presence of a negative charge. A previous study demonstrated that the leader sequence of F1-βATPase could tolerate the mutational introduction of glutamic acids as single and double point mutations (10). However, in another study, negative charges introduced in the same leader interfered with import (11). The conflicting results may be interpreted using arguments based on amphiphilicity. In the case in which negative charges interfered with the import of F1-βATPase, amphiphilicity was not maintained. This was also observed in a mutant of malate dehydrogenase in which a Leu to Glu mutation placed the negative charge on what appears to be the hydrophobic portion of the helix (12). Conversely, the negative charges that were tolerated in F1-βATPase were placed in a proper amphiphilic position. An alternative explanation for the different results may reside in a model of structural compensation for charge deficiencies to be developed below.

We have shown that not all of the positive charges in the

**TABLE II**

| Peptide | 20% TFE, pH 5.2 | 40% TFE, pH 5.2 | 20% TFE, pH 7.2 | 40% TFE, pH 7.2 |
|---------|----------------|----------------|----------------|----------------|
| pALDH   | -4,896 (14%)   | -7,420 (21%)   | -4,896 (14%)   | Not determined |
| S7D     | -6,806 (19%)   | -9,691 (27%)   | -9,676 (27%)   | -12,000 (34%)  |
| S7E     | -10,000 (28%)  | -14,875 (42%)  | -9,806 (28%)   | -14,594 (41%)  |
| R3Q(S7E)| -9,454 (27%)   | -17,126 (48%)  | -12,193 (34%)  | -17,580 (49%)  |
| S7E(R14E)| -12,080 (29%) | -18,503 (52%)  | -12,972 (36%)  | -19,344 (54%)  |
| pALDH-RGP | -14,959 (42%) | -19,586 (55%)  | Not determined  | Not determined  |

*Values were previously determined from Ref. 15.*
leader of pALDH are required for import; R3Q, R10Q, R14Q, R17Q, and R14Q(R17Q) are all functional leaders under in vitro conditions (15). The double mutant R3Q(R10Q) was import impaired and led to the suggestion of the necessity of at least one positive charge in the N-helical segment. Both Arg$^3$ and Arg$^{16}$ are required for the S7E mutant to function properly. A likely explanation for the ability of the leader to tolerate the S7E substitution is that the N-helical segment retains a sufficient, net positive charge. As summarized in Table I, the S7E mutant is equivalent in N-helical segment charge to the mutants of R3Q and R10Q. The mutants R3Q(S7E) and R10Q(S7E) are equivalent to that of R3Q(R10Q) in that all three display a net charge of 0 in the N-terminal helical segment.

Arguments for a net positive charge requirement in the N-terminal helical segment were strengthened by the results obtained from the S7E(R11E) and S7E(R14E) mutants. For both the S7E(R11E) and S7E(R14E) mutants, the overall charge of the leader was reduced from +5 to +2. The S7E(R14E) mutant displayed greater import and even surpassed the import level of the linker-deleted structure. The introduction of the two negative charges in the S7E(R14E) mutant may have potentially improved the stability of the leader through a network of electrostatic interactions within the leader, as illustrated in Fig. 3 and suggested by the circular dichroism experiments. In the S7E(R11E) mutant, the presence of a negative charge was more likely influencing the net charge of the N-terminal helical segment, even though it is not part of the structure determined from the native leader determined by NMR (16).

Removing both positive charges from either the N-terminal helical segment or the C-terminal helical segment of the aldehyde dehydrogenase leader sequence impaired import. However, deleting the linker from the R3Q(R10Q) or R14Q(R17Q) mutants restored import. This was also found for the R3Q(S7E) and R10Q(S7E) mutants. The more stable helix, produced by removing the linker, appeared to overcome charge deficits in that it restored import when arginines were substituted by glutamines. Although the linker-deleted leader could compensate for the reduction of positive charges, the level of import depended upon whether a positive or negative charge was the first charged residue in the sequence.

The S7D mutant of pALDH was not imported as well as the S7E mutant. A structural investigation on a peptide corresponding to the S7D leader showed no apparent loss in the ability of the peptide to be induced to a helical structure as compared with the native peptide. There are two plausible explanations for the observation that the S7D precursor mutant is import deficient, yet structurally stable. Firstly, the glutamic acid residue of S7E is capable of extending the charge toward a hydrophilic environment, but the charge on the shorter aspartate residue is unable to extend away from what may be a hydrophobic environment. If the N-terminal helix of pALDH begins to interact amphipathically with the receptor proteins, the negative charge on the aspartate residue may be placed in an unfavorable hydrophobic environment. The glutamic acid and arginine residues are capable of proper extension and potential ion pairing, which may shield opposing charge effects and add a favorable, albeit small, energetic contribution to helix stability. An alternative, related explanation for the reduced import of the S7D mutant could be that the charge was not effectively shielded through potential electrostatic interactions with the two arginines in the leader.

From the results of this study, we propose a model to explain how negative charges may be tolerated in the leader sequence of pALDH. Introducing a negative charge into the N-terminal helical segment reduces the net charge of the helix in much the same manner that was observed when the positive charges were substituted with glutamines in absence of negative charges. Provided that a net positive charge is maintained in the N-helical segment, negative charges are tolerated, and import is maintained. When the presence of a negative charge reduces the net charge of the N-terminal helical segment to 0, import is impaired but can be restored by increasing the length and stability of the leader. Although more structural information and import properties are needed from other leader sequences that naturally contain negative charges, the model is consistent with our previous studies that have shown structure to be an important contributor to offset any reduction in positive charge. If our model is correct, mitochondrial leader sequences that naturally contain a number of negative charges such as isopropyl malate synthase may have a greater capacity to form a longer, more stable helix than those that are richer in positive charges.

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