The Role of Positive Charges and Structural Segments in the Presequence of Rat Liver Aldehyde Dehydrogenase in Import into Mitochondria*  
(Received for publication, April 5, 1996, and in revised form, June 5, 1996)

Philip K. Hammen, Mary Waltner, Birger Hahnemann, Thomas S. Heard, and Henry Weiner‡
From the Department of Biochemistry, Purdue University, West Lafayette, Indiana 47907-1153

Most mitochondrial proteins are nucleus-encoded and translated in the cytosol. They have an N-terminal presequence that allows recognition by the mitochondrial import apparatus and subsequent import into mitochondria. These presequences are rich in positive charges, mainly arginines. The role of these positive charges in the 19-amino acid presequence of rat liver aldehyde dehydrogenase was investigated by systematically replacing them with the polar but uncharged residue, glutamine. The single substitution of any of the four Arg residues in the helical segments did not affect import. Substitution of both Arg residues in the N-terminal segment (R3Q/R10Q) caused a dramatic decrease in import competence. This could be restored by using the mutant lacking the three-amino acid (RGP) linker that separates the two helical domains, determined by two-dimensional NMR (Thornton, K., Wang, Y., Weiner, H., and Gorenstein, D. G. (1993) J. Biol. Chem. 268, 19906-19914). CD and NMR spectra of the peptide corresponding to the linker-deleted presequence showed that it was substantially more prone to helix formation than the native peptide over its entire length. A similar analysis of the peptide corresponding to the R3Q/R10Q presequence revealed that this peptide was only somewhat more helical than the native peptide and that the greater helicity did not include the residues near the N terminus. It is concluded that positively charged residues in the presequence play a vital role in the import of precursor aldehyde dehydrogenase. One of the positive charges in the N-terminal helical segment of the presequence is necessary for import competence. However, if both positive charges are removed, import competence can be retained as long as the presequence is capable of forming a relatively more stable \(\alpha\)-helix near its N terminus.

Most mitochondrial matrix proteins are encoded in the nucleus and, after translation, imported into mitochondria. These precursor proteins possess specific, N-terminal targeting sequences that are required for translocation across mitochondrial membranes and are removed by a matrix space-processing protease. The mechanism of how these presequences direct the import of proteins into mitochondria remains, to some extent, obscure. Since mitochondrial presequences lack a common primary structure, there must be other shared characteristics that contribute to their function. Based on sequence comparison among a variety of presequences, a net positive charge is one common attribute (1). Positive charges in the presequence could interact with the negatively charged surface of the mitochondrial membrane. There is mounting evidence that mitochondrial membrane proteins that must be present for import to occur contain negatively charged surfaces (2–4). In addition, MPP has been shown to be highly desirable for an import-competent presequence (6, 7). Secondary structures of presequences are known only for the corresponding synthetic peptides. All synthetic peptides that have been studied display the tendency to forms \(\alpha\)-helices (8–12) in the presence of either structure-promoting organic cosolvents or membrane-mimetic media.

Point mutations have been made in presequences to determine the consequences of replacing the positive charge of arginine residues. When both Arg-14 and Arg-15 in the presequence of malate dehydrogenase were replaced by Ala, import competence was impaired by more than 50% (13). Subsequently, Arg-14 and Arg-15 were shown to be part of the helical structure formed when the presequence peptide was dissolved in trifluoroethanol (11). The role of Arg-23 in the presequence of ornithine transcarbamylase has been investigated (14). A mutation to glycine resulted in a precursor protein that was not imported. Replacement of Arg-23 with other amino acids produced precursors that were imported. The extent of import was shown to increase with the propensity of the amino acid residue to be found in an \(\alpha\)-helix. These results suggest that, in addition to charge, secondary structure can be a factor in determining whether a presequence is import-competent.

The presequence of rat liver mitochondrial \(p\)ALDH contains 19 amino acids. In a micellar environment (9), the corresponding synthetic peptide forms two \(\alpha\)-helical segments separated by a three-residue linker, which disrupts the continuity of the \(\alpha\)-helical structure. The N-terminal helix is formed by residues 4–10, while the C-terminal helix spans residues 14–19, and

* This work was supported in part by NIAAA, National Institutes of Health, Grant AA05812. This is Journal Paper 15086 from the Purdue University Agriculture Experiment Station. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Recipient of a Senior Scientist Award AA00028 from the NIAAA, National Institutes of Health. To whom correspondence should be addressed: Dept. of Biochemistry, Purdue University, West Lafayette, IN 47907-1153. Tel.: 317-494-1650; Fax: 317-494-7697; E-mail: Weiner@biochem.purdue.edu.

1 The abbreviations used are: MPP, mitochondrial processing peptidase; ALDH, aldehyde dehydrogenase; \(p\)ALDH, precursor ALDH; SUVs, small unilamellar vesicles; TOCSY, two-dimensional total coherence spectroscopy; NOESY, two-dimensional nuclear Overhauser effect spectroscopy; DQF-COSY, two-dimensional double-quantum filtered correlation spectroscopy; PAGE, polyacrylamide gel electrophoresis.
Effect of Positive Charges on Import of pALDH

Extends into residues that are part of the mature portion of the protein. The linker segment, residues 11–13, has the sequence RGP. When this three-residue segment is removed, the resulting precursor protein is import-competent but is not proteolytically processed (15). In micelles or aqueous trifluoroethanol mixtures, the corresponding peptide forms a single helix, which spans its entire length. Both the native sequence and the sequence with the RGP deletion contain four Arg residues within their helical regions. In each case, the Arg side chains can be aligned on one side of the helix, so the structure obtains a positively charged surface. Leucine and alanine side chains project from the opposite surface, producing the amphiphilic helix.

It has been shown that for import to occur, the N-terminal segment must have the ability to form an α-helix, and that, with pALDH, the C-terminal helix stabilizes the N-terminal one (16). When the C-terminal segment was replaced by one that did not form a helix, both membrane binding and the ability to be imported were lost. Previous mutation studies have not investigated the need for positive charge within the first 10 residues of the precursor. The presence of the Arg residues in the sequence could be necessary for the stabilization of the amphiphilic α-helices. It has also been demonstrated that the C-terminal helical segment binds preferentially to lipid bilayers (9, 15). The suggestion has been made that positively charged side chains play a role in the initial binding of presequences to the mitochondrial membrane (17, 18). In the present study, we address questions concerning the necessity of positive charges in the pALDH presequences for binding to the membrane and/or for translocation into mitochondria.

**MATERIALS AND METHODS**

Site-directed Mutagenesis—Rat liver mitochondrial pALDH, previously cloned into pGEM-3Z (Promega) (19), was used as the template and vector plasmid for all polymerase chain reaction-directed mutagenesis. In native pALDH, all of the presequence Arg residues are coded by CGG. Mutations to glutamine were achieved by oligonucleotide primers in which the Arg codon was changed to CAG. Both the vector plasmid and polymerase chain reaction products were digested with SphI and NsiI and ligated. The mutations were verified by sequencing and SphI digestion. DNA sequence analysis was performed according to Sanger et al. (20) or by the Purdue DNA Sequencing Center.

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 (19, 21). Proteins radiolabeled with [35S]methionine were made using the TNT coupled transcription translation system (Promega).

To quantify import, the band intensities of polyacrylamide gels 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 total translated protein. The import experiments were performed on mitochondria isolated from three different rats and were averaged.

In Vitro Processing Activity—A solution consisting of both MPP (1 mg/ml) and mitochondrial intermediate peptidase (0.6 mg/ml) was a gift from Dr. Frantisek Kalousek (Yale University School of Medicine). Processing assays were performed as described previously (23). Samples were subjected to SDS-PAGE. The amount of processed protein was determined using phosphor imaging. The percent processing was calculated as the amount of processed protein divided by the total amount of protein in the assay.

Trypsin Digestion of Translated Proteins—The rate of folding in rabbit reticulocyte lysate was examined as described previously (22, 23). Briefly, protein was translated at 30 °C for either 30 or 60 min. Trypsin digestion was started by the addition of 1 μl of 0.3 mg/ml L-1-tosylamido-2-phenylethyl-chloromethyl ketone-treated trypsin to the diluted translation product, and the incubation proceeded for 1, 5, 10, and 20-min time periods at 0 °C. After each time period, 2.5 μl was removed, diluted into SDS-PAGE treatment buffer, and boiled. The samples were analyzed on a 12.5% polyacrylamide gel, and proteins were visualized and quantified by phosphor imaging.

Oligonucleotide and Peptide Synthesis and Purification—All oligonucleotides and peptides were synthesized at the Laboratory for Macro-molecular Structure in the Purdue University Biochemistry Department. The 5′ and 3′ oligos were purified on NENSORB PREP columns using the diluted translation product, and the incubation proceeded for 1, 5, 10, and 20-min time periods at 0 °C. After each time period, 2.5 μl was removed, diluted into SDS-PAGE treatment buffer, and boiled. The samples were analyzed on a 12.5% polyacrylamide gel, and proteins were visualized and quantified by phosphor imaging.

Oligonucleotide and peptide synthesis were performed at the Laboratory for Macro-molecular Structure in the Purdue University Biochemistry Department. The 5′ and 3′ oligos were purified on NENSORB PREP columns.

**RESULTS**

Mutations in the ALDH Presequence—As shown in Fig. 1, the presequence of ALDH consists of two putative α-helical seg-

![Fig. 1. ALDH Leader Sequence. Schematic representation of the ALDH leader sequence with the helical regions denoted by cylinders. The boldface residue labels in the sequence are the residues mutated in this study.](image-url)

(DuPont). The R17Q peptide was prepared using an ABI 430A solid state peptide synthesizer. The native ALDH, R30/R10Q, and R14Q peptides were prepared using an ABI 433 Synergy solid state peptide synthesizer. The linker-deleted presequence was synthesized as described previously (15). All peptides were purified using a semi-preparative Vydac C18 reverse-phase column (20 × 1-cm diameter) high pressure liquid chromatography column. Peptide elution was achieved by the application of a 0–60% acetonitrile gradient. Peptide authenticity was determined by mass spectroscopy at the Purdue University Campus-wide Mass Spectrometry Center (M, = 2460 for native ALDH, and M, = 2432 and 2404 for mono- and di-Gln-substituted peptides, respectively) and was verified by amino acid analysis in the laboratory of Prof. Michael Laskowski in the Purdue University Department of Chemistry. Peptide concentrations in aqueous buffer were determined from the amino acid analysis or from tyrosine absorbance measurements.

Circular Dichroism—Circular dichroism spectra were obtained on a Jasco J-600 spectropolarimeter (24). The samples were typically scanned from 350 to 200 nm at 25 °C, with a path length of 0.1 cm. The buffer was 50 mM phosphate, pH 5.2. Base line spectra for each solvent were obtained prior to the peptide spectra. Peptide concentrations for these measurements were in a range from 8 to 20 μM. Helical content was estimated using ~35,600 as the value of [θ]222 for 100% helix (25).

Nuclear Magnetic Resonance—NMR spectra were obtained on a Varian Unity+ 600 spectrometer. Peptide concentrations were approximately 2 mM for native ALDH and R30/R10Q (and due to low solubility 1 mM for R17Q). The peptides were dissolved in 0.28 mM trifluoroethanol and 0.42 mL of 50 mM phosphate buffer, pH 5.2. Chemical shifts were referenced to an internal standard of perdeuteriosodium 3-(trimethylsilyl)-1-propane sulfonate. All NMR spectra were acquired at 21 °C.

Two-dimensional spectra were obtained with spectral width of 6000 Hz in both f1 and f2 in 2,028 complex data sets. A total of 300 t2 increments were acquired using the hypercomplex method to achieve quadrature detection (26). Fourier transformations were weighted with a shifted sine bell function. For the native and R30/R10Q peptides, resonance assignments were made from TOCSY (27, 28) data that were obtained with a 30-ns MLEV-17 mixing sequence. To make specific resonance assignments for the R17Q peptide, a DQF-COSY experiment (29, 30) was performed. For NOESY experiments, mixing times of 200 ms were used for the native and R17Q peptides and 100 ms for the R30/R10Q peptide. Artefacts resulting from zero-quantum coherence were removed by randomizing the mixing time within the range of ±20 ms. The solvent resonance was suppressed by low power irradiation during the recycle delay and during NOESY mixing times. Data sets were processed on a SPARC20 workstation using VNMR software (Varian Associates, Inc.).

Liposomes—Liposomes were prepared as described previously (26). The one exception was in the cardiolipin solution, which was 10 mg/ml in CHCl3. The SUVs contained 20% cardiolipin, by weight.

Fluorescence Spectroscopy—Fluorescence spectra were obtained using a Hitachi F-2000 Fluorescence spectrophotometer, as described previously (7, 18). The Tyr excitation wavelength was 272 nm. Emission was detected from 290–350 nm, with maxima between 304 and 308 nm. The peptide concentrations were 1–3 μM in 50 mM phosphate buffer, pH 5.2. Aliquots of solution containing SUVs were added to the quartz cuvette and stirred at 25 °C.

Miscellaneous—pGEM-3Z plasmid was from Promega. Protease K and pyruvate kinase were from Boehringer Mannheim. Reagents for polymerase chain reaction were from Perkin-Elmer. SDS-polyacrylamide gel electrophoresis was performed according to Laemmli (31).


**Effect of Positive Charges on Import of pALDH**

Fig. 2. *Mitochondrial import of precursor proteins.* In vitro import was performed as described under "Materials and Methods." Each translated protein (lane T) was incubated with rat liver mitochondria for 30 min at 30°C. A 50-μl aliquot was left untreated (lane -P.K), and another 50-μl aliquot was treated with proteinase K to digest nonimported protein (lane +P.K).

![Image](http://www.jbc.org/)

**TABLE I**

| Mutant         | Presequence | Import* (%) | Processing† |
|----------------|-------------|-------------|-------------|
| pALDH          | MLRAALSTARGPRLSRL | 100 ± 9     | +++         |
| R3Q            | MLRAALSTARGPRLSRL | 94 ± 4      | +++         |
| R10Q           | MLRAALSTARGPRLSRL | 91 ± 29     | +++         |
| R3,10Q         | MLRAALSTARGPRLSRL | 6 ± 5       | ++          |
| R14Q           | MLRAALSTARGPRLSRL | 101 ± 28    | +++         |
| R17Q           | MLRAALSTARGPRLSRL | 101 ± 16    | -           |
| R14Q/R17Q      | MLRAALSTARGPQLSLL | 48 ± 28     | -           |
| pALDH (RGP)    | MLRAALSTARGPRLSRL | 163 ± 76    | -           |
| R3Q/R10Q (RGP) | MLRAALSTARGPRLSRL | 58 ± 9      | -           |
| R14Q (RGP)     | MLRAALSTARGPRLSRL | 172 ± 4     | -           |
| R110/R140 (RGP)| MLRAALSTARGPQLSLL | 154 ± 47    | -           |

* Percentage of import is reported relative to pALDH, which was arbitrarily given the value of 100%. The uncertainties reported are the standard error of measurement for n = 3, with the exception of pALDH (RGP), for which n = 5.

† The number of plus signs refers to the relative case of processing in the import assay, while a minus signs mean that the precursor was not processed.

Fig. 3. *Import of ALDH mutants relative to native pALDH.* Bar heights show the ratio of percentage import for the mutant relative to that of pALDH obtained from quantitative analysis of SDS gel band intensities. Each point is the average of three observations. Error bars correspond to one standard deviation from the mean value. When (-L) appears in a label, it indicates the deletion of the RGP linker segment from the sequence.

(R14Q and R17Q) were imported with an efficiency similar to that of wild type. When both charged residues in the C-terminal segment were replaced (R14Q/R17Q), import was only partially impaired. Therefore, significant impairment of import function was not observed until two Arg residues were replaced. The effect was most apparent when mutations were in the N-terminal segment of the presequence.

Mutations in Linker-deleted pALDH—When the three-residue segment, RGP, that forms the interhelix linker was removed from the native presequence, an enhancement of import was observed (Fig. 3). The presequence peptide of the linker-deleted mutant has been shown to form a single, continuous helical segment (15), suggesting that the increased propensity for helix formation may increase the ability of the presequence to function in import. The linker was removed from the doubly mutated presequences to determine if import competence could be restored or enhanced by removal of these residues. The import efficiency of the R3Q/R10Q (-RGP) mutant rose to
nearly 60% that of the native sequence, but did not attain the level of the linker-deleted mutants. Therefore, it appeared that a presequence that was able to form a longer, more stable helix could overcome the loss of import competence resulting from the replacement of the two positively charged residues in the N-terminal segment. When the linker was removed from the mutant (R14Q/R17Q (R11Q/R14Q (−RGP))), the import efficiency was enhanced to a level greater than that of the native presequence. To observe the effect on import when the linker was removed from a single Arg to Gln mutant, it was deleted from the R17Q mutant, forming R14Q (−RGP). As with the R11Q/R14Q (−RGP), an increased level of import was observed. Thus, the linker-deleted precursor protein remains import-competent despite the loss of both positively charged residues at either end of the presequence.

Processing of R/Q mutants—From the SDS gel images shown in Fig. 2, it can be seen that the native precursor was processed, but mutants lacking either Arg-17 or the linker were not processed in the in vitro import assay. The loss of processing with linker-deleted pALDH was consistent with previous work (15). In a statistical study, Arg was commonly found 2 or 3 residues before the processing site of several mitochondrial precursor proteins (32). The results with Arg-17 mutants are consistent with this residue being involved in the processing site of pALDH, which is between Leu-19 and Ser-20 (33). Since the R3Q/R10Q precursor was imported into mitochondria very poorly, it was necessary to assess processing by incubating the mutant with the isolated processing protease, MPP. The MPP was able to cleave this mutant, but not the R17Q mutant (data not shown).

Trypsin Digests of Import-incompetent Precursor Proteins—The loss, or partial loss, of import competence of the R3Q/R10Q and R14Q/R17Q mutants could be the result of interactions between the presequence and the mature part of ALDH that caused them to be unrecognized by their target on the mitochondrial surface. To be assured that these mutants were equally accessible to the mitochondrial import apparatus, they, along with native pALDH, were treated with trypsin followed by SDS-PAGE. The intensity corresponding to the undigested protein was plotted versus digestion time (Fig. 4A), as was done previously (22, 23). These plots show that both mutants were about as susceptible to trypsin digestion as the native precursor. Closer inspection of the gels revealed that trypsinolysis of the native and R3Q/R10Q mutant precursors produced a fragment that was 1–2 kDa smaller than the full-length protein (Fig. 4B). This fragment was not observed on the gel derived from the trypsin digestion of R14Q/R17Q. Therefore, it appears that pALDH and R3Q/R10Q were cleaved after either Arg-14 or Arg-17 as expected if their N termini were available to interact with components of the mitochondrial import system.

Binding to Mitochondrial Membranes—One reason for lack of import competence could be that a precursor protein did not interact with the mitochondrial surface. Import assays that were not treated with proteinase K appeared to show, after

![Fig. 4. Trypsin digest of import-incompetent precursor proteins. R3Q/R10Q and R14Q/R17Q, mutant precursors that were poorly imported, and pALDH were translated for 30 min and then treated with trypsin at 0 °C. Digestion was halted after 1, 5, 10, and 20 min, and the solutions were run on SDS-PAGE along with a 0 min control. A, the loss of undigested protein as a function of time for pALDH (●), R3Q/R10Q (∅), and R14Q/R17Q (○). B, profile view of gels for pALDH, R3Q/R10Q, and R14Q/R17Q after a 1-min exposure to trypsin. The origin of the distance scale was varied with each plot to allow the peaks to be aligned. The large peak at 3.5 mm is due to the remaining undigested protein. The smaller peak at 4.5 mm in the pALDH and R3Q/R10Q plots indicates the presence of a digestion product that is approximately 2 kDa smaller than the full-length protein. A comparable peak is not apparent in the R14Q/R17Q plot.](http://www.jbc.org/Downloaded from)
peptides bound SUVs with higher affinity. Therefore, the R3Q/R10Q peptide cannot be distinguished from the other peptides that represent functional presequences on the basis of lipid binding ability.

Liposome binding of the ALDH(−RGP) peptide was also measured because the ALDH(−RGP) precursor was imported better than the native precursor. In both 50 mM and 5 mM phosphate, the ALDH(−RGP) peptide had a 2-fold greater affinity for SUVs than the other peptides.

Circular Dichroism Studies of Leader Peptides—To determine whether intrinsic conformational preferences could be associated with the loss of import competence by R3Q/R10Q or the loss of processing by R17Q, synthetic peptides corresponding to these sequences were analyzed by CD spectroscopy. For comparative purposes, peptides corresponding to the native sequence, ALDH(−RGP), and the R14Q mutant were also analyzed. Each peptide displayed the spectrum of a predominantly random coil peptide in aqueous buffer, consistent with what we have previously shown by CD and NMR spectroscopy (9, 26). When CD spectra were obtained as a function of trifluoroethanol concentration, an absorbance maximum at approximately 192 nm, a minimum at approximately 204 nm, and a pronounced shoulder at 222 nm appeared as trifluoroethanol concentration increased. These spectral features are associated with an α-helical conformation. With the exception of the spectra in 0% trifluoroethanol, the series of spectra for each peptide had isodichroic points near 203 nm, as we have shown before for similar peptides (21). The isodichroic point is indicative of a two-state equilibrium between unfolded and folded structures.

For each of the peptides, the mean residue ellipticity at 222 nm ([θ]222) increased in magnitude as the proportion of trifluoroethanol was increased. The mean residue ellipticity and corresponding helicity estimated in 0, 20, and 40% trifluoroethanol for the peptides are shown in Table II. As the percentage of trifluoroethanol increased, the R3Q/R10Q and R14Q peptides appeared to become somewhat more helical than the native peptide. In general, though, the peptides displayed only small differences in helix-forming ability with the exception of the ALDH(−RGP) peptide. The ALDH(−RGP) mutant had greater import efficiency than native pALDH, suggesting that enhanced helix propensity in the presequence could also enhance import function. The R14Q and R17Q mutants were imported, and the R3Q/R10Q mutant was not imported, yet these three peptides demonstrated similar abilities to form secondary structure.

Sequence-Specific Secondary Structure Formation—Circular dichroism spectroscopy provides information on the gross secondary structure of a polypeptide. Differences in helicity of the presequence peptides could be in one or both helical segments. Since import competence has been related to the presence of a stable helical structure near the N terminus, it was desirable to determine where the differences detected in the CD spectra of
the R3Q/R10Q peptide occurred. To detect sequence-specific differences in the helical structure of the native ALDH, R3Q/R10Q, and R17Q peptides, NMR spectra were acquired in 40% trifluoroethanol. Chemical shifts were determined from TOCSY and NOESY spectra.

The "fingerprint" regions of the NOESY spectra for each peptide, showing interactions between NH and H-α protons, are compared in Fig. 6. The spectra showed that the largest NH chemical shift differences occurred in the Gln residues or in residues three or four positions from them. The largest change observed was for Gln-3 NH in the R3Q/R10Q peptide, which moved 0.36 ppm downfield. Typically, Arg and Gln NH chemical shifts are of the same magnitude in both peptides and proteins (36). The large downfield move of the Gln-3 NH resonance was due to a significant change in the electronic environment of this amide proton and could indicate a change in conformational preference of the R3Q/R10Q peptide near the N terminus. The chemical shift of Arg-14 NH was moved upfield by the replacement of Arg-10 in the R3Q/R10Q peptide but was moved downfield by the replacement of Arg-17 in the R17Q peptide. The spacing of 3 or 4 residues indicates that these Arg side chains exerted some influence on the environment of the amide protons in adjacent turns of the helical structure.

The NOESY spectra of each peptide in 40% trifluoroethanol showed that the secondary structures were similar to that which has been reported for the ALDH peptide in the presence of micelles (9). However, the three NOESY spectra did not have a sufficient number of resolved resonances in common to use NOEs for a thorough comparison of secondary structure characteristics. To assess helical content as a function of sequence, -proton chemical shifts (dH-α) for each residue in the ALDH, R17Q, and R3Q/R10Q peptides were compared with the value expected to be found in a random coil (36). For peptides in a helical conformation, dH-α has been shown to be upfield from the random coil value, leading to a negative difference between the experimental dH-α and the random coil value for a particular amino acid (ΔdH-α). A series of consecutive negative values in the amino acid sequence has been used to identify helices in peptides and proteins (37). The results of these comparisons for the R3Q/R10Q and R17Q peptides are plotted in Fig. 7 along with comparative values for the native peptide. Because of the series of consecutive negative ΔdH-α values than the native peptide from Thr-8 to Arg-14, suggesting that the helical structure may be somewhat longer than that of the other peptides. The ΔdH-α values of the R17Q peptide did not display large differences from the native peptide. These results are consistent with the CD data.

It was shown previously (15) that the peptide without the RGP linker segment was capable of forming a longer, more stable helix than the native peptide in the presence of micelles. Data previously obtained for the ALDH(-RGP) peptide (38) were included in Fig. 7, confirming that this peptide was clearly the most helical of the peptides studied in 40% trifluoroethanol. According to the chemical shift data, the greater tendency for helix formation extended from Arg-3 to the C terminus. The differences displayed by the R3Q/R10Q peptide were not as large as those of the ALDH(-RGP) peptide and were in the center of the peptide rather than near the N terminus. These observations support the hypothesis that helical structure near the N terminus is essential for import. As shown by the R3Q/R10Q peptide, the location of increased...
helicity was important. An increase in helicity that did not involve the residues near the N terminus was unable to overcome the loss of function caused by replacement of the two Arg residues.

**DISCUSSION**

The presence of positively charged residues is a common feature of mitochondrial presequences. These charges are distributed rather uniformly over the presequence, which typically consists of 20–30 amino acid residues. A data base of 33 known matrix protein presequences was built from native sequences we have used and a selection of published sequences (39). An analysis of these presequences reveals that there are, on average, 3.6 Arg (18%) and 1.8 Lys (9%) residues in the first 20 amino acids. The Swiss Protein Data Bank (version 32) shows that Arg and Lys make up 5.2 and 5.9% of the total amino acid composition, respectively. These statistics show that the average presequence is unusually rich in positively charged residues, especially Arg. What, then, is the contribution of the positive charges in making a functional mitochondrial presequence? It could be to bind to the negatively charged surface of the mitochondrial membrane (40). Alternatively, the presequence could interact with negatively charged regions of import receptor/translocator proteins such as Tom20 or Tom22 (2–4). The extent of import of precursor proteins that require Tom20 for import has been shown to depend inversely on the concentration of salt in the import assay solution (3). Salt concentration dependence was not found for precursor proteins that require the Tom37-Tom70 heterodimer. These results suggest that positive charge is important in one class of mitochondrial presequences but is not a universal requirement. More recently, the deletion of Tom22 and Tom22 domains that carry a negative charge has been shown to lead to a loss of import function (4). It has also been suggested that MPP contains a negatively charged surface, which is critical for the binding of precursor proteins and subsequent cleavage of the presequence (5).

Recently, a R10P mutation in the presequence of human mitochondrial pyruvate dehydrogenase E1 α has been identified (41). A protein attached to the mutant presequence was imported in vitro at a reduced rate when compared with the native presequence. The presequence of pyruvate dehydrogenase also contains positively charged residues at positions 2 and 3. The effect on import could be due to the loss of the positive charge and/or a change in presequence conformation.

Since mitochondrial membranes possess a negative surface charge, the positive charges in the presequence could function by directing precursor proteins to the organelle. It has been argued that the extent of binding of model peptides to negatively charged membranes is governed by electrostatic interactions and subsequently stabilized by hydrophobic interactions (16, 40). Negative surfaces have been identified on several proteins that appear to be involved in import, so presequence recognition could be on the basis of electrostatic attraction. An alternative mode of action of positive charges is that they assist in the induction of helical structure. All of the presequences that have been studied show a tendency to form helices when placed in an environment that favors the adoption of secondary structure. It is possible that membrane-binding induces helix formation, so that if the helix is the bioactive conformation of the presequence, it would be allowed to interact correctly with import receptors.

In this study positive charges at different positions in the presequence of mitochondrial ALDH have been removed to study the effect on translocation of pALDH into rat liver mitochondria. The replacement of either Arg by Gln in the N-terminal segment did not have a significant effect on import competence. Replacement of both N-terminal Arg residues (R3Q/R10Q) produced a precursor that was poorly imported. Therefore, one charge in the N-terminal portion of the ALDH presequence appears to be necessary for import to occur. Elsewhere, we have shown that the C-terminal portion of the ALDH presequence, which contains three Leu residues, binds preferentially to artificial membranes (9, 15). The replacement of either Arg by Gln in the C-terminal segment did not have a significant effect on import competence, while replacement of both Arg residues led to a significant loss of import competence. The R14Q/R17Q mutation, which occurs in the putative membrane-binding region, could result in a loss of import competence that is due to the inability of the precursor to interact with the mitochondrial membrane. Although the data were inconclusive, the fraction of R14Q/R17Q precursor that reached the mitochondria was consistently among the lowest of all the mutants studied. Therefore, it is possible that membrane binding ability is a distinguishing factor for this mutant, as it was with an ALDH- cytochrome c oxidase chimeric presequence (18).

Based on previous work with chimeric presequences, we concluded that a stable N-terminal α-helix is required for import. Moreover, in the case of ALDH, the C-terminal helix stabilizes the N-terminal one (16). In this study, we used different approaches to provide evidence to support the helix stability argument. First, import competence was substantially restored to the R3Q/R10Q mutant and increased in the R14Q/R17Q mutant by the removal of the linker segment from the presequence. The RGP deletion was expected to allow the presequence to form a longer, more stable α-helix (15), which could overcome the effects resulting from the loss of two charged side chains. Secondly, both the CD and NMR data showed that the helix-forming potential of the ALDH(−RGP) peptide was greater than that of the other sequences. The NMR data revealed that the relative helix stabilization was nearly the entire length of the peptide. Although CD spectra showed the R3Q/R10Q peptide to have a slightly higher helix propensity than the native peptide, the NMR data indicated that the stabilization occurred in the middle of the peptide, and not near the N terminus. Thus, we conclude that not only is helix length important for import, but the helix location is important as well.

The role of secondary structure requires further definition as a result of these studies. The polypeptide segments do not appear capable of forming significant amounts of secondary structure unless it is induced by environmental conditions. In mitochondrial import, the necessary environment could be a protein of the outer membrane or the membrane itself. The ability of a sequence to adopt a helical structure when induced may determine whether or not import will occur. As we have shown, removing the RGP linker segment leads to an increase in helix-forming ability and a coincident increase in import competence. Additionally, the removal of the linker enabled the precursor to overcome the loss of import competence resulting from the replacement of two charged residues. This presequence did not contain a positive charge until Arg-11, thereby calling into question the absolute need for the N terminus of the presequence to contain a positive charge. In light of this observation, the role of negatively charged surfaces on import receptors may have to be studied in greater detail.

Consensus sequences for presequence processing have been proposed (32). The presence of an arginine two or three residues from the cleavage site appears to be significant. We found that both the R17Q and R14Q/R17Q mutants were not processed by isolated MPP. Therefore, Arg-17 may be a necessary residue for the recognition of the processing site by MPP.

Different regions of the presequence appear to contain unique information that is necessary for its function. For
ALDH it appears the N-terminal segment is needed for translocation through the membrane, while the C-terminal segment seems to be involved in both binding to membranes and interaction with the processing enzyme. Elsewhere,2 data will be presented to show that the processing information does indeed lie in the C-terminal segment. All presequences do not possess distinct domains as does ALDH. Conceivably, the information necessary for binding, translocation, and processing still resides in different portions of these presequences.

Mitochondrial protein import is a complex process. It is generally agreed that proteins must retain a loosely folded conformation to traverse biological membranes. In recent years, several ATP-dependent cytosolic factors have been identified that stabilize precursor proteins in the cytosol, preventing their folding or aggregation (42). The specific recognition of import-competent precursor proteins by receptor proteins in the outer mitochondrial membrane is apparently sufficient for correct import. An alteration of the presequence disfavors any one of these interactions. Nonetheless, we have found that each, or pairs, of the positive charges in the N terminus. We have presented evidence, here and elsewhere (16), to suggest that the reduction of helicity in the N-terminal portion of the ALDH presequence leads to a neutralizing charge that is not competent for mitochondrial import but still binds and is processed.

REFERENCES

1. von Heijne, G., Steppuhn, J., and Herrmann, R. G. (1989) Eur. J. Biochem. 180, 535-545
2. Nargang, F. E., Künkele, K.-P., Mayer, A., Ritzel, R. G., Neupert, W., and Lill, R. (1995) EMBO J. 14, 1099-1108
3. Hauke, V., Lithgow, T., Rospert, S., Hahne, K., and Schatz, G. (1995) J. Biol. Chem. 270, 5565-5570
4. Bolliger, L., Junne, T., Schatz, G., and Lithgow, T. (1995) EMBO J. 14, 6318-6326
5. Pace, V., Rosenberg, L. E., Fenton, W. A., and Kalousek, F. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 5355-5358
6. Rôse, D., Theiler, F., Horvath, S. J., Tomich, J. M., Richards, J. H., Allison, D. S., and Schatz, G. (1988) EMBO J. 7, 649-653
7. Hammel, P. K., Gorenstein, D. G., and Weiner, H. (1996) Biochemistry 35, 3772-3781
8. Endo, T., Shimada, I., Roise, D., and Inagaki, F. (1989) J. Biochem. 106, 396-400
9. Karslake, C., Piotto, M., Pak, Y. K., Weiner, H., and Gorenstein, D. G. (1990) Biochemistry 29, 9872-9878
10. Hoyt, D. W., Cyr, D. M., Gierasch, L. M., and Douglas, M. G. (1991) J. Biol. Chem. 266, 21693-21699
11. MacLachlan, L., Haris, P. I., Reid, D. G., White, J., Chapman D., Lucy, J. A., and Austen, B. M. (1994) Biochem. J. 303, 657-662
12. Jarvis, J. A., Ryan, M. T., Hoogenraad, N. J., Craik, D. J., and Høj, P. B. (1995) J. Biol. Chem. 270, 1323-1331
13. Chiu, T. W., Grant, P. M., and Strauss, A. W. (1987) J. Biol. Chem. 262, 12806-12811
14. Horwich, A. L., Kalousek, F., Fenton, W. A., Pollock, R. A., and Rosenberg, L. E. (1986) Cell 44, 451-459
15. Thornton, K., Wang, Y., Weiner, H., and Gorenstein, D. G. (1993) J. Biol. Chem. 268, 19096-19102
16. Wang, Y., and Weiner, H. (1993) J. Biol. Chem. 268, 4759-4765
17. Tamm, L. K., and Bartoldus, I. (1990) FEBS Lett. 272, 29-33
18. Wang, Y., and Weiner, H. (1994) Biochemistry 33, 12860-12867
19. Wang, T. Y., Farré, J., and Weiner, H. (1989) Arch. Biochem. Biophys. 272, 440-449
20. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 5463-5467
21. Pak, Y. K., and Weiner, H. (1990) J. Biol. Chem. 265, 14298-14307
22. Mattingly, J. R., Youssef, J., Iriarte, A., and Martinez-Carrion, M. (1993) J. Biol. Chem. 268, 3925-3927
23. Wallner, M., and Weiner, H. (1995) J. Biol. Chem. 270, 26311-26317
24. Hammen, P. K., Gorenstein, D. G., and Weiner, H. (1994) Biochemistry 33, 8610-8617
25. Park, S.-H., Shalongo, W., and Stellwagen, E. (1993) Biochemistry 32, 12901-12905
26. States, D. J., Haberkorn, R. A., and Rubeen, D. J. (1982) J. Magn. Reson. 48, 286-292
27. Bax, A., and Davis, D. G. (1985) J. Magn. Reson. 65, 355-360
28. Braunschweiler, L., and Ernst, R. R. (1983) J. Magn. Reson. 53, 521-528
29. Pietrini, U., Sørensen, O. W., and Ernst, R. R. (1992) Am. Chem. Soc. 104, 6800-6801
30. Rance, M., Sørensen, O. W., Bodenhausen, G., Wagner, G., Ernst, R. R. (1983) Biochim. Biophys. Res. Commun. 117, 479-485
31. Lammli, U. K. (1970) Nature 227, 680-685
32. Gavel, A., and von Heijne, G. (1990) Protein Eng. 4, 33-37
33. Zhou, J., Bai, Y., and Weiner, H. (1995) J. Biol. Chem. 270, 16689-16693
34. Swanson, S. T., and Roise, D. (1992) Biochemistry 31, 5746-5751
35. Joles, J. A., and Gierasch, L. M. (1994) Biochem. J. 297, 1546-1561
36. Wishart, D. S., Sykes, B. D., and Richards, F. M. (1991) J. Mol. Biol. 222, 311-333
37. Wishart, D. S., Sykes, B. D., and Richards, F. M. (1992) Biochemistry 31, 1647-1651
38. Thornton, K. H. (1983) 2D NMR Structural Studies of Peptides in Micelle and 40% TFE. Ph.D. dissertation, Purdue University, West Lafayette, IN
39. Hartl, F. U., Pfanner, N., Nicholson, D. W., and Neupert, W. (1989) Biochim. Biophys. Acta 988, 1-45
40. de Kroon, A. T. P., Diederik, J., and de Kruijf, B. (1993) in New Comp. Biochemistry, Vol. 25 (Watts, A., ed) pp. 107-127, Elsevier Science Publishers, Amsterdam
41. Takakubo, F., Cartwright, P., Hoogenraad, N., Thorburn, D. R., Collins, F., Lithgow, T., and Dahl, H. H. (1995) Am. J. Hum. Genet. 57, 722-730
42. Langer, T., and Neupert, W. (1994) Chaperoning Mitochondrial Biogenesis: The Biology of Heat Shock Proteins and Molecular Chaperones (Morimoto, R. I., Tissieres, A., and Geogopoulos, C., eds) pp. 53-83, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

2 M. Waltner and H. Weiner, submitted for publication.
The Role of Positive Charges and Structural Segments in the Presequence of Rat Liver Aldehyde Dehydrogenase in Import into Mitochondria
Philip K. Hammen, Mary Waltner, Birger Hahinemman, Thomas S. Heard and Henry Weiner

J. Biol. Chem. 1996, 271:21041-21048.
doi: 10.1074/jbc.271.35.21041

Access the most updated version of this article at http://www.jbc.org/content/271/35/21041

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 40 references, 13 of which can be accessed free at http://www.jbc.org/content/271/35/21041.full.html#ref-list-1