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2D NMR and Structural Model for a Mitochondrial Signal Peptide Bound to a Micelle†

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ABSTRACT: The 19 amino acid signal peptide of rat liver aldehyde dehydrogenase, possessing a lysine substitution for an arginine and containing 3 extra amino acid residues at the C terminus, was studied by two-dimensional NMR in a dodecylphosphocholine micelle. In this membrane-like environment, the peptide contains two α-helical regions, both of which are amphiphilic, separated by a hinge region. The helix located closer to the C terminus is more stable than is the helix located near the N terminus. This suggests that the hydrophobic face of the C-terminal helix is buried within the hydrophobic region of the micelle. On the basis of these results a general model for protein translocation is presented in which the C-terminal amphiphilic helix of the signal region in the preprotein first binds to the mitochondrial membrane and then diffuses to the translocation receptor. The receptor then recognizes the N-terminal helix of the signal region, which is not anchored to the membrane. To explain how this signal peptide was imported into isolated mitochondria in the absence of energy or receptor protein [Pak, Y. K., & Weiner, H. (1990) *J. Biol. Chem. 265*, 14298–14307], a model for signal peptide translocation across a membrane barrier without the need for auxiliary membrane proteins is proposed. In this model the faces of the two helices fold upon each other, resulting in the mutual shielding of positively charged residues by the complementary hydrophilic face of the other amphiphilic helix.

Most mitochondrial preproteins are coded by nuclear genes so they are synthesized in the cytosol as preproteins and translocated to the destined location. Preproteins are composed of the mature protein plus an N-terminal extension of a signal peptide containing 17–50 amino acid residues (von Heijne et al., 1989; Watson, 1984). The signal peptide contains the information necessary for the proteins to be transported into mitochondria. In fact, when the signal sequence of a mitochondrial protein is attached to a cytosolic protein, the new fusion protein is transported into the mitochondria (Hurt et al., 1984). No amino acid homology exists between signal peptides, though they are typically rich in basic and hydroxylated amino acids and lack acidic residues (Roise et al., 1988; von Heijne, 1986).

Although the exact mechanism of precursor protein import is not known, it is thought that the precursor initially binds to the mitochondrial outer membrane and then migrates to a receptor/translocator to be imported. The structure of the signal peptide is thought to be important for these events to occur. Little detailed structural information, however, has been presented for the signal peptides. To investigate the potential structure of related synthetic peptides, circular dichroism (CD) (Briggs & Gierasch, 1984; Shinnar & Kaiser, 1984), attenuated total reflectance–Fourier transform infrared spectroscopy (Cornell et al., 1989), and more recently nuclear magnetic resonance spectroscopy (NMR) (Bruch et al., 1989) has been studied. With mitochondrial signal peptides CD data showed that in aqueous solutions the peptides exist in a random conformation, while in hydrophobic solvents they can form α-helices or β-sheets (Roise et al., 1988; Pak & Weiner, 1990). Helical wheel construction revealed that the helix is amphiphilic and has a high hydrophobic moment (von Heijne, 1986).

Unfortunately, while CD provides an estimate of the fraction of various secondary structural elements, it does not provide any information on the location of these structures. Recently two-dimensional NMR has been used to identify structural elements of a number of small peptides in aqueous solution (Kim & Baldwin, 1984; Roongta et al., 1989; Shoemaker et al., 1987). In general, most small linear peptides exhibit little stable secondary structure in purely aqueous solution (Epand & Scheraga, 1968; Taniuchi & Anfinsen, 1969). Indeed, the Zimm–Bragg theory (Zimm & Bragg, 1959) would suggest that peptides shorter than 20 residues should not form much α-helical structure in aqueous solution. Thus, it is important that the structure of the signal peptide be determined in a membrane-like environment to mimic what might be occurring on the surface of the bilayer. Organic solvents such as trifluoroethanol or methanol have been used in both CD and NMR studies to induce secondary structure (Bazzo et al., 1988) and to simulate the hydrophobic environment of the bilayer membrane (Bruch et al., 1989). A phospholipid micelle would appear to be a better environment for studying the structure. The presence of micelles, though, increases the difficulty of studying peptide structure by NMR since it increases the line width of the ^{1}H signals. However, NMR has been able to define the solution structure of both mellitin (Brown et al., 1982; Inagaki et al., 1989) and glucagon (Wider et al., 1982) bound to dodecylphosphocholine micelles.

One of our laboratories has been studying the import of prealdehyde dehydrogenase into mitochondria. The preprotein possesses a 19-residue signal peptide (Farres et al., 1988) necessary for its transport into the mitochondrial matrix, the site of its activity. The import of both the precursor protein and the synthetic signal peptide into mitochondria has been

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studied (Pak & Weiner, 1990; Wang et al., 1989). Unlike precursor protein, the synthetic peptide is imported in the absence of the receptor/translocator (Pak & Weiner, 1990). Thus, the question arises as to how such a hydrophilic peptide with five basic residues can be transported across a hydrophobic membrane.

In this paper, we report the structure of a 22-residue synthetic prealdehyde dehydrogenase (ALDH) peptide with the sequence NH_2-MLRAALSTARKGPRLSRLLSYA-$CONH_2$. This signal sequence is based upon the rat prealdehyde dehydrogenase protein in which three additional residues at the C terminus have been added to simulate the beginning of the mature protein. The structure of this peptide has been studied by two-dimensional NMR in a dodecylphosphocholine micelle to understand how the signal peptide crosses the mitochondrial membrane. On the basis of these results a general model is presented for preprotein binding to mitochondrial as well as other possible cell membranes and recognition by protein translocation receptors.

Materials and Methods

Peptide Synthesis and Purification. The signal peptide of rat aldehyde dehydrogenase containing three extra residues at the C terminus was synthesized by the Purdue Peptide Synthesis Facility. A semipreparative Vydac C-18 reverse-phase HPLC column was utilized for purification. Purity was confirmed by amino acid analysis, FAB mass spectrometry, and amino acid sequencing. Purity was also confirmed by the detailed ^1H NMR assignments and observation of only a few low-intensity signals integrating for less than 10% of the main peaks (see below). This level of impurities did not compromise the conformational analysis.

Circular Dichroism. Studies were performed on a Jasco 600 CD/ORD. Cellular path lengths were either 0.1 or 1 mm at 25 °C. Dodecylphosphocholine was obtained from Avanti Polar Lipids, ethanol was from Fisher Scientific, and myristic acid and SDS were from Sigma Chemical. The data were analyzed with the aid of a secondary structure deconvolution program (Yang et al., 1986).

NMR. Typically, 63.6 mg of deuterated dodecylphosphocholine (MSD Isotopes) and 8.5 mg of ALDH peptide were dissolved in 0.7 mL of 50 mM NaP_i, pH 6.0, buffer, treated with Chelex 100 ion-exchange resin, and repeatedly lyophilized in either D_2O or 90%/10% H_2O/D_2O. The NH-exchange NOESY (States et al., 1982) experiments were recorded at pH 3.3 (uncorrected). Spectra were recorded at ambient temperature on either a Varian VXR 500 or 600 spectrometer operating at proton frequencies of 500 and 600 MHz, respectively. The sweep width was set to 6000 Hz for experiments run on the VXR 500. All spectra were referenced to HDO at 4.76 ppm. Water suppression was attained by irradiation of the HOD signal during the relaxation delay of all NMR experiments as well as the mixing period of the pure absorption phase NOESY experiments. The two-dimensional experiments were recorded with 16 transients and resolution of 256 points in the t_1 dimension and 2048 points in the t_2 dimension. Zero-filling to 2K by 2K was performed on all experiments. A sine-bell window was used in both dimensions except for the NH titration experiment, which employed Gaussian functions in both dimensions for processing. The mixing times for the NOESY experiments were 50, 100, 150, 200, and 300 ms. The TOCSY experiments (Bax & Davis, 1985; Braunschweiler & Ernst, 1983) had mixing times of 50 and 100 ms. Data sets were processed on SUN 4 workstations utilizing VNMR software from Varian.

Minimization and Molecular Dynamics. A model structure for the ALDH peptide was built and manipulated on a Silicon Graphics Iris 3030 workstation utilizing UCSF MIDAS software (Langridge & Ferrin, 1984). Minimization and molecular dynamics were performed by using a modified version of AMBER (Weiner & Kollman, 1981), which contains a flat well pseudopotential instead of a harmonic potential to constrain the peptide with the NOE distances (Nikonowicz et al., 1989). A total of 44 interresidue NOEs derived from a 50-ms NOESY spectrum was used to constrain the ALDH peptide. These NOEs were categorized into strong (<2.5 Å), medium-strong (2.5–3.0 Å), medium (3.0–4.0 Å), and weak (>4.0 Å) according to the averaged intensity of volumes on both sides of the diagonal.

Results

Circular Dichroism. It was previously shown that the CD spectrum for the ALDH signal peptide in aqueous buffer was that of a random coil but in SDS or TFE was that of a 45% α-helical peptide (on the basis of the molar ellipticity at 222 nm; Pak & Weiner, 1990). Computer deconvolution analysis of the CD spectrum of a solution of 0.1 mg/mL ALDH peptide in 20 mM SDS and 10 mM NaP_i suggests that the peptide is ca. 25% helical (data not shown). Several other micelle-forming lipids were tested to determine the feasibility of using a micellar environment in which to study the structure of ALDH peptide. Addition of ALDH peptide to myristic acid caused precipitation of the micelles. Even after heating to 60 °C, precipitate remained. Indeed, amphiphilic helices are known to possess fusogenic properties when added to micelles (Briggs et al., 1985). Dodecylphosphocholine did not precipitate upon addition of ALDH peptide. Further studies were conducted to determine the ratio of ALDH peptide to dodecylphosphocholine that would produce the highest α-helical content. Molar ratios of 1:5, 1:10, 1:20, 1:40, and 1:100 ALDH peptide/dodecylphosphocholine were evaluated. A 1:40 molar ratio resulted in the highest α-helical content (26%) as well as limited the NMR signals from the undeuterated lipid. Since the 1:40 ratio produced the best results, this ratio was used for future experiments, and the CD spectrum of the peptide in this micelle is shown in Figure 1. The spectrum was unaltered (±2.5%) over an 8-fold dilution with buffer. This approximate ratio was also used for studying melittin and glucagon in dodecylphosphocholine micelles (Inagaki et al., 1989; Wider et al., 1982).

NMR Assignments. Most of the ^1H NMR signals of the ALDH peptide in the micelle were assigned through pure absorption phase 2D NMR spectra in H_2O and D_2O by using the sequential assignment methodology developed by Wüthrich and co-workers (Wüthrich, 1986). Significantly, the NOEs were large and negative, indicating that the peptide was not a random coil in free solution and that a significant population of the peptide was bound to the micelle. Additional data supporting the non-random-coil structure for the peptide fragment in the micellar solution derived from the chemical shifts of the molecule. As shown in Figure 2, the chemical shifts of many of the residues differ significantly from the "random" coil conformation chemical shifts (Wüthrich, 1986). Most of the chemical shifts of the peptide in the micellar solution are upfield of the chemical shifts of the random coil conformation. In contrast, the chemical shifts of the assigned signals of the ALDH peptide in purely aqueous solution were quite similar to those of the residues in a random coil conformation (data not shown).

We have used both the TOCSY (through bond) and NOESY (through space) NMR spectra in H_2O to first identify the various spin systems of the different amino acid

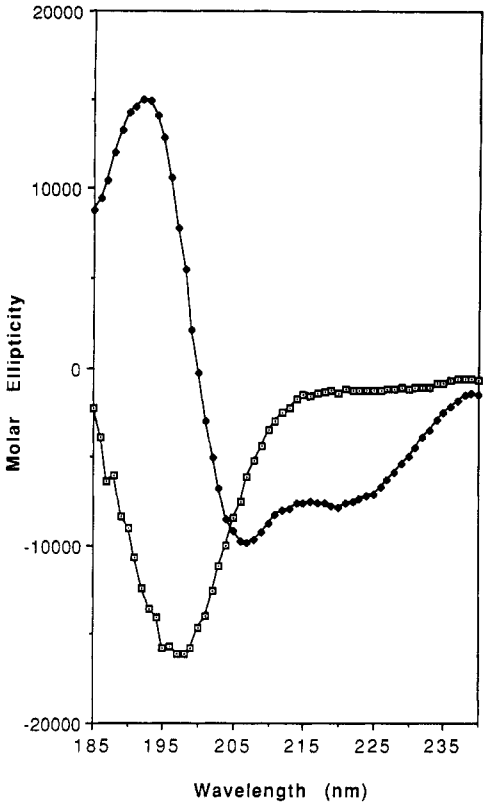

FIGURE 1: Circular dichroic spectra of the N-terminal 22-residue peptide fragment of prealdehyde dehydrogenase (ALDH peptide) in aqueous buffer (□) and in a dodecylphosphocholine micelle (1:40 molar ratio of peptide to dodecylphosphocholine), at 25 °C, 50 mM sodium phosphate, pH 6.0 (◆).

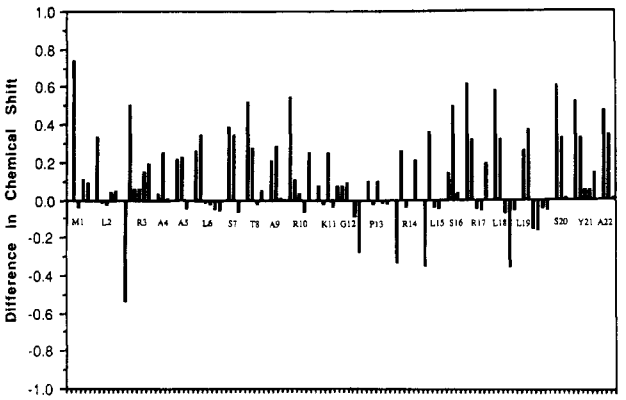

FIGURE 2: Difference between the ^1H chemical shifts of ALDH peptide in a dodecylphosphocholine micelle and the predicted chemical shifts in a random coil conformation plotted against sequence. The order of entry of the bar plots for each residue follows NH_α, CH_α, CH_β, CH_γ, CH_δ, and CH_ϵ (where appropriate) with a blank entry indicating the start of a new residue. Missing assignments (see Table I) are also represented by a blank. Positive differences indicate the chemical shift of the ALDH peptide proton in the micelle is upfield of the chemical shifts of the random coil conformation.

residues in the peptide. The TOCSY spectra show connectivity between the $N_\alpha H_i$, $C_\alpha H_i$, $C_\beta H_i$, and $C_\gamma H_i$ protons (Wüthrich, 1986). A portion of the pure absorption phase H_2O TOCSY and NOESY spectra in the important "fingerprint" $N_\alpha H$ and $C_\alpha H$ region is shown in Figure 3.

The sequential connectivities across the peptide bond and assignments of the amino acid spin systems have been established by these 2D NMR spectra. Thus, while there is no J (and hence no TOCSY) connectivity between $C_\alpha H_i$ and $N_\alpha H_{i+1}$ protons and between $C_\beta H_i$ and $N_\alpha H_{i+1}$ protons, in many conformations of the oligopeptide, these protons are often within 4 Å of each other and hence show NOESY crosspeaks.

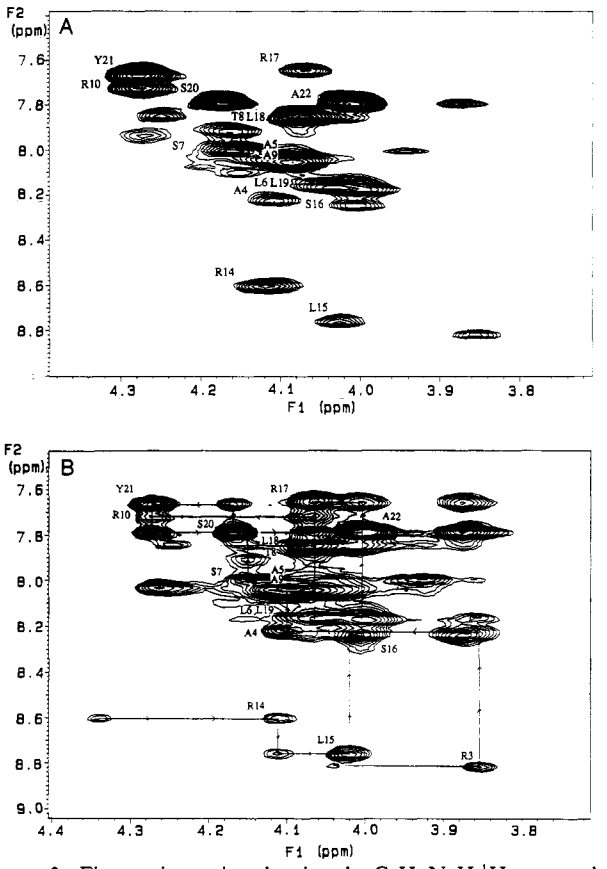

FIGURE 3: Fingerprint region showing the $C_\alpha H$–$N_\alpha H$ ^1H crosspeaks of the pure absorption phase H_2O 2D TOCSY (A) and NOESY (B) spectra of the N-terminal 22-residue peptide fragment of prealdehyde dehydrogenase in dodecylphosphocholine micellar solution, pH 6.0. The sequential connectivity and assignments are diagrammed in (B).

In this fingerprint region (Figure 3), we can walk along the peptide backbone since both NOESY and TOCSY crosspeaks in general exist between intraresidue $C_\alpha H_i$ and $N_\alpha H_i$ protons. Only the NOESY fingerprint spectrum shows crosspeaks between the $C_\alpha H_i$ and $N_\alpha H_{i+1}$ residues. By this sequential connectivity between the TOCSY and NOESY crosspeaks in Figure 3 and various other spectra not shown, we have assigned the proton signals of the fragment. The $C_\beta H_i$ to $N_\alpha H_{i+1}$ to H_2O NOESY crosspeaks (spectra not shown) were particularly helpful in confirming the sequential assignments. The proton assignments are listed in Table I.

Secondary Structure. The NOESY spectrum provides strong support for elements of secondary structure in the peptide. The NH/NH NOESY region (Figure 4) is particularly informative. As shown in Figure 4, a striking network of crosspeaks is observed from residues 14 to 21 as well as a second network of $N_\alpha H_i$–$N_\alpha H_{i+1}$ crosspeaks from residues 3 to 10.

This pattern of NOE crosspeaks between the NH protons is consistent with two α-helical segments in the peptide. In a regular α-helix the distance between the $C_\alpha H_i$ and the $N_\alpha H_{i+1}$ protons is near the maximum possible separation of 3.5 Å, while the $N_\alpha H_i$–$N_\alpha H_{i+1}$ distance is 2.8 Å. In an extended conformation such as β-sheet, the $C_\alpha H_i$–NH_{i+1} distance is 2.2 Å and the NH_i–NH_{i+1} distance is 4.3 Å (Wüthrich, 1986); in random coil regions, $N_\alpha H_i$–$N_\alpha H_{i+1}$ connectivities are rarely observed (Roongta et al., 1989).

Thus, two separate $N_\alpha H_i$–$N_\alpha H_{i+1}$ α-helical connectivities were noted, one being of much greater intensity than the other. These NH connectivities aided in the initial sequential assignments because Y21 had been unambiguously assigned, and

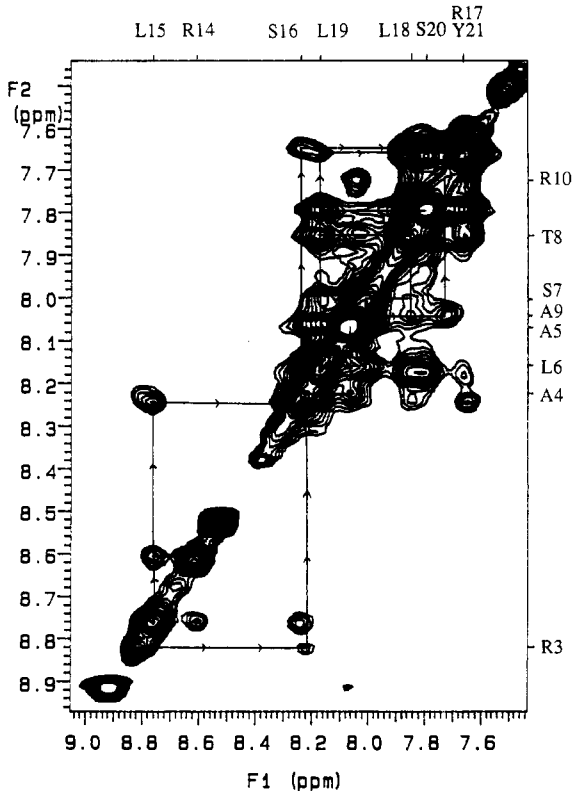

FIGURE 4: Amide NH region of the H_2O pure absorption phase NOESY spectrum of ALDH peptide. $N_\alpha H_i$–$N_\alpha H_{i+1}$ connectivities are noted between indicated residues of the N-terminal and the C-terminal helix, suggestive of α-helical character.

thus the connectivity of the C-terminal segment was assigned to the higher intensity sequential set of $N_\alpha H_i$–$N_\alpha H_{i+1}$ crosspeaks while the N-terminal segment was assigned to the lower intensity set. Finally, these assignments agreed with those obtained by the sequential assignment method.

Another criterion often used to confirm secondary structure is the three bond coupling constant, $^3J_{HN-C\alpha H}$. For a regular α-helix, 3_{10}-helix, antiparallel β-sheet, and parallel β-sheet the values are 3.9, 4.2, 8.9, and 9.7, respectively (Wüthrich, 1986). Unfortunately, these spin–spin coupling constants could not be measured due to the extensive line broadening observed in the micellar environment.

The $C_\alpha H_i$–$N_\alpha H_{i+3}$ and $C_\alpha H_i$–$N_\alpha H_{i+4}$ distances (3.4 and 4.2 Å, respectively) are also short in α-helices (Wüthrich, 1986). Unfortunately, many $C_\alpha H_i$ to $N_\alpha H_{i+3}$ or $N_\alpha H_{i+4}$ crosspeaks, indicative of α-helical structure, overlapped with other crosspeaks but several were well resolved [R3 αH to L6 NH (i, i+3), S16 βH to L19 NH (i, i+3), and R3 αH to S7 NH (i, i+4)]. Additional support for the two α-helical sections is provided by the $C_\beta H_i$–$N_\alpha H_{i+1}$ connectivities, where often these crosspeaks are more intense in α-helical regions (Hahn & Ruterjans, 1985). Either S16 β to R17 (i, i+1) or S20 β to Y21 (i, i+1) displayed strong crosspeaks. Because these crosspeaks are strong even in the 50-ms mixing time NOESY spectrum, these are probably primary NOEs. Thus, the observation of $N_\alpha H_i$–$N_\alpha H_{i+1}$ connectivities and a few nonoverlapping $C_\beta H_i$–$N_\alpha H_{i+1}$, $C_\alpha H_i$–NH_{i+3}, and $C_\alpha H_i$–NH_{i+4} suggest a significant degree of α-helical structure between residues 3–10 and residues 14–21.

An NH-exchange experiment performed at pH 3.3 and room temperature showed that while most of the backbone amide protons were exchanged within 30 min when D_2O was added (spectra not shown), a number of peaks remained in the spectrum considerably longer. In fact, two protons were still present even after 1 day at room temperature. Because of NH overlap in the 1D spectrum, unambiguous identification of the peaks could not be made in the 1D spectra, although these two amide protons were believed to be L18 and L19. These assignments were confirmed by a series of five time-dependent 2D NOESY experiments performed at pH 3.3 and room temperature. The fingerprint region and assignments of remaining unexchanged crosspeaks are shown in Figure 5. Each NOESY experiment lasted 160 min. The 16-min NOESY (Figure 5A) exhibited amide protons for A5, L15 (weak), S16, R17, L18, L19, S20, Y21, and A22. These residues compose almost the entire C-terminal helix. Amide protons were observed for L18, L19, Y21, and A22 in the 178-min NOESY (Figure 5B). These residues are located on the same face of the helix. The 340-, 502-, and 802-min NOESY spectra contained only L18 and L19 crosspeaks (spectra not shown). These experiments allowed us to build a map of the approximate exchange rates of the amide protons and definitely establish that L18 and L19 are the very slowly exchanging residues. The half-life for NH exchange of each residue is listed on the helix wheel representation for the peptide shown in Figure 6.

Table I: Assignments of ALDH Peptide with Dodecylphosphocholine

residue	NH	αH	βH	other
M1	n.o.[a]	3.78	2.04	γCH_2 2.53 ϵCH_3 2.04
L2	n.o.	4.04	1.66	γH 1.66 δCH_3 0.85, 0.90
R3	8.80	3.87	1.83	γCH_2 1.64 δCH_2 3.13 NH n.a.[b]
A4	8.22	4.10	1.38	–
A5	8.03	4.12	1.43	–
L6	8.16	4.03	1.78	γH 1.78 δCH_3 0.85, 0.90
S7	7.99	4.15	3.94	–
T8	7.84	4.07	4.24	CH_3 1.18
A9	8.04	4.06	1.38	–
R10	7.72	4.27	1.76	γCH_2 1.86 δCH_2 3.07 NH n.a.
K11	n.o.	4.28	1.51	γCH_2 1.48, 1.37 δCH_2 1.62 ϵCH_2 2.93 ϵNH_3^+ n.a.
G12	n.o.	4.05, 4.24	–	–
P13	–	4.34	2.30, 1.92	γCH_2 2.04 δCH_2 3.70
R14	8.60	4.12	1.92	γCH_2 1.70 δCH_2 3.11 NH n.a.
L15	8.76	4.02	1.68	γH 1.68 δCH_3 0.85, 0.90
S16	8.24	4.01	3.85	–
R17	7.66	4.06	1.93	γCH_2 1.75 δCH_2 3.13 NH n.a.
L18	7.84	4.06	1.72	γH 1.93 δCH_3 0.85
L19	8.16	4.01	1.80	γH 1.80 δCH_3 0.85, 0.90
S20	7.78	4.17	3.87	–
Y21	7.66	4.27	3.08	2,6H 7.10 3,5H 6.72
A22	7.78	4.01	1.38	–

[a] n.o., not observed. [b] n.a., not assigned.

Discussion

CD and NMR Spectra. The circular dichroic spectra of ALDH peptide (Figure 1; Pak & Weiner, 1990) indicate that the peptide has significant α-helical content only in TFE-like or aqueous micellar solution. This is consistent with the ob-

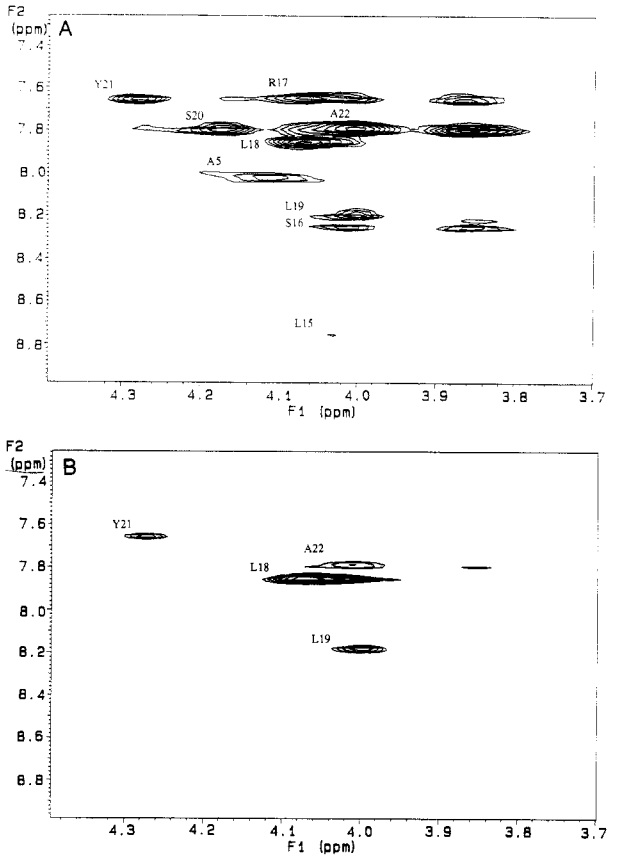

FIGURE 5: Fingerprint region of the pure absorption phase NOESY spectrum of ALDH peptide after addition of D_2O. Remaining $C_\alpha H_i$–$N_\alpha H_i$ crosspeaks are labeled. Each NOESY experiment lasted 160 min and is referred to by its starting time in minutes. (A) 16-min NOESY; (B) 178-min NOESY.

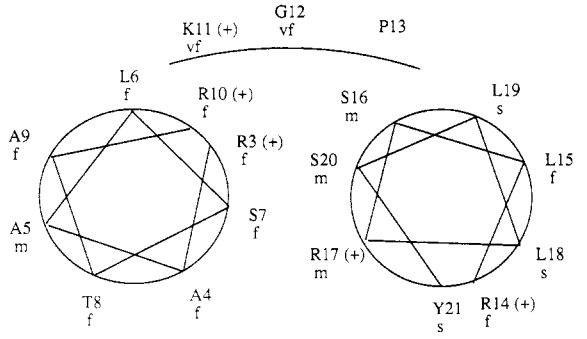

FIGURE 6: Helical wheel representation of the ALDH peptide showing the two α-helical segments and the linking residues. The approximate half-life (τ) for NH exchange derived from Figure 5 and the 1D spectra is identified for each residue: s (slow, 150 min $< \tau$), m (medium, $150 > \tau > 20$ min), f (fast, $20 > \tau > 0$ min), and vf (very fast, NH not observed).

servation that, in general, most small linear peptides exhibit little stable secondary structure in purely aqueous solution. Addition of solvents such as trifluoroethanol or methanol is often required to stabilize secondary structures (Brazzo et al., 1988).

The molar ellipticity at 222 nm ($[\theta]_{222}$) for the peptide is −7500 deg cm^2/dmol at 25°C in aqueous dodecylphosphocholine micelle containing solution. On the basis of the $[\theta]_{222}$ of −26 500 deg cm^2/dmol for 100% helix formation of a 13-residue C-peptide from ribonuclease (Shoemaker et al., 1987), we estimate that the helical content of the peptide is ca. 25%. A computer deconvolution of the CD spectra of the ALDH peptide in the micelle also indicates a comparable amount of α-helix. Analysis of the 2D NOESY spectra (Figures 3 and 4) and other criteria described under Results indicate that residues 3–10 and 14–21 (72% of the total) are in α-helical-like conformations. The CD and NMR results indicate that these two segments are on average ca. 40% in an α-helical state when bound to the micelle. This assumes that the other residues are in a random coil conformation and that the helical segments are in fast exchange with a random coil conformation. Note that both of these segments conform to amphiphilic α-helices (Figure 6).

Generally, the NOESY crosspeak volumes of residues in the C-terminal helix (helix C) are at least twice as large as those in the N-terminal helix (helix N) (cf. parts A and B of Figure 5). This suggests that helix C has a higher α-helical content than helix N. In addition, the rates of NH exchange of residues in helix C are much slower than those in the other helical segment (Figures 5 and 6). Remarkably, the NH exchange of residues in helix C was slow enough to obtain a time series of 2D NOESY spectra of the peptide after addition of D_2O. While this is consistent with the higher helical content of the C-terminal helix, it could also be attributed to a greater shielding of the NH protons of this segment from the solvent. If helix C were buried in the micelle, exchange of the amide protons with water would also be slower. We assume in this analysis that water cannot penetrate the region of the micelle in which the slowly exchanging portion of the helix is buried. Exchange is expected to occur through transient passage of the helix into a random-coil-like conformation in either the aqueous solution or the polar interface between the lipid and water.

It is important to note (Figure 6) that even the hydrophilic residues of the amphiphilic helix C exchange more slowly than any of the residues in helix N with the exception of residue A5. This supports the interpretation that the micelle stabilizes the entire C-terminal helix and that the slow NH exchange is not simply attributable to sequestering of the hydrophobic residues from the solvent. The NH signals of the helix C leucines (L18 and L19) exchange unusually slowly for a small linear peptide, with the NH signal remaining even after 24 h. These results are in dramatic contrast to the observations of O'Neil and Sykes (1989), which demonstrate that solubilization of a hydrophobic tripeptide into a detergent micelle does *not* alter the minimum rate of peptide backbone NH exchange. A detergent micelle, however, has been shown to slow the rate of backbone NH exchange in micelle-bound M13 coat protein (Henry et al., 1987a,b).

A probable explanation for the inaccessibility of the signal peptide protons is that these leucines are more firmly embedded in the surface of the micelle than are the remaining hydrophobic side chains of helix C. However, even the N terminus of helix C is "anchored" to the micelle, albeit less so than the opposite end. This anchoring of the signal peptide to micelle is in good agreement with the previous results (Pak & Weiner, 1990) which showed that it was associated with inner membrane after import into mitochondria.

The 2D NMR spectra indicate that the N-terminal segment is also in an α-helical conformation in micellar solution. However, only A5 appears to be significantly shielded from the solvent as monitored by its slow rate of NH exchange. A5 is at the approximate same position on its helix as L18 is on its helix. In contrast to helix C, the NH proton of the two leucines (L2 and L6) in helix N exchanges very rapidly with solvent. Thus, the interaction of helix N is considerably different (and weaker) than that of helix C.

These results are in contradiction to the prediction of secondary structure stability by Chou–Fasman analysis (Chou

& Fasman, 1978). Helix propensity factors by Chou–Fasman analysis for the N-terminal and C-terminal helices are 1.11 and 0.97, respectively. Generally, values of α-helix propensity (P_α) greater than 1.1 indicate a good probability that the sequence can form a stable α-helix. Therefore, helix N, which is predicted to have good helical propensity, shows only moderate stability, while helix C, which is predicted to have little helical propensity, has the greatest stability.

Residues 11–13 which link the two helical segments are predicted to be in a flexible coil or turn conformation because both glycine (12) and proline (13) are known to be helix breakers. In fact, these two amino acids have the lowest α-helical propensity of all amino acids according to Chou–Fasman calculations. The NMR data indicate that these residues are in a flexible coil conformation which is accessible to the aqueous environment. Thus, the amides of both K11 and G12 do not show NOESY crosspeaks to neighboring residues. Also, the crosspeak between P13 $C_\alpha H$ and the amide proton of R14 is extremely weak. It is not possible to determine if this region is a type I or II turn.

When P13 of prealdehyde dehydrogenase was mutated to Q13 by site-directed mutagenesis, the import ability of precursor protein was not altered (unpublished data). Interestingly, the Chou–Fasman prediction for the structure of the Q13 signal peptide was very similar to that of the natural P13 signal peptide even though Pro is a helix breaker and Gln is a helix former. However, the peptide containing a helix former at the position of G12 was predicted to abolish the turn structure. Therefore, G12 might play an important role in forming the helix–turn–helix structure of the signal peptide. Almost all known signal peptides contain Gly residues which could form the helix-turn-helix structure.

Signal Peptide Models. von Heijne et al. (1989) proposed the existence of two domains in mitochondrial signal peptides that have different amphiphilic properties on the basis of Chou–Fasman and hydrophobic moment calculations. They suggested that these two domains might be involved in two steps of signal peptide cleavage after import. Haldi and Guarente (1989) showed by deletion study that δ-aminolevulinate synthetase signal peptide could be divided into two domains. The presence of the N-terminal nine amino acids only was sufficient to achieve 60% import. A fusion protein with a signal peptide lacking the N-terminal domain was imported, but poorly.

Our own data on the ALDH signal peptide are consistent with the two-domain hypothesis, which perhaps can be described as a helix–"turn"–helix model (Figure 6). The turn may only be a flexible linker rather than a true type I or II turn, although the presence of the Gly-Pro sequence at the end of the N-terminal helix would strongly support a turn. Although NOE crosspeaks between residues on the two helices were not observed, signal overlap does not allow us to rule out the presence of significant stabilization of interhelical interaction. It is unlikely, though, that these helices are folded upon one another when bound to the micelle into a single stable structure.

It is quite unusual that this peptide crosses vesicle membranes without other proteins such as receptors being present. How can such a charged molecule cross such a hydrophobic environment? We have utilized the NMR information and the molecular modeling program MIDAS (Langridge & Ferrin, 1984) to model build a helix–turn–helix conformation. Using the AMBER (Weiner & Kollman, 1981) molecular mechanics energy minimization program, we first calculated a minimum energy structure for the peptide consistent with the NMR

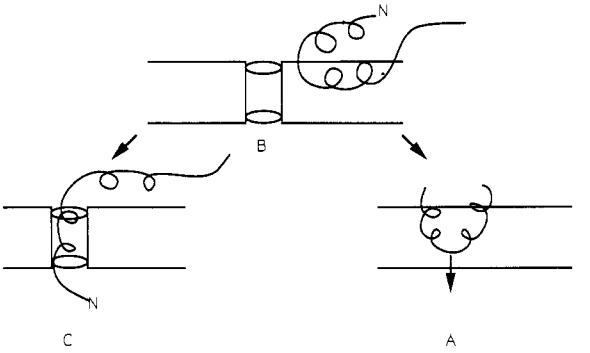

FIGURE 7: (A) Model for translocation of the helix–turn–helix ALDH signal peptide. By sheltering the charged polar residues in the interior of the peptide, the peptide may translocate across the membrane. (B) Model for preprotein translocation and initial anchoring of the C-terminal amphiphilic α-helix (or β-sheet) of the preprotein to the membrane. (C) Diffusion of the C-terminal helix to the receptor followed by insertion of the N-terminal helix into the preprotein receptor, leading to translocation.

results for two separated helices. NOESY distance restraints (for the helices) were then included in an additional flat well modified pseudo energy minimization. Amphiphilic helices in soluble proteins normally pack on the hydrophobic faces. However, in the model of Figure 6 we have oriented the two hydrophilic sides of the helices toward each other and reminimized the model. We emphasize again that we have no data supporting any specific interaction between the helices.

The hydrophilic faces of each of the helices in the peptide contain two arginines. If these residues are sequestered from the hydrophobic environment of the micelle, then the signal peptide in this helix–turn–helix model could more easily translocate across the membrane as shown in the model of Figure 7A. This "inside-out" organization may also explain the helical packing of membrane-spanning proteins (Cohen & Parry, 1990). The antiparallel arrangement of the two helices would also provide additional stabilization of the model by a favorable orientation of the helix dipoles.

Conclusions. NMR of the prealdehyde dehydrogenase signal peptide has suggested that the peptide contains two helical regions, both of which are amphiphilic, separated by a flexible, hinge region. The helix located closer to the C terminus is more stable than the helix located near the N terminus. This indicates that the hydrophobic face of the C-terminal helix is buried within the hydrophobic region of the micelle.

If the signal peptide attached to passenger protein can also be anchored to the outer membrane by the helix C, then the signal peptide could diffuse on the surface of the mitochondrial membrane until it meets an appropriate receptor. Our helix–turn–helix model proposes the following sequence of events leading to protein import (Figure 7B,C). First, the signal peptide is initially anchored to the membrane via a C-terminal amphiphilic structure, which in the ALDH peptide is an α-helix. By anchoring the signal peptide to the membrane, it can diffuse across the surface of the membrane until it meets an appropriate receptor. Diffusion upon the surface of the membrane would allow the peptide to search in two-dimensional space rather than the three-dimensional space of the cytosol. This decrease in the dimensionality of the search has been used to explain how the *lac* repressor protein can locate its operator DNA faster than the rate of diffusion (in three-dimensional space; Berg et al., 1982).

The receptor then recognizes the N-terminal helix of the signal peptide, which is not anchored to the membrane. Indeed, deletion studies have determined that the first nine

residues of another signal sequence are of particular importance for efficient transport (Haldi & Guarente, 1989). Most signal sequences have been proposed to contain this N-terminal helix (von Heijne et al., 1989). After the receptor and peptide form a complex, the protein can be transported. The proteolytic protein may also recognize this helix–turn–helix structure for processing.

Finally, this helix–turn–helix model in which the polar faces of the amphiphilic helices fold back upon one another can explain the unusual translocation of the free, highly charged peptide across the lipid bilayer in the absence of membrane potential or receptor proteins (Pak & Weiner, 1990). The membrane potential of the inner membrane (inside negative) would not be required to drive this shielded peptide into the mitochondria. This charged peptide with only hydrophobic residues facing the lipid bilayer then could simply diffuse across the membrane without the involvement of a receptor.

Registry No. ALDH, 122653-67-2; NH_2MLRAALSTARKGPRLSRLLSYACONH_2, 129266-53-1.

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