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Secondary Structure and Topology of a Mitochondrial Presequence Peptide Associated with Negatively Charged Micelles. A 2D ^{1}H-NMR Study†

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ABSTRACT: In this study the secondary structure and topology of the peptide, corresponding to the presequence of cytochrome oxidase subunit IV (p25) in a negatively charged membrane-mimetic environment, were assessed by circular dichroism and two-dimensional nuclear magnetic resonance. The micelles used consisted of dodecylphosphoglycol (DPG), a mild anionic detergent with a headgroup resembling that of phosphatidylglycerol. The secondary structure was analyzed by interresidue nuclear Overhauser enhancement measurements and chemical shifts of backbone protons. The data revealed α-helix formation of the peptide upon interaction with the micelles, both in the N- and in the C-terminal halves, which are separated from each other by the proline residue at position 13. The topology of the peptide was studied by determining the effect of spin-labeled 12-doxylstearate on the line widths of the peptide proton resonances. This method revealed the insertion of hydrophobic residues of both the N- and the C-terminal halves of p25 into the hydrophobic environment of the micelles, demonstrating the orientation of the amphiphilic helix.

The majority of the mitochondrial proteins are synthesized in the cytosol as precursors carrying an amino-terminal extension, the presequence. Presequences contain the information to target precursor proteins to mitochondria and transport them across the two mitochondrial membranes into the matrix [for a review on mitochondrial protein import, see Kiebler et al. (1993)]. In the matrix, the presequence is proteolytically removed. The molecular mechanism by which the presequence can fulfill its functions is not clear. Although hardly any sequence homology exists among presequences from different proteins, they are generally enriched in positively charged, hydroxylated, and apolar amino acid residues (Von Heijne, 1986). This suggests that some similarity in the secondary structure must be more important for their function than a specific sequence. A common feature among presequences that has been suggested to play a role in the translocation process is the potential to form an amphiphilic α-helix (Von Heijne, 1986). CD[1] [reviewed in Tamm (1991)] and 2D NMR studies of different presequence peptides have revealed that these peptides can adopt an α-helical conformation in membrane mimetic environments (Endo et al., 1989; Karslake et al., 1990; Bruch

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[1] Abbrevations: CD, circular dichroism; cmc, critical micelle concentration; 2D, two-dimensional; DPC, dodecylphosphocholine; DPG, dodecylphosphoglycol; DPG-d_{29}, [$^2H_{29}$]dodecylphosphoglycol; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser enhancement; NOESY, 2D nuclear Overhauser enhancement spectroscopy; p25, peptide corresponding to the presequence of cytochrome oxidase subunit IV; PG, phosphatidylglycerol; TOCSY, 2D total correlation spectroscopy; TPPI, time-proportional phase increment.

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& Hoyt, 1992; Thornton et al., 1993; Hammen et al., 1994; Jarvis et al., 1995; Chupin et al., 1995a). However, the topology of the α-helical peptides in a membrane-bound state was never addressed in these studies.

In the present study, the secondary structure and topology of the peptide, corresponding to the presequence of yeast cytochrome oxidase subunit IV (p25) associated with lipid micelles, were investigated by CD and 2D ^{1}H-NMR. Because of their small size, micelles undergo rapid isotropic motion, yielding a high-resolution NMR spectrum for any associated peptide. Until now, in 2D NMR studies on mitochondrial presequence peptides the zwitterionic detergent DPC or aqueous trifluoroethanol solutions have been used as membrane mimetic environment. Since presequences strongly interact with lipid membranes, with a preference for anionic lipids [see for a review Tamm (1991)], we used dodecylphosphoglycol (DPG) as a representative for anionic phospholipids. This monoacyl lipid contains a negatively charged headgroup resembling that of PG. To assess the topology of the peptide associated with the micelles, spin-labeled 12-doxylstearic acid was used to produce selective broadening of the residues buried into the hydrocarbon region (Brown et al., 1982; Papavoine et al., 1994; Chupin et al., 1995b).

MATERIALS AND METHODS

Materials. Dodecanol-d_{23}, sodium acetate-d_3, ethylene glycol-d_4, methanol-d_4, and ^2H_2O were from Isotec Inc. (Miamisburg, OH). 12-Doxylstearic acid was obtained from Sigma (St. Louis, OH). 3-(Trimethylsilyl)propionate-d_4 was from Merck (Germany). Perdeuterated DPG-d_{27} was synthesized as described previously (de Jongh & de Kruijff, 1990). The peptide corresponding to the presequence of yeast cytochrome oxidase subunit IV (p25, H_3N^+-Met^1-Leu-Ser-Leu-Arg^5-Gln-Ser-Ile-Arg-Phe^{10}-Phe-Lys-Pro-Ala-Thr^{15}-Arg-Thr-Leu-Cys-Ser^{20}-Ser-Arg-Tyr-Leu-Leu^{25}-$CONH_2$) was prepared as described previously (de Kroon & McConnell, 1994).

NMR Measurements. For the preparation of the NMR samples p25 was dissolved in buffer containing 20 mM sodium acetate-d_3, 3 mM dithiothreitol, 0.1 mM (ethylenedinitrilo)tetraacetate, and 0.1 mM 3-(trimethylsilyl)propionate-d_4 in either 90%H_2O/10%^2H_2O or ^2H_2O, pH 4.6 (direct reading), and mixed with a DPG micelle solution in the same buffer. The cmc of DPG is 0.6 mM (de Jongh & de Kruijff, 1990). The final concentrations of p25 and DPG were 3 mM (based on weight) and 180 mM, respectively. The molar ratio of p25/detergent, 1/60, was used to obtain an approximate occurrence of one peptide molecule per micelle [based on the number of SDS molecules per micelle as determined by Papavoine et al. (1994)]. DPG in micelles is negatively charged at pH 4.6, as concluded from ^{31}P-NMR titration measurements (data not shown), which showed that the pK_a of DPG is 3.0. At higher pH values the quality of the NMR spectra in the NH region was significantly reduced due to line broadening. All NMR experiments were performed at 25 °C in an argon atmosphere to avoid oxidation. NMR spectra were recorded on a Bruker AMX spectrometer operating at 500 MHz. Chemical shifts were measured relative to 3-(trimethylsilyl)propionate-d_4. TOCSY spectra were recorded using a clean MLEV-17 mixing sequence (Bax & Davis, 1985; Griesinger et al., 1988) with total mixing times of 30−50 ms which include the delays of the clean TOCSY pulse scheme. NOESY spectra (Jeener et al., 1979; Kumar et al., 1981) were recorded with mixing times of 100, 200, and 300 ms. All 2D spectra were recorded in a phase-sensitive absorption mode using TPPI in t_1 (Marion & Wütrich, 1983). The cross-peaks around 4.6 ppm, which are normally irradiated together with the water line, were made observable using the SCUBA method (Brown et al., 1988). In order to assess the Hα−Hβ cross-peaks under the water line, spectra were recorded in ^2H_2O. Two-dimensional spectra were collected as a 400−512 (t_1) real and 1024 (t_2) complex point time domain matrix with a spectral width of 5000 Hz in both dimensions and 80−160 scans per t_1 increment. Data were transformed, after zero-filling in the F1 dimension, into 1024 and 1024 real points in the F1 and F2 dimension frequency-domain spectra. Sixth-order polynomial base line corrections in each domain were applied after the double Fourier transformation was completed (Boelens et al., 1985). The data were processed with the "Triton" software library (Bijvoet Center for Biomolecular Research, Utrecht University, The Netherlands). Three-dimensional structures were built using the program Insight II on an Indy workstation (Silicon Graphics Inc., Mountain View, CA).

To determine the insertion of the presequence into micelles, the effect of spin-labeled 12-doxylstearic acid on the ^{1}H presequence peptide resonances was investigated. After recording the 2D NMR spectra in the absence of the spin-labeled acid, the NMR sample (450 μL) was mixed with 10 μL of the 12-doxylstearic acid in methanol-d_4 to yield a 60:1 molar ratio of detergent to 12-doxylstearic acid and the NMR spectra were recorded. The effect of the 12-doxylstearic acid was measured by comparing the intensities of the cross-peaks in the presence and in the absence of the spin-labeled acid. The spin label can be expected to be present in the micelle interior according to Papavoine et al. (1994). Moreover, the narrow singlet peak of DTT at 3 ppm in the NMR spectra was not affected by the presence of 12-doxylstearic acid in the micelle solution (data not shown), indicating that the doxyl group is not in contact with the buffer solution.

Circular Dichroism. CD measurements were carried out on a JASCO 600 spectropolarimeter, with a 1 nm bandwidth, 0.1 nm resolution, 1 s response time, a scan speed of 20 nm/min, and using a 0.2 mm path length cell. Typically eight scans were added and averaged, followed by subtraction of the CD signal of peptide-free micelle solutions recorded under the same conditions. Spectra shown are from NMR samples diluted to a final peptide concentration of 100 μM. Identical results were obtained when samples were prepared freshly. The helix content was estimated by the method of Greenfield and Fasman (1969).

RESULTS

CD was used to get a first indication about the secondary structure induced in the peptide in the absence and presence of DPG micelles (Figure 1). The CD spectrum of p25 in aqueous solution is consistent with that of a random coil and does not show any features characteristic for an α-helix. P25 in a micelle environment gives rise to a CD spectrum typical of a highly α-helical conformation. From a computer deconvolution of the CD spectrum of p25 in the presence of DPG micelles the helical content of the peptide was estimated to be 70%.

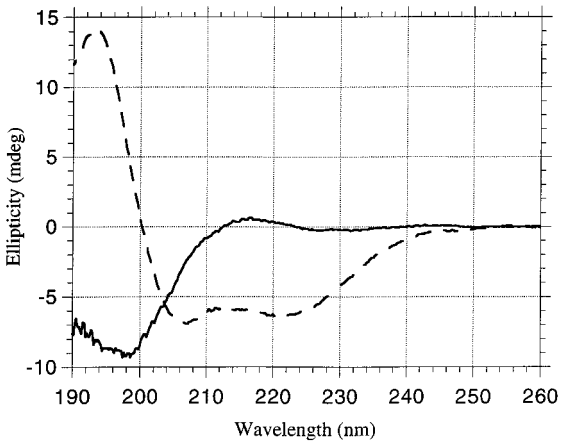

FIGURE 1: CD spectra of p25 in water (—) and upon interaction with DPG (- - -) micelles at 25 °C.

For 2D NMR data analysis, the standard method proposed by Wüthrich (1986) was used to carry out the sequential assignment of all the proton resonances of the presequence peptide in a DPG environment. TOCSY spectra were used to identify spin systems, and NOESY spectra were used to obtain interresidue connectivities and to distinguish equivalent spin systems. Unambiguous assignments were obtained with the help of sequential NH(i) − NH(i + 1), Hα(i) − NH(i + 1), and other NOE connectivities characteristic of α-helical conformation. Figures 2 and 3 show the Hα−NH region of the TOCSY (A) and NOESY (B) spectra in water and in the presence of DPG micelles, respectively. In Figure 3, some selected Hα-NH cross-peaks (TOCSY spectrum, A) and corresponding sequential Hα(i)−NH(i, i + 1) cross-peaks (NOESY spectrum, B) are indicated. All the protons of the peptide in DPG micelles and the chemical shift values of the Hα and NH protons of the peptide in water were assigned (see supporting information).

The determination of the secondary structure of p25 in a DPG micellar environment involved identifying NOE cross-peaks between protons on non-neighboring residues and chemical shifts of backbone protons. The NOE connectivity pattern for an α-helix consists of medium Hα(i)−Hβ(i + 3) and weak Hα(i)−NH(i + 3) medium-range contacts. The medium-range Hα(i)−Hβ(i + 3), Hα(i)−NH(i + 3) and sequential NOE connectivities for the peptide in DPG micelles are summarized in Figure 4A. They reveal that α-helical conformation is present along the full length of the peptide.

Hα proton chemical shift values were used as an additional tool for structural analysis. It has been shown that the difference between the Hα proton chemical shifts in a protein structure and the chemical shifts in a random coil also correlates with protein secondary structure. An α-helix structure is indicated by an upfield chemical shift of more than 0.1 ppm with respect to random coil chemical shift values (Wishart et al., 1992). For p25 in the presence of DPG micelles, the differences between the Hα chemical shift values with respect to those in water, are given in Figure 4B as a function of the residue position. The overall negative values reflect the α-helical conformation of the peptide. The reason why Ser^3 gives a positive instead of a negative difference value is not clear to us.

To determine the insertion of p25 into DPG micelles, spin-labeled 12-doxylstearic acid was used to produce selective broadening of the residues buried into the hydrocarbon region (Brown et al., 1982; Papavoine et al., 1994; Chupin et al., 1995b). The effect of the 12-doxylstearic acid was measured by comparing the intensities of the 2D NMR cross-peaks in the absence (Figure 5A) and presence (Figure 5B) of the 12-doxylstearic acid. The difference spectrum between these spectra, which was normalized to the intensities of the Gln^6 Hα−Hβ, Hγ cross-peaks, is shown in Figure 5C and reveals that the intensities of many cross-peaks are reduced in the presence of 12-doxylstearic acid.

DISCUSSION

The aim of this work was to study the conformational behavior of p25 in micelles with a negative surface charge. Both the CD and the NMR measurements reveal that the peptide is random coiled in water. The CD spectrum of p25 in water is typical for a random coil peptide (Figure 1). The range of the chemical shifts in the TOCSY spectrum of p25 in water (Figure 2A) is rather narrow which also argues for a random coil conformation. Moreover, the NOESY spectrum (Figure 2B) shows only a small number of NOE's with relatively weak intensities, indicative for a structureless conformation of the peptide.

Upon addition of DPG micelles to p25, both CD and NMR data demonstrate that the interaction of p25 with the micelles induces α-helix formation in the peptide. This is in agreement with earlier CD reports on the secondary structure of p25 in interaction with anionic phospholipids (Roise et al., 1986; Tamm & Bartoldus, 1990). The CD spectra of p25 in the presence of DPG micelles are typical for a highly α-helical conformation of the peptide (Figure 1). The changes in both the TOCSY and the NOESY spectrum of p25 upon addition of DPG micelles are clear when comparing Figures 2 and 3. The Hα chemical shift values are shifted upfield (Figure 3A, compared with Figure 2A). Moreover, Figure 3B clearly reveals that both the number of the interresidue NOE cross-peaks and their intensities are increased in the NOESY spectrum of p25 in the presence of micelles, compared to that in water (Figure 2B), suggesting that the peptide has acquired a specific structure. Indeed, both the medium-range Hα(i)−Hβ(i + 3), Hα(i)−NH(i + 3), and sequential NOE connectivities (Figure 4A) and the chemical shift differences of Hα resonances of the peptide in DPG solution, with respect to those in water (Figure 4B) reveal that the peptide adopts a largely α-helical conformation, starting at Ser^3 or Leu^4 and present along the full length of the peptide. The number and intensities of the observed NOE's are higher for the N-terminal half of the peptide, compared with the C-terminal half, indicating that the C-terminal part of p25 has a less regular or more flexible structure compared with the N-terminus. On average it should be noticed that the intensities of the medium-range NOE cross-peaks of p25 are low compared with, for instance, those of the bacterial signal peptide of PhoE associated with similar micelles (Chupin et al., 1995b), which is probably due to the dynamical nature of the p25 conformation [see also Chupin et al. (1995a)]. The chemical shift differences show that the helix of the presequence peptide is destabilized at Pro^{13}, with again the helical content being higher at the N-terminus and lower at the C-terminus of p25. The helix of the C-terminal half is destabilized at Ser^{20}, Ser^{21}. These results are comparable with the results of two previous 2D NMR studies on p25 (Endo et al., 1989; Chupin et al.,

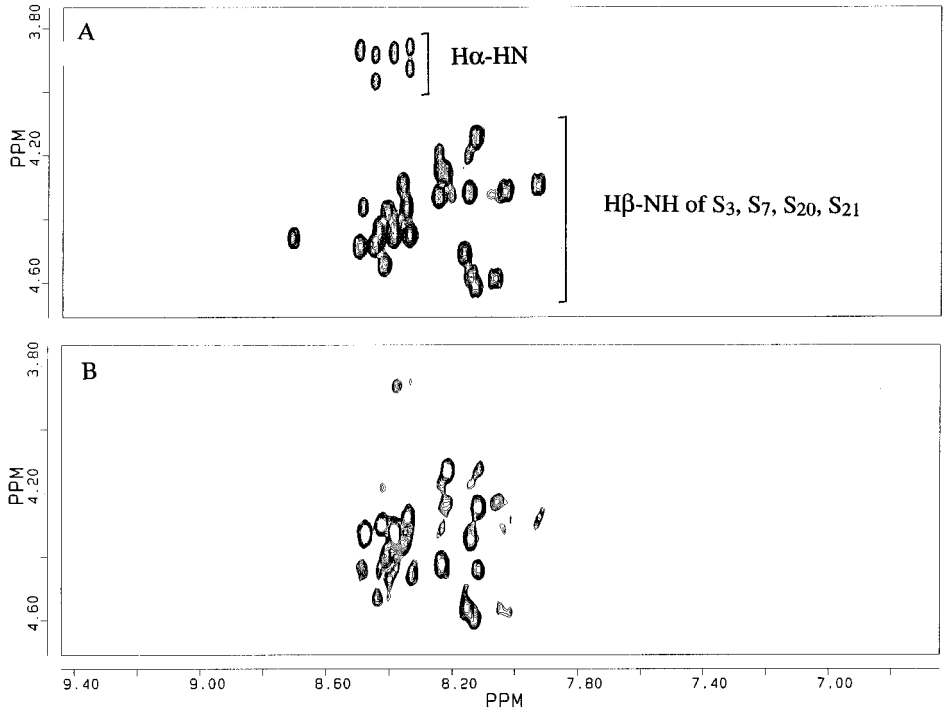

FIGURE 2: Hα−NH region of the TOCSY (A) and NOESY (B) spectra of p25 in water (90% H_2O/10% D_2O).

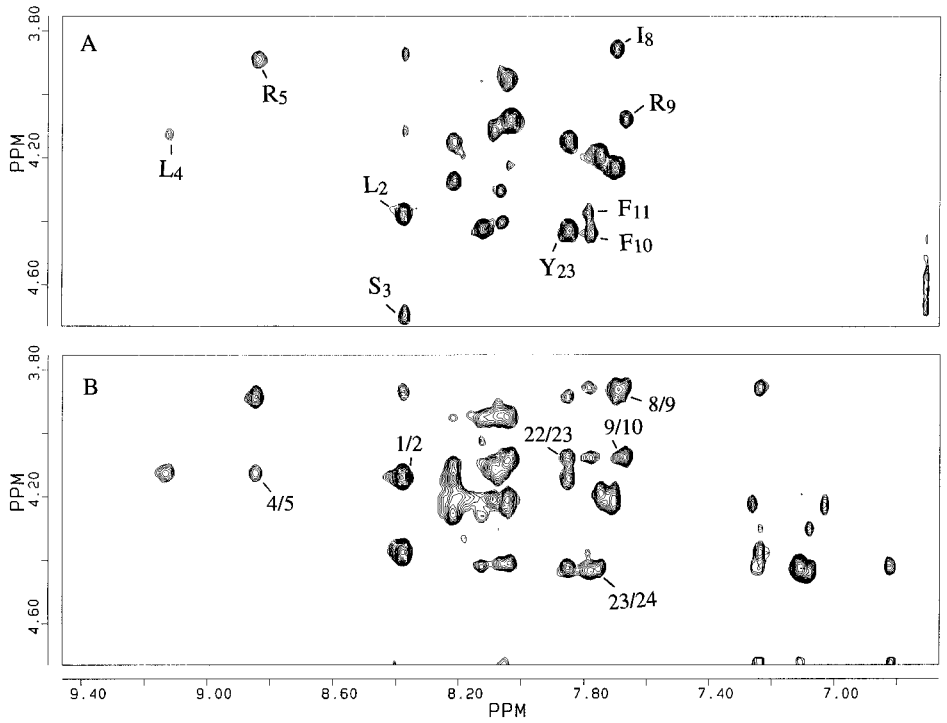

FIGURE 3: Hα−NH region of the TOCSY (A) and NOESY (B) spectra of p25 upon interaction with DPG micelles. Selected Hα-NH (A) and corresponding sequential Hα(i)−NH(i + 1) (B) connectivities are indicated.

1995a). In the study of Chupin et al. (1995a) the early investigations on p25 associated with DPC micelles of Endo et al. (1989) were extended and a helix−turn−helix motif was found, with the N-terminal helix being more stable than the C-terminal helix. The helical content of p25 in the presence of DPG micelles is slightly higher than in the presence of DPC micelles, which is due to an increased helicity in the C-terminal half.

For the investigation of the insertion of p25 into the negatively charged micelles, spin-labeled 12-doxylstearic acid was incorporated in the DPG micelles. The spin label in 12-doxylstearic acid is covalently attached to the stearic acid at position 12, locating the free radical in the center of the micelle. The 12-doxylstearic acid/DPG molar ratio used was 1/60 to obtain an average occurrence of 1 molecule of the relaxation reagent per micelle. The presence of the 12-doxylstearic acid drastically reduced the intensities of the p25 NMR cross-peaks of the hydrophobic amino acids of both the N- and the C-terminal helical parts of the presequence (Leu[2], Ser[3], Leu[4], Ile[8], Phe[10], Phe[11], Ala[14], Thr[17],

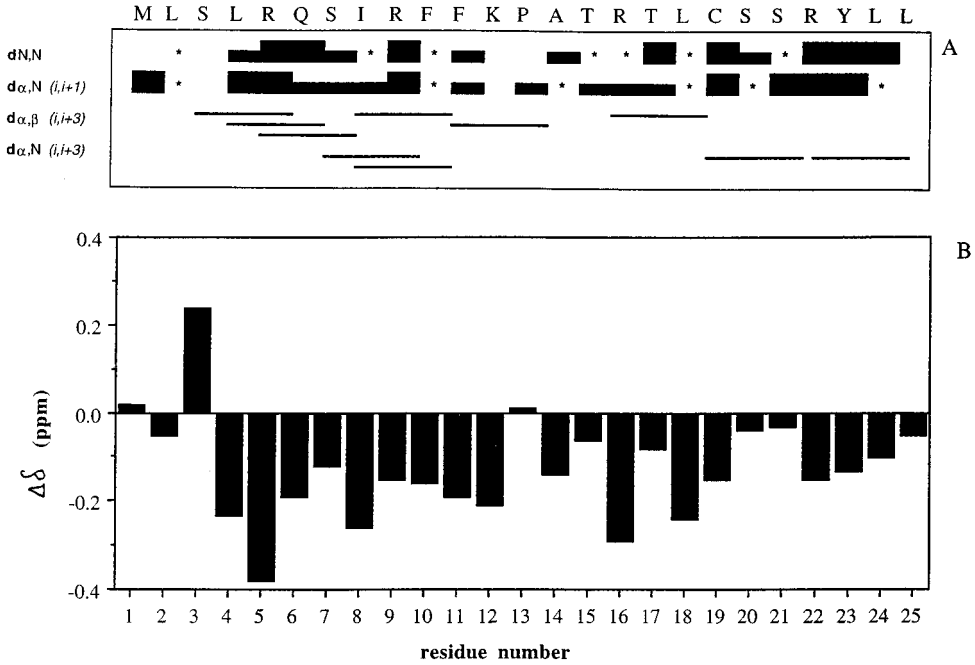

FIGURE 4: Secondary structure determination of p25 associated with DPG micelles. (A) Sequential and medium-range NOE contacts observed in the NOESY spectra of p25 in DPG micelles. The thickness of the horizontal bars is a qualitative indication of the relative intensity of the sequential NOEs as observed in NOESY spectra. Solid bars indicate NOEs that have been identified, while asterisks indicate NOEs whose presence or absence cannot be assessed due to overlap. (B) The difference between the Hα chemical shifts observed in DPG micelles and the random coil chemical shifts determined in water solution is represented as a function of residue position for p25.

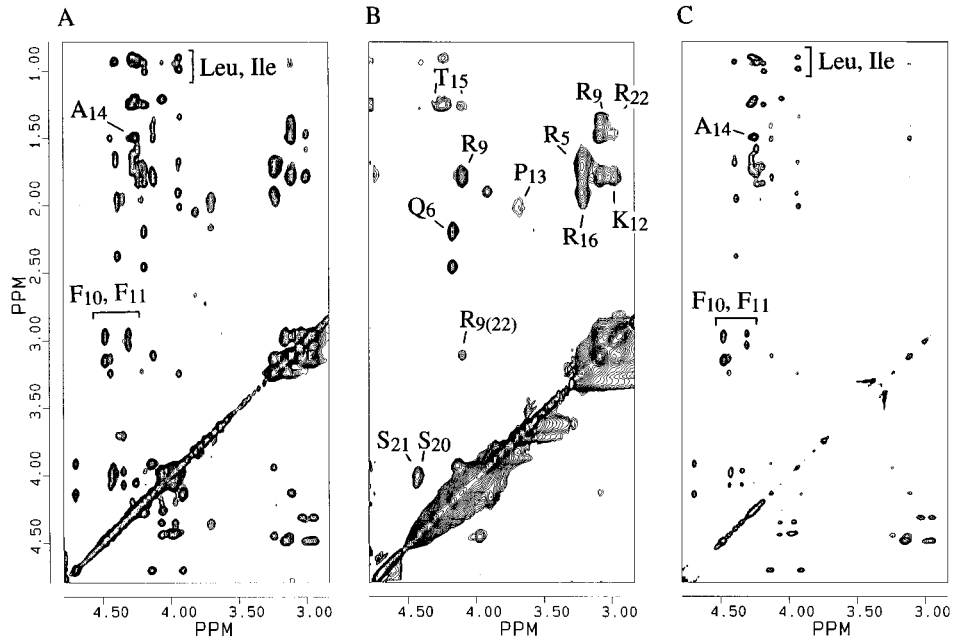

FIGURE 5: Comparison of expanded region of NOESY spectra of p25 in DPG micelles in the absence (A) and presence (B) of 12-doxylstearic acid. The difference between (A) and (B) is represented on the right panel (C), in which resonances are indicated which were affected by the presence of 12-doxylstearate. Resonances which were not affected by the presence of 12-doxylstearate are indicated in panel B.

Leu[24], and Leu[25]), while the intensities of most hydrophilic residues (Arg[5], Gln[6], Arg[9], Lys[12], Arg[16], Ser[20(21)], and Arg[22]) are not affected (compare spectrum in the absence, Figure 5A, with that in the presence of spin label, Figure 5B). The difference spectrum (Figure 5C) shows residues, that are located in the hydrocarbon core of the micelles. These data are consistent with an amphiphilic structure of the helix and a buried location for the hydrophobic side of the α-helix of p25 in the micelle interior. The potential of presequences to form an amphiphilic α-helix has been proposed to be an important functional property among mitochondrial presequences. This is to our knowledge the first time that the orientation of the native p25 peptide is demonstrated. In a related study, a series of single- and double-cysteine-substituted p25 peptides, labeled with nitroxide spin labels was used, in combination with electron paramagnetic resonance spectroscopy (Yu et al., 1994). Together with energetic considerations the authors suggested that the hydrophobic residues were solvated by the acyl chains, with the charged side chains extending towards the polar membrane surface, consistent with the experimental results obtained in the present study.

In conclusion, we have demonstrated that in the negatively charged DPG micellar environment p25 adopts a largely

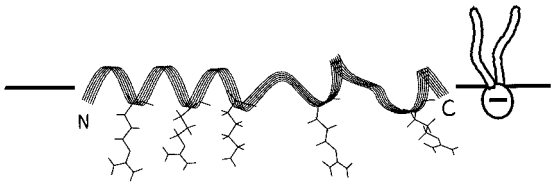

FIGURE 6: Molecular model of p25 associated with a lipid membrane as an amphiphilic α-helix parallel to the membrane surface. For clarity only the peptide backbone and its positively charged side chains are depicted (in an arbitrary conformation).

α-helical conformation, stretching from the N- to the C-terminus. The α-helix orients as an amphiphilic structure parallel to the membrane surface. A molecular model of p25 associated with a lipid membrane is schematically depicted in Figure 6.

SUPPORTING INFORMATION AVAILABLE

Assignments for all the protons of the peptide in DPG micelles are given as well as the chemical shift values of the Hα and NH protons of the peptide in water (1 page). Ordering information is given on any current masthead page.

REFERENCES

Bax, A., & Davis, D. G. (1985) *J. Magn. Reson. 65*, 355−366.
Boelens, R., Scheek, R. M., Dijkstra, K., & Kaptein, R. (1985) *J. Magn. Reson. 62*, 378−386.
Brown, L. R., Braun, W., Kumar, A., & Wütrich, K. (1982) *Biophys. J. 37*, 319−328.
Brown, S. C., Weber, P. L., & Muller, L. (1988) *J. Magn. Reson. 77*, 166−169.
Bruch, M. D., & Hoyt, D. W. (1992) *Biochim. Biophys. Acta 1159*, 81−93.
Chupin, V., Leenhouts, J. M., de Kroon, A. I. P. M., & de Kruijff, B. (1995a) *FEBS Lett. 373*, 239−244.
Chupin, V., Killian J. A., Breg, J., de Jongh, H. H. J., Boelens, R., Kaptein, R., & de Kruijff, B. (1995b) *Biochemistry 34*, 11617−11624.
de Jongh, H. H. J., & de Kruijff, B. (1990) *Biochim. Biophys. Acta 1029*, 105−112.
de Kroon, A. I. P. M., & McConnell, H. M. (1994) *J. Immunol. 152*, 609−619.
Endo, T., Shimada, I., Roise, D., & Inagaki, F. (1989) *J. Biochem. 106*, 396−400.
Greenfield, N., & Fasman, C. D. (1969) *Biochemistry 8*, 4108−4116.
Griesinger, C., Otting, G., Wüthrich, K., & Ernst, R. R. (1988) *J. Am. Chem. Soc. 110*, 7870−7872.
Hammen, P. K., Gorenstein, D. G., & Weiner, H. (1994) *Biochemistry 33*, 8610−8617.
Jarvis, J. A., Ryan, M. T., Hoogenraad, N. J., Craik, D. J., & Hoj, P. B. (1995) *J. Biol. Chem. 270*, 1323−1331.
Jeener, J., Meier, B. H., Bachmann, P., & Ernst, R. R. (1979) *J. Chem. Phys. 71*, 4546−4553.
Karslake, C., Piotto, M. E., Pak, Y. K., Weiner, H., & Gorenstein, D. G. (1990) *Biochemistry 29*, 9872−9878.
Kiebler, M., Becker, K., Pfanner, N., & Neupert, W. (1993) *J. Membr. Biol. 135*, 191−207.
Kumar, A., Wagner, G., Ernst, R. R., & Wüthrich, K. (1981) *J. Am. Chem. Soc. 103*, 3654−3658.
Marion, D., & Wüthrich, K. (1983) *Biochem. Biophys. Res. Commun. 113*, 967−974.
Papavoine, C. H. M., Konings, R. N. H., Hilbers, C. W., & Van de Ven, F. J. M. (1994) *Biochemistry 33*, 12990−12997.
Roise, D., Horvath, S. J., Tomich, J. M., Richards, J. H., & Schatz, G. (1986) *EMBO J. 5*, 1327−1334.
Tamm, L. K. (1991) *Biochim. Biophys. Acta 1071*, 123−148.
Tamm, L. K., & Bartoldus, I. (1990) *FEBS Lett. 272*, 29−33.
Thornton, K., Wang, Y., Weiner, H., & Gorenstein, D. G. (1993) *J. Biol. Chem. 268*, 19906−19914.
Von Heijne, G. (1986) *EMBO J. 5*, 1335−1342.
Wishart, D. S., Sykes, B. D., & Richards, F. M. (1992) *Biochemistry 31*, 1647−1651.
Wüthrich, K. (1986) in *NMR of Proteins and Nuclear Acids*, John Wiley & Sons, New York.
Yu, Y. G., Thorgeirsson, T. E., & Shin, Y.-K. (1994) *Biochemistry 33*, 14221−14226.

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