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Structures of Wild-Type and Mutant Signal Sequences of *Escherichia coli* Ribose Binding Protein

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ABSTRACT The structure of a chemically synthesized 25-residue-long functional signal peptide of *Escherichia coli* ribose binding protein was compared with that of a nonfunctional mutant-signal peptide using circular dichroism and two-dimensional ^{1}H NMR in solvents mimicking the amphiphilic environments. The functional peptide forms an 18-residue-long α-helix starting from the NH_2-terminal region and reaching to the hydrophobic stretch in a solvent consisting of 10% dimethylsulfoxide, 40% water, and 50% trifluoroethanol (v/v). The nonfunctional mutant peptide, which contains a Pro at position 9 instead of a Leu in the wild-type peptide, does not have any secondary structure in that solvent but forms a 12-residue-long α-helix within the hydrophobic stretch in water/trifluoroethanol (50:50, v/v) solvent. It seems that the Pro-9 residue in the nonfunctional peptide disturbs the helix propagation from the hydrophobic stretch to the NH_2-terminal region. Because both of these peptides have stable helices within the hydrophobic stretch, it may be concluded that the additional 2 turns of the α-helix in the NH_2-terminal region of the wild-type signal peptide is important for its function.

INTRODUCTION

The translocation of newly synthesized proteins through the plasma membrane of *Escherichia coli* has been the subject of intensive studies by diverse investigators ranging from microbial geneticists to biophysicists (Gierasch, 1989; Randall and Hardy, 1989; Wickner and Lodish, 1985). All the proteins exported from *E. coli* have a signal sequence attached to the NH_2 terminus of the mature domain. Although the function of the signal sequence has not been unequivocally determined so far, targeting and modulation of the folding of the mature part seem to be involved. Although all known signal sequences reveal no region of conservation (von Heijne, 1985), the importance of signal sequence in the translocation or folding of nascent protein may derive from overall features such as hydrophobicity and conformation.

One of the main advantages of studying protein translocation in prokaryotes such as *E. coli* over eukaryotes is its easy access to the translocation-incompetent mutants, which can be compared with the wild type. There have been reports on the mutation in the signal sequence that abolish the translocation completely (Iida et al., 1985). It is of interest to see whether the translocation incompetence originates from the targeting or folding modulation. In any event, the comparative studies on the wild type and mutant signal sequences seem to be of importance.

Gierasch (1989) pointed out that the signal sequence may be relatively free of interaction with the rest of the nascent chain due to the fact that it can be attached to a number of different mature parts and still function. This suggests that the studies on the interaction of signal sequence alone, detached from the mature part, with an amphiphilic surface may give important information on the targeting process. The approach of studying the isolated signal sequence was taken by Gierasch and her coworkers in a series of elegant investigations of wild-type and various mutant signal sequences of LamB protein (Bruch et al., 1989; Bruch and Gierasch, 1990; Mcknight et al., 1989; Briggs, 1986) and OmpA protein (Rizo et al., 1993). They compared the wild-type and mutants where several residues in the hydrophobic stretch are deleted or one of the residues is replaced with another. These sequences are in the form of a random coil in aqueous solution, but for other signal sequences β-structure was also observed (Randall and Hardy, 1989). However, the α-helix becomes the predominant structure in 20 mol % trifluoroethanol (TFE) solution, which mimics an amphiphilic environment. The results from combined circular dichroism (CD) and two-dimensional (2D) NMR studies indicate that both the length and the stability of the α-helix stretch are important.

In the present investigation, the CD and 2D NMR experiments were carried out to elucidate the conformations of the wild-type signal sequences of *E. coli* ribose binding protein (Met-Asn-Met-Lys-Lys-Leu-Ala-Thr-Leu-Val-Ser-Ala-Val-Ala-Leu-Ser-Ala-Thr-Val-Ser-Ala-Asn-Ala-Met-Ala) and the signal sequence of a mutant, which does not exhibit any translocation (Iida et al., 1985), in aqueous solution containing TFE. The mutant peptide contains a Pro at position 9 instead of Leu in the wild-type peptide.

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MATERIALS AND METHODS

Synthesis and purification

Wild-type and mutant signal peptides were synthesized by a solid phase method on a MilliGen (Burlington, MA) 9060 automated peptide synthesizer. The peptide was purified by reverse-phase high performance liquid chromotography using Z-module/radial-pak μ-Bondapak C_{18} column (10

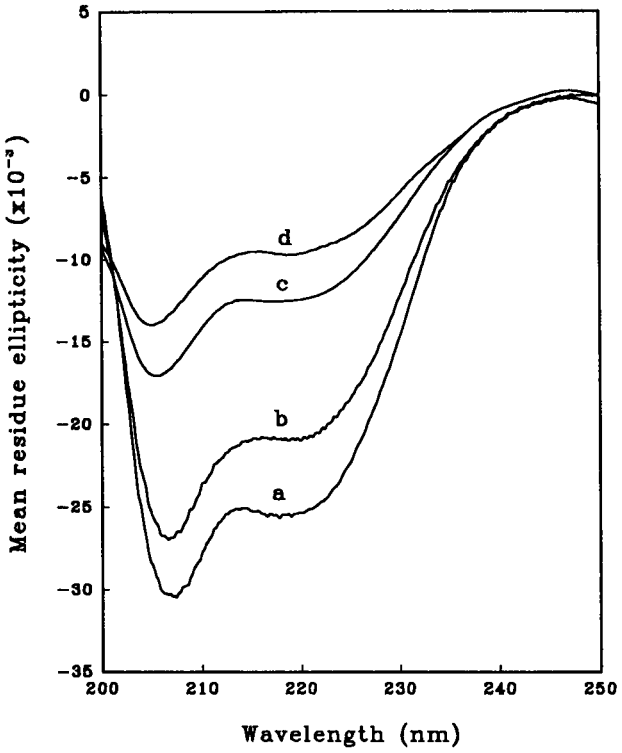

FIGURE 1 CD spectra of RBP peptides in 20 mol % TFE/water solution at pH 3. The estimated α-helix content is 69% in wild peptide at 25°C (*a*), 56% in wild peptide at 50°C (*b*), 33% in mutant peptide at 25°C (*c*), and 28% in wild peptide at 50°C (*d*).

cm × 0.8 cm), elution being made with a water-acetonitrile linear gradient containing 0.1% trifluoroacetic acid. The gradient was from 24–30% of acetonitrile for the mutant signal peptide and from 36–44% for the wild-type signal peptide. The sequences of the peptides were confirmed by a MilliGen/Biosearch 6600 Prosequencer.

Circular dichroism

CD spectra were obtained on a Jasco (Tokyo, Japan)J-600 spectropolarimeter using a 1-mm cell. The concentrations used were 9.5–19 μM for the wild-type signal peptide and 36–420 μM for the mutant signal peptide. The peptide concentration was determined by quantitative amino acid analysis. The temperature was regulated either at 25 or 50°C by a NESLAB (Portsmouth, NH) RTE-210 temperature controller. The mixed solvent used contained 50% unbuffered water and 50% 2,2,2-TFE (Sigma Chemical Co., St. Louis, MO) v/v (equivalent to 20 mol % TFE), and its pH was adjusted to 3.0 with 0.1 *N* HCl solution at 25°C. All CD spectra obtained were the average of seven consecutive scans from 250–200 nm and were base-line corrected and smoothed.

NMR spectroscopy

NMR spectra were obtained at 500 MHz on a Bruker (Karlsruhe, Germany) AMX 500 spectrometer at 25°C and pH 3. The peptide concentrations used were 1.5 mM for the mutant signal peptide and 0.8 mM for the wild-type signal peptide. The mutant signal peptide was dissolved in a mixed solvent of 50% TFE-d_3 (Cambridge Isotope Laboratories, Boston, MA) and 50% unbuffered water (v/v). Because the solubility of the wild-type signal peptide in H_2O/TFE is low, a mixed solvent consisting of 10% dimethylsulfoxide-d_6, (DMSO-d_6, Aldrich), 50% TFE-d_3, and 40% unbuffered water (v/v) was used. Any aggregated matter in the NMR sample preparations was removed by centrifugation, and the clear solution was used for the experiments.

Sequential assignments were obtained by total correlation spectroscopy (TOCSY) (Davis and Bax, 1985), double quantum-filtered (DQF) correlation spectroscopy (COSY) (Rance et al., 1983), and two-dimensional nuclear Overhauser effect spectroscopy (NOESY) (Macura et al., 1981). TOCSY were collected with a mixing time of 75 ms. An MLEV-17 composite pulse (Bax and Davis, 1985) was used for spin locking. The trim pulse was not used to prevent phase distortion in the case of water suppression. The mixing time of NOESY experiments was 240 ms. The water resonance was suppressed by preirradiation during the relaxation delay for TOCSY and DQF-COSY. In the experiments of NOESY, the hydrogen-deuterium oxide (HDO) resonance was suppressed by irradiation during the relaxation delay and mixing period. The relaxation delay was 1.3 s in all experiments, and

TABLE 1 Chemical shift for wild type signal peptides of *E. coli* ribose binding protein in water/TFE at 25°C*

Residue	NH	αH	βH	γH	Others
Met-1					
Asn-2	8.49	4.81	2.96, 3.06		γNH_2 6.99, 7.91
Met-3	8.73	4.41	2.14	2.68	ϵCH_3 1.97
Lys-4	8.08	4.07	1.92	1.50	δCH_2 1.56;ϵCH_2 3.01; ϵNH_3^+ 7.61
Lys-5	7.85	4.02	1.93	1.46	δCH_2 1.56; ϵCH_2.3.01; ϵNH_3^+ 7.61
Leu-6	7.81	4.13	1.92	1.66	δCH_3 0.98
Ala-7	8.35	3.99	1.54		
Thr-8	7.84	3.87	3.97	1.30	
Leu-9	7.92	4.23	1.87	1.55	δCH_3 0.97, 1.09
Val-10	8.56	3.62	2.16	0.97, 1.08	
Ser-11	7.88	4.16	3.98, 4.08		
Ala-12	8.01	4.13	1.60		
Val-13	8.39	3.58	2.27	0.97, 1.12	
Ala-14	8.44	4.08	1.55		
Leu-15	8.44	4.15	1.81	1.55	δCH_3 0.97, 1.09
Ser-16	8.15	4.13	4.03, 4.14		
Ala-17	8.44	4.13	1.55		
Thr-18	7.91	4.03	4.47	1.28	
Val-19	8.27	3.86	2.22	1.00, 1.08	
Ser-20	8.01	4.25	3.89, 4.04		
Ala-21	7.93	4.23	1.53		
Asn-22	7.92	4.71	2.83		γNH_2 6.83, 7.63
Ala-23	7.96	4.26	1.51		
Met-24	7.85	4.44	2.09, 2.16	2.59, 2.68	ϵCH_3 2.04
Ala-25	7.81	4.32	1.48		

* Chemical shifts are in ppm relative to 3.88 ppm for trifluoroethanol methylene resonance. The estimated error is ± 0.02 ppm.

TABLE 2 Chemical shift for mutant type signal peptides of *E. coli* ribose binding protein in water/TFE at 25°C*

Residue	NH	αH	βH	γH	Others
Met-1					
Asn-2	8.64	4.60	2.79, 2.91		γNH_2 6.73, 7.53
Met-3	8.42	4.43	2.02, 2.10	2.55, 2.62	ϵCH_3 2.05
Lys-4	8.07	4.20	1.70, 1.80	1.36	δCH_2 1.67; ϵCH_2 2.96; ϵNH_3^+ 7.53
Lys-5	7.85	4.32	1.72, 1.84	1.43	δCH_2 1.67; ϵCH_2 2.96; ϵNH_3^+ 7.53
Leu-6	7.71	4.34	1.60	1.44	δCH_3 0.84, 0.92
Ala-7	7.74	4.33	1.44		
Thr-8	7.77	4.33	4.25	1.22	
Pro-9		4.44	2.17, 2.29	1.94	δCH_2 3.73
Val-10	7.54	3.89	2.02	0.94	
Ser-11	7.79	4.27	3.90, 4.00		
Ala-12	7.96	4.14	1.44		
Val-13	7.69	3.66	2.09	0.90, 1.00	
Ala-14	7.89	4.08	1.43		
Leu-15	8.19	4.13	1.74	1.50	δCH_3 0.84, 0.93
Ser-16	8.02	4.13	3.94, 4.05		
Ala-17	8.34	4.14	1.50		
Thr-18	7.87	4.04	4.37	1.22	
Val-19	8.24	3.82	2.14	1.00, 1.08	
Ser-20	7.97	4.25	3.89, 3.97		
Ala-21	7.91	4.20	1.50		
Asn-22	7.88	4.60	2.88		γNH_2 6.67, 7.50
Ala-23	8.01	4.23	1.44		
Met-24	7.81	4.46	2.04, 2.13	2.55, 2.63	ϵCH_3 2.06
Ala-25	7.83	4.37	1.38		

* Chemical shifts are in ppm relative to 3.88 ppm for trifluoroethanol methylene resonance. The estimated error is ± 0.02 ppm.

the carrier frequency was set on the HDO resonance. The 2D experiments were recorded with 512 t_1 measurements and 2048 data points in the t_2 dimension. Eighty-eight to three hundred fifty-two transients were collected for each increment of t_1 in the NOESY experiments, and 80 transients were acquired in the TOCSY experiments. The DQF-COSY was recorded with 512 t_1 transients and 4096 data points in the t_2 dimension to determine the J-coupling constants.

All 2D data were processed with the Bruker program, UXNMR, on a Bruker X-32 workstation or Felix2.10 on an IRIS (Mountain View, CA) 4D-20 silicon graphics workstation. All 2D data sets were collected in the phase-sensitive mode, using the time-proportional phase incrementation method (Marion and Wüthrich, 1983). The data were zero-filled to 1 K in the t_1 dimension. Before the Fourier transform, a 60° shifted-squared sine bell function was multiplied to free induction decays in the NOESY, DQF-COSY, and TOCSY experiments. A polynomial base-line correction was applied to the entire spectral range except for the water resonance region. The spectra were analyzed in both unsymmetrized and symmetrized forms, and the data sets presented are in unsymmetrized form.

RESULTS

Circular dichroism measurement of peptides

No discernible structure of the mutant signal peptide could be seen in water. Because of the limited solubility of the wild-type signal sequence, a CD experiment in water was not performed. These peptides showed practically identical spectra at different concentrations and at pH 3 and 7 (in 50% TFE and 50% 50 mM phosphate buffer by volume), indicating that the conformation is not sensitive to these parameters. Therefore, CD spectra were collected only at pH 3. Fig. 1 presents the temperature-dependent conformational change for both wild-type and mutant signal sequences. All the CD spectra show a minimum at 206–208 nm, with a shoulder around 222 nm, which is indicative of the presence of a α-helical structure. The CD spectra were curve-fitted by the least squares method into the reference CD spectra of four conformations, α-helix, β-sheet, turn, and random structure, based on five proteins, myoglobin, lactate dehydrogenase, lysozyme, papain, and ribonuclease (Yang et al., 1986). The estimated value of the α-helix of the wild-type peptide was 69 and 56% at 25 and 50°C, respectively. For the mutant peptide, the helical content was much lower (33% at 25°C and 28% at 50°C). The isodichroic point observed at 200–202 nm between wild and mutant peptides is consistent with a helix-random coil interconversion. Although the α-helical contents for both wild-type and mutant peptides decreased significantly with increasing temperature, the fractional decreases for both cases were about the same.

Resonance assignments of the peptides

The ^1H resonances in the NMR spectra of the wild-type peptide (Table 1) and mutant peptide (Table 2) were assigned by the sequential assignment methodology (Billeter et al., 1982; Wüthrich, 1986). First, the complete spin system of amino acid residues was identified using the TOCSY spectra (Fig. 2). Next, the sequential connectivities of the backbone were established by following the fingerprint region of the NOESY spectra (Fig. 3).

Secondary structure of wild-type and mutant signal peptides

The NOESY spectrum of the wild-type peptide in 10% DMSO water/TFE solution at 25°C is shown in Figs. 3 *A* and 4 *A*. The NOESY spectra provide a strong support for the presence of a secondary structure in the peptide (Wüthrich et al. 1984; Wüthrich, 1986). A striking network

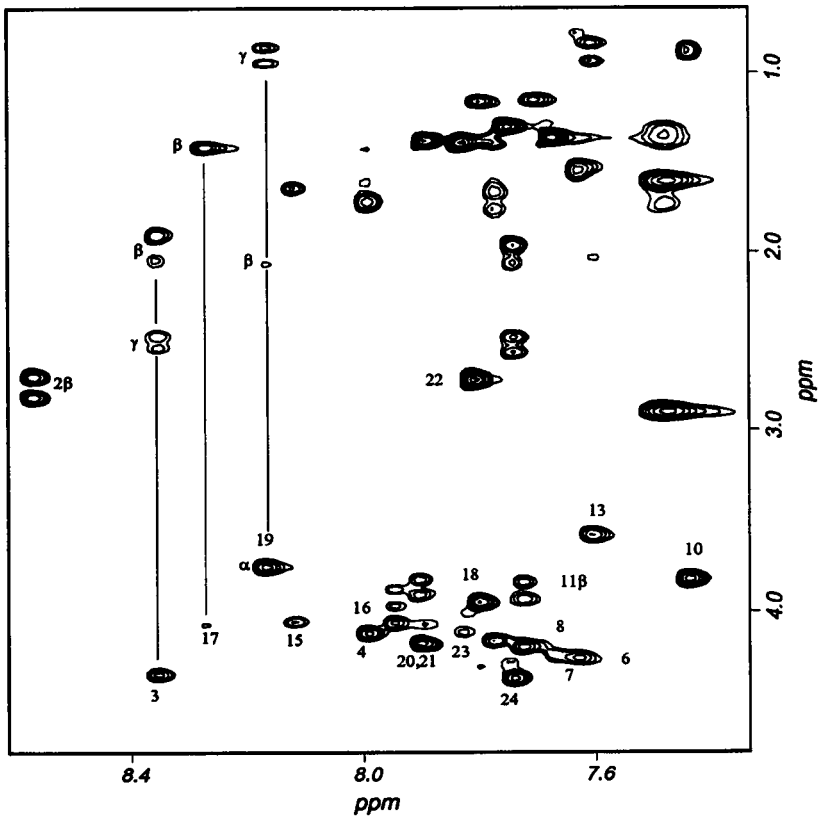

FIGURE 2 A region of the TOCSY spectrum of mutant signal peptide in 10% DMSO, 50% TFE, and 40% water (v/v), pH 3, at 25°C. The CαH(*i*)/NH(*i*) cross peaks are labeled by the number in the sequence. The cross peaks between NH and CαH, CβH, and other protons present the typical correlation pattern of each amino acid (The spin systems of Met-3, Ala-17, and Val-19 are marked).

of cross peaks is observed from residues 3–20. The strong NH(*i*)/NH(i + 1) connectivity of NOE cross peaks is consistent with the presence of α-helical segments in the peptide. The NOE cross peak intensities of NH(*i*)/NH(i + 1) were about 5 times stronger than those of CαH(*i*)/NH(i + 1). Medium range interactions, CαH(*i*)/NH(i + 3), which are also typical of a helical conformation, were clearly observed in Fig. 3 *A*. In addition, the $^3J_{HN\alpha}$ values estimated from the DQF-COSY spectrum were found to be less than 6Hz all along, indicating the polypeptide chain was in the form of a helix. The NH(*i*)/NH(i + 2) and CαH(*i*)/NH(i + 4) interactions were not observed. It is generally difficult to observe NOEs between protons more than 3.5 Å apart in small peptides having less than 30 residues because of the short correlation time in solution. The distances of NH(*i*)/NH(i + 2) and CαH(*i*)/NH(i + 4) are about 4.2 Å in the α-helix.

The NOESY spectra of the mutant peptide in 20 mol % TFE/H_2O measured at 25°C is shown in Figs. 3 *B* and 4 *B*.A strong NH(*i*)/NH(i + 1) as well as CαH(*i*)/NH(i + 1) and CαH(*i*)/NH(i + 3) connectivities are present from Val-10 to Ala-21. The medium range interactions of CαH(*i*)/NH(i + 3) clearly indicate that the α-helical conformation exists in the central part of the peptide. The small $^3J_{HN\alpha}$ values, less than 6Hz in the hydrophobic core region, are also indicative of helical conformation.

Addition of 10% DMSO was found to destabilize the helical conformation of the mutant peptide, as observed by the significant diminution in the magnitude of the NH(*i*)/NH(i + 1) connectivities and by reduced chemical shift of the NH resonances. The crowded overlap of NH resonances is expected from a peptide with an unordered structure. None of the medium and weak interactions were observed in this solution.

Schematic diagrams summarizing the various connectivities observed in the NOESY spectra of the wild-type and mutant peptides are shown in Fig. 5.

DISCUSSION

The translocation machinery in the plasma membrane of *E. coli* with which the signal sequence interacts is a very complicated entity containing several integral proteins, a peripheral protein, and phospholipids. Although the precise nature of interactions between the signal sequence of a precursor protein and these diverse membrane components is yet to be worked out, a common denominator may be that they provide an amphiphilic and/or a hydrophobic environment for the signal sequences. Signal sequences of a large number of precursor proteins of *E. coli*, which interact with the same translocation system, have no conservation of primary sequence. This implies that a common feature of conformation rather than the sequence-specific character of each signal peptide is essential to the translocation. Therefore, structural studies in one or more amphiphilic or nonpolar environments may give a picture close to that of the in vivo situation. The two most commonly used systems that provide these environments for peptides are water/TFE solution and SDS suspension. Earlier, it was reported that the structure of peptides in water/TFE solution is qualitatively similar to a membrane-mimetic environment such as SDS micelles and phospholipid

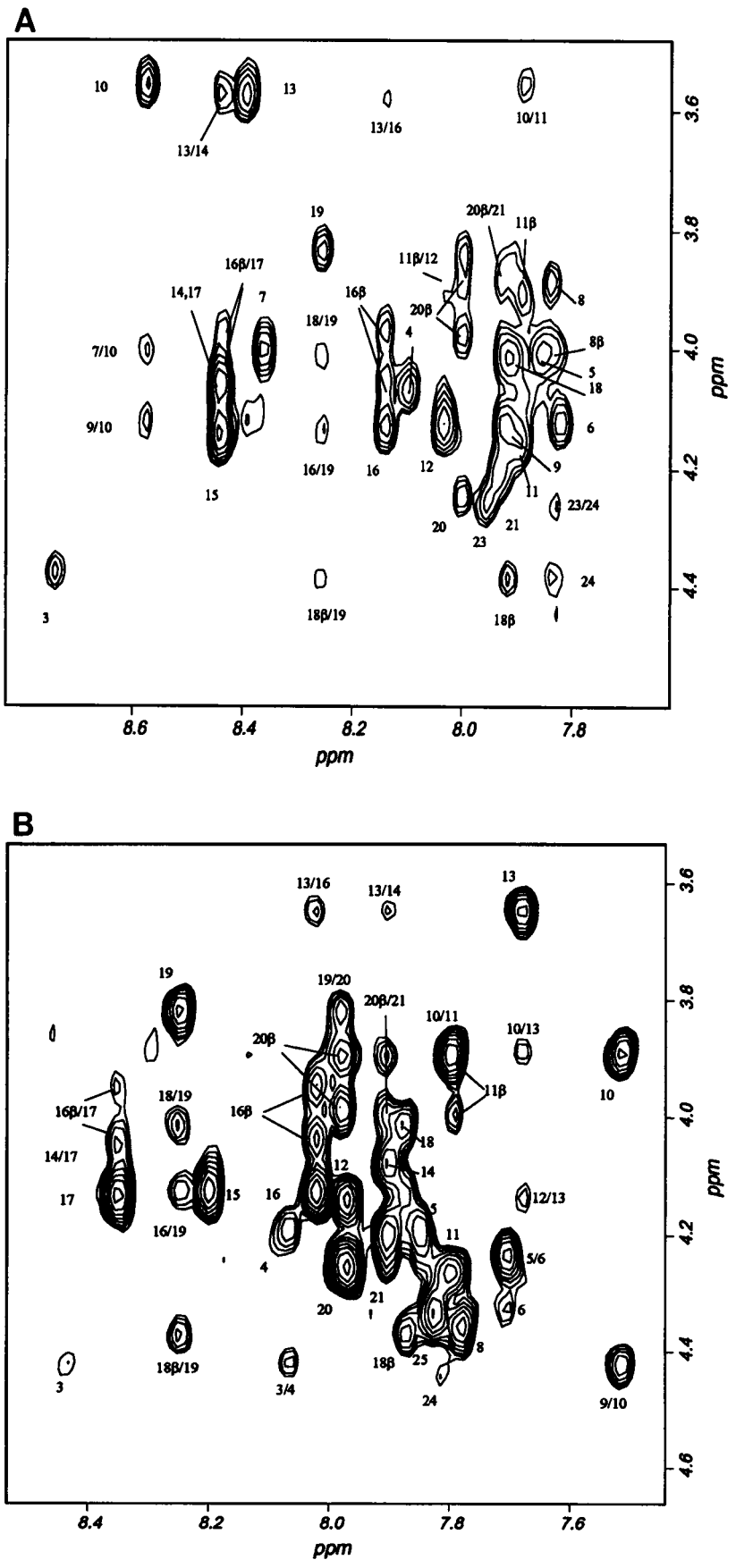

FIGURE 3 Fingerprint regions of NOESY spectra. (*A*) The spectrum of the wild-type peptide in 50% TFE and 40% water (v/v), pH3, at 25°C. (*B*) The spectrum of the mutant peptide in 10% DMSO, 50% TFE, and 40% water (v/v), pH3, at 25°C. The CαH(*i*)/NH(*i*), CαH(*i*)/NH(*i*+3), and CβH(*i*)/NH(*i*+1) cross peaks are labeled by the numbers of both amino acids in the sequence.

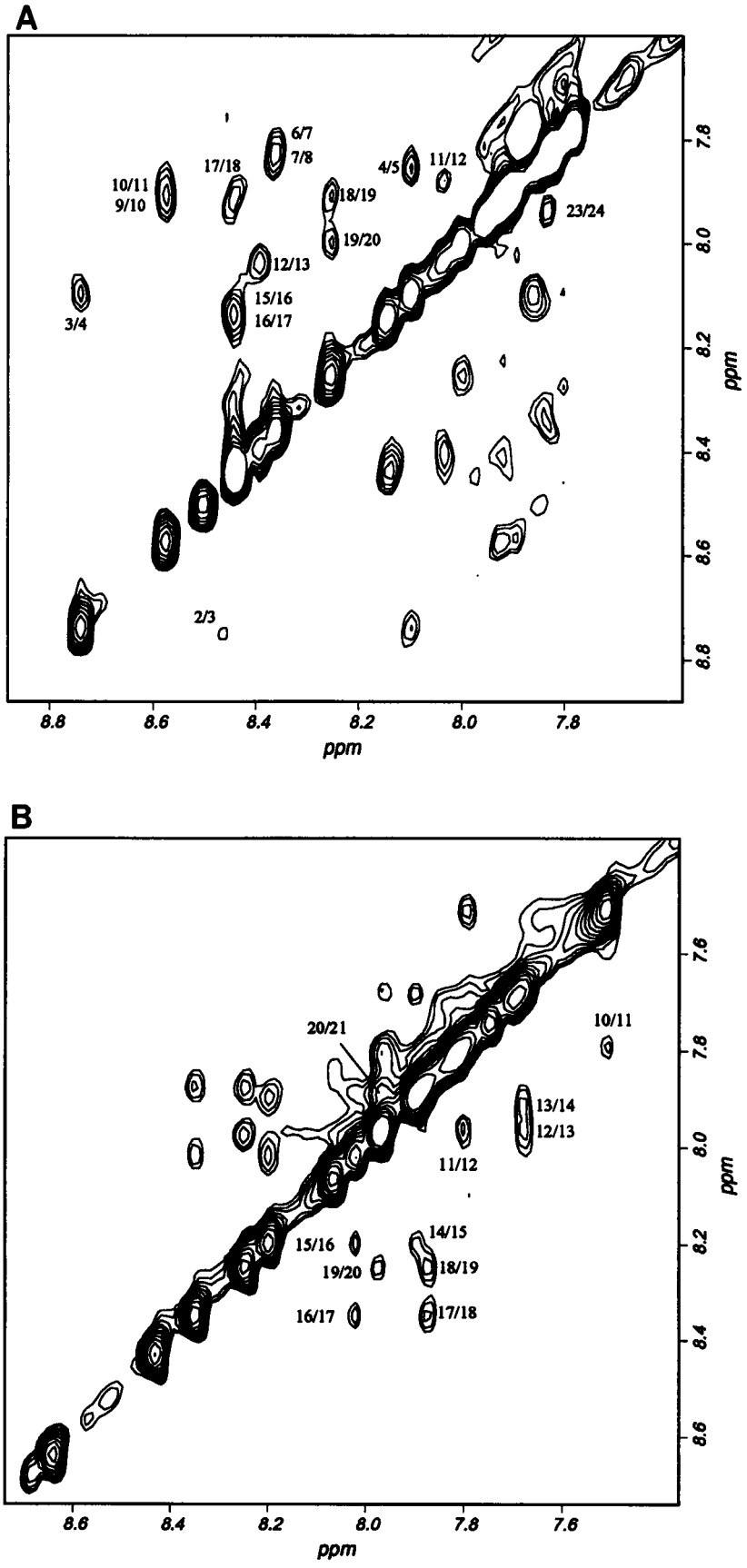

FIGURE 4 The NH region of NOESY spectra of wild-type (*A*) and mutant (*B*) peptides at pH3, 25°C. The solvent composition was the same as the above experiments. The cross peaks between two NHs are marked by their numbers in the sequence.

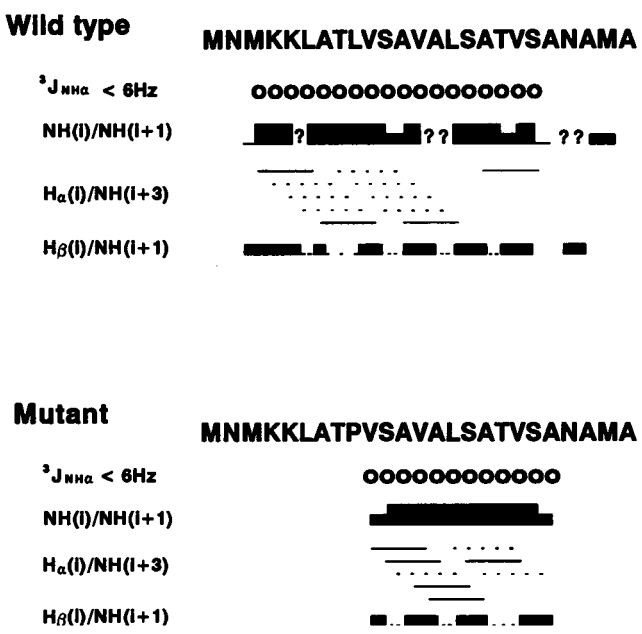

FIGURE 5 Schematic representation of the summary of NMR data. The residues that have $^3J_{HN\alpha}$ value less than 6 Hz are represented by open circles. The thickness of the lines represents the intensity of NOE, and the question marks indicate the indistinguishable cross peaks because of their proximity to the diagonal. The dashed lines in the medium range NOEs are the cross peaks overlapped by the other cross peaks such as intraresidue cross peaks. (*A*) Wild-type peptide in 10% DMSO and 50% TFE/water (by volume) solution and the (*B*) mutant peptide in 20 mol % TFE/water solution.

vesicles (McKnight et al., 1989; Briggs, 1986). A recent critical comparision of structures of *E. coli* OmpA signal peptides in TFE solution and SDS micelle solution, however, showed some differences between these solutions; the SDS micelle suspension inducing more α-helix structure (Rizo et al., 1993). Still, it is difficult to assess exactly what kind of environment the signal peptide will encounter during the translocation steps. It may be that the signal peptide goes through several different environments during the whole translocation process, from polar cytosolic solution through amphiphilic membrane surface into the hydrophobic interior of the membrane, etc. Its interaction with the peripheral and integral protein of the transloction machinery will give additional complication. Under this uncertain situation, we chose the water/TFE solution as the first stage of our research, because it provides a simple system to compare critically the structure of wild-type and mutant signal peptides. The addition of DMSO into the water/TFE solution was needed to dissolve the wild-type peptide. DMSO disturbed the water/TFE environment and broke the α-helix of the mutant peptide. But the effect of DMSO on the wild-type peptide was small, and this peptide still maintained the helical structure. There is also a question about the propriety of studying signal peptides detached from the rest of the precursor protein. In a sense, this is unnatural, because the COOH-terminal end of the isolated signal peptide is usually charged, and the effect of the charge is difficult to assess. However, this may be justified when the aim of the study is to see the structural difference between the wild-type and the mutants, inasmuch as the end-charge effect should be the same for both of these peptides. We are currently preparing the signal peptides with a 30-residue extension into the mature domain.

The data have delineated the regions of α-helix structure in both functional and nonfunctional RBP signal peptides. The functional peptide has an α-helix of 18 residues (Met-3 to Ser-20) including the NH_2-terminal region in 10% DMSO water/TFE solution. The α-helix in the nonfunctional peptide has only 12 residues from Val-10 to Ala-21 (Fig. 5). It seems that the helix propagation from the hydrophobic core region to the COOH terminus was disturbed by the helix breaker Asn-22 in both peptides (Chou and Fasman, 1974). In the nonfunctional mutant peptide, another strong helix breaker, Pro-9, apparently hinders the formation of the helix in the NH_2-terminal region (Chou and Fasman, 1974). The positive charges in the NH_2-terminal region (Lys-4 and Lys-5) did not affect the helix formation of the wild-type signal peptide of RBP. This differs from LamB signal peptides (Bruch and Gierasch, 1990), which have a helix through the hydrophobic core to the COOH terminus (17 residues from Leu-8 to Met-24) in the functional peptide and only in the hydrophobic core (7 residues from Val-14 to Ser-20) in nonfunctional peptides in water/TFE solution at 25°C. Although positive residues in the NH_2-terminal region were suggested to destabilize α-helical conformation (Shoemaker et al., 1987), it is possible that a Pro at position 9 in both the wild-type and mutant signal peptides of LamB limits the area of the helix to the NH_2-terminal region. However, the interaction between the helix dipole and charged groups, which was thought to destabilize a secondary structure (Shoemaker et al., 1987), did not break the helix formation of the RBP signal peptide at the NH_2-terminal. We were able to observe the NH(i)/NH(i + 1) NOE between residues 23 and 24 and C_βH(i)/NH(i + 1) NOE between residues 20 and 21 and 22 and 23. Because the connectivity may not be continuous and there is no intermediate NOE between C_αH(i)/NH(i + 3), a helix structure in the region is unlikely. The obvious difference in the location of the helices and similar length of helices in both functional signal peptides of LamB and RBP suggests that the general requirements for the function of the signal peptide may not be the location of the helix but the proper length of the helix. Similar studies on human lysozyme (Yamamoto et al., 1990), mitochondrial aldehyde dehydrogenase (Karslake et al., 1990), and *E. coli* OmpA (Rizo et al., 1993) signal peptides suggest the importance of the α-helical structure for the translocation, although no definite general pattern has emerged as to the locality and stability of α-helical segments in relation to their function. However, it was shown that the synthetic signal peptides of chicken lysozyme and some *E. coli* proteins form the β-structure in nonpolar solvents (Reddy and Nagaraj, 1989). It is clear, therefore, that additional studies on a variety of signal peptides under a wide range of conditions are needed before the relationship between the structure of the signal peptides during the translocation steps and translocation efficiency becomes apparent.

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