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A Peptide Corresponding to Residues Asp177 to Asn208 of Human Cyclin A Forms an α-Helix

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Cyclins are essential activators of eukaryotic cell cycle-regulating enzymes called cyclin-dependent kinases (CDKs). The binding of cyclins to CDKs is mediated by a structural motif comprising a five-helix bundle called the cyclin fold and an additional helix (the N-terminal α-helix) located N-terminal to the cyclin fold. In this work, we examine, using CD and NMR spectroscopy, the structure of a 32-residue synthetic peptide derived from the segment (Asp177 to Asn208) corresponding to the N-terminal α-helix of human cyclin A. CD spectroscopic analysis of the peptide revealed that trifluoroethanol (TFE) can induce the peptide to assume a stable α-helix conformation. Two-dimensional ^{1}H NMR spectroscopy showed that the α-helix is formed by the Asp181 to Cys193 segment of the peptide. The α-helical structure of the peptide in the TFE/H_2O cosolvent was found to be identical to that in the crystal structure of intact cyclin A. Taken together, these results suggest that the N-terminal α-helix of cyclins may exist as an independent structural unit that plays essential functional roles in activating CDKs.

Key Words: **cyclin A; cyclin fold; N-terminal α-helix; NMR; peptide.**

Cyclin-dependent kinases (CDKs) play pivotal roles in the coordination of the eukaryotic cell cycle. Formation of binary complexes with specific cyclins is mandatory for the activities of CDKs. The activities of CDKs are further regulated by an intricate mechanism of protein–protein association and phosphorylation/dephosphorylation (1). The recent elucidation of the three dimensional structures of CDK2 in three different states [cyclin A-free (inactive); cyclin A-bound, Thr160 unphosphorylated (partially activated); and cyclin A-bound, Thr160 phosphorylated (fully activated)] provided a prototype model of CDK activation by association with a cyclin and phosphorylation of the activation loop (2–4).

In the absence of cyclin A, the PSTAIRE helix of CDK2 is located outside of the active site of the enzyme. The binding of cyclin A induces the PSTAIRE helix to move into the active site, resulting in the alignment of the side chain of Glu51 with side chains of Lys33 and Asp145 to form the catalytic triad. The binding of cyclin A also melts the αL12 helix of free CDK2 into a β-strand, and at the same time directs the T-loop away from the entrance of the catalytic cleft (3). Phosphorylation of Thr160 further locks the T-loop in a rigid conformation by extensive electrostatic and hydrogen-bonding interactions between the phosphate group and a number of Arg residues in the vicinity, thereby creating a substrate binding surface on the enzyme.

The crystal structures of cyclin A (3–5) and cyclin H (6, 7) have shown that each of these members of the cyclin family contains two conserved "cyclin folds" which share very little sequence identity. Within each cyclin fold, three helices (α1, α2, and α3) form a three-helix bundle, and the additional two helices (α4 and α5) pack on two sides of the bundle. Cyclin A exhibits a rigid structure, and it retains its conformation upon binding to CDK2 (3–5). The cyclin folds are also found in the crystal structures of transcription factor TFIIB (8, 9) and the A-pocket and B-pocket domains of the tumor suppressor retinoblastoma protein (Rb) (10, 11), even though no significant sequence identity can be found among cyclins, TFIIB, and Rb. The crystal structures of these proteins show that the wedged surface formed between α3, α4, and α5 act as the binding site for their respective targets (3, 9, 11). Therefore, it is likely that the cyclin fold is a general adapter motif

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Abbreviations used: CD, circular dichroism; CDK, cyclin-dependent kinase; DTT, dithiothreitol; HMQC, heteronuclear multiple quantum correlation; NOE, nuclear Overhauser enhancement; NOESY, NOE spectroscopy; TFE, 2,2,2-trifluoroethanol; TOCSY, total correlation spectroscopy; $[\Theta]_{222}$, mean ellipticity at 222 nm.

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employed for protein-protein and protein-DNA recognition.

Apart from the five cyclin fold-forming helices, cyclin fold-containing proteins usually contain extra helices at both their N- and C-termini. These helices are often found to be an indispensable part of these proteins in both structural and functional senses. For example, deletion of part of the N-terminal α-helix of cyclin A completely abolishes the binding of the protein to CDK2 (12); likewise, an N-terminal α-helix deletion mutant of cyclin H is incapable of activating CDK7 (6). We postulate that the N-terminal α-helix of cyclins may function as a relatively independent structural unit. To address this hypothesis, we have studied the solution structure of a 32-residue synthetic peptide encompassing the N-terminal α-helix of cyclin A.

MATERIALS AND METHODS

Peptide synthesis. A 32-residue peptide (DYHEDIHTYLREMEVKCKPKVGYMKKQPDITN) corresponding to the sequence Asp177 to Asn207 of human cyclin A was synthesized with an Applied Biosystems Model A431 automated peptide synthesizer using Fmoc-based chemistry. The crude peptide was purified by gel filtration chromatography using a Sephadex G-10 column followed by a reverse-phase C_{18} HPLC column. The purity of the peptide was found to exceed 95% by analytical reverse phase C_{18} HPLC, and the authenticity of the peptide was confirmed by measurement of its molecular mass by matrix-assisted laser desorption ionization time-of-flight–mass spectrometry.

CD experiments. CD spectra of the cyclin A peptide were collected on a JASCO J-720 CD spectropolarometer at 25°C. A cell pathlength of 1 mm was used throughout the experiments. The concentration of the cyclin A peptide stock solution was determined by the ultraviolet absorption at 280 nm of the three Tyr residues in the peptide. For CD measurements, the peptide was dissolved in 20 mM sodium acetate buffer (pH 3.0, 4.0, and 5.0) or 10 mM Mes buffer (pH 6.0, and 7.0) containing various concentrations of 2,2,2-trifluoroethanol (TFE). Dithiothreitol (DTT) with a concentration of 1 mM was included in the sample buffer to prevent oxidation of the single Cys residue in the cyclin A peptide. The final concentration of the peptide was 50 μM.

NMR experiments. To study the conformation of the peptide in aqueous solution by NMR, the cyclin A peptide was dissolved in 0.5 ml of unbuffered 90% H_2O/10% D_2O solution, and the pH value of the sample was adjusted to 4.0. Another two NMR samples, one in 70% H_2O/30% (*v*/*v*) deuterated TFE-d_3 and another in 70% D_2O/30% (*v*/*v*) deuterated TFE-d_3, were prepared to investigate the conformation of the peptide in aqueous/TFE mixtures. The concentration of the peptide was 3 mM in all three samples. To prevent oxidation of the single Cys residue in the peptide, deuterated DTT-d_{10} with a concentration of 2 mM was included in the NMR samples. All NMR spectra were acquired on a Varian INOVA 500 spectrometer at a ^{1}H frequency of 500.11 MHz at 20°C. Two dimensional TOCSY (13) and NOESY (14) spectra were acquired with a spectral width of 5500 Hz in both dimensions. FID data matrices were composed of 512 × 2048 ($t_1 \times t_2$) for both types of experiments. The mixing times used for NOESY experiments were 150 and 300 ms. MLEV17 spin lock pulses (40 and 80 ms) were used in TOCSY experiments. The (^{1}H,^{13}C)-HMQC spectrum of the natural abundance cyclin A peptide in 70% D_2O/30% TFE-d_3 was recorded using a pulse sequence described by Bax *et al.* (15). All NMR spectra were processed and displayed using the nmrPipe software package (16).

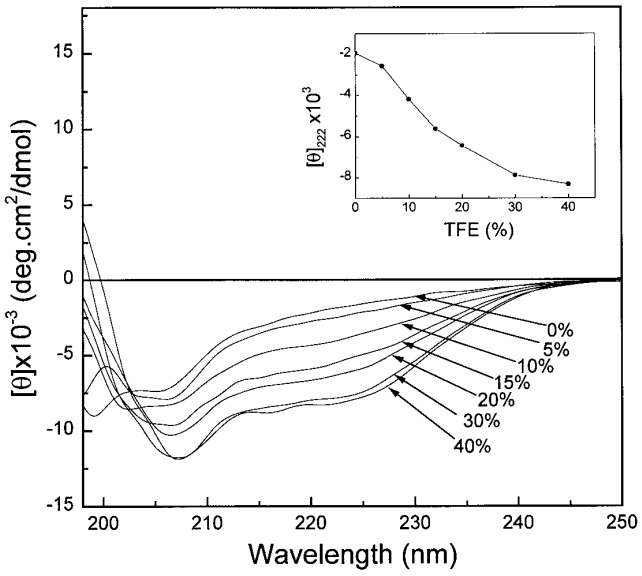

FIG. 1. Ultraviolet circular dichroism spectra of the cyclin A peptide (50 μM) in various concentrations (% *v*/*v*) of TFE. The peptide was dissolved in 20 mM sodium acetate buffer at 25°C, pH 4.0. The insert shows the change in the mean ellipticity of the peptide at 222 nm as a function of TFE concentration.

RESULTS

The cyclin A peptide adopts an unordered structure in aqueous solution. The 32-residue synthetic peptide studied here corresponds to amino acid residues Asp177 to Asn208 of human cyclin A; this region includes the N-terminal α-helix of the first cyclin fold of the protein (3, 5). A combination of two dimensional TOCSY and NOESY experiments of the peptide in pure H_2O allowed sequence specific backbone ^{1}H resonance assignment of the peptide (data not shown). Analysis of the NOESY spectra (150 and 300 ms mixing times) as well as $^1H^{\alpha}$ chemical shift values of the peptide demonstrated that the cyclin A peptide adopts an extended conformation in aqueous solution (data not shown). To study whether the peptide has a pH dependent conformational preference, we have performed similar NMR analysis of the peptide at different pH values (pH 5.0 and 6.0). No regular secondary structure could be observed under all pH conditions (data not shown). The CD spectrum also showed that the peptide adopts a random coil structure in pure H_2O at 25°C (Fig. 1).

CD spectra of the cyclin A peptide in TFE/H_2O mixtures. To investigate the secondary structure-forming propensity of the cyclin A peptide, the helix-promoting solvent TFE was used in subsequent studies. With increasing concentration of TFE, the CD spectra of the peptide showed greater negative ellipticities at 208 and 222 nm, indicating the formation of α-helical conformations. The increases in negative ellipticities of the peptide reached a maximum value at a TFE concentra-

tion of ≈30% (see Fig. 1, inset); this corresponds to an approximately 20% of α-helix overall in the peptide using the value $\Theta_{222} = -40{,}000$ deg · cm^2 dmol^{-1} to represent 100% α-helix (17). Since the cyclin A peptide contains a number of charged residues with pKa values ranging between 3 and 7, we have analyzed the structure of the peptide at different pH values with 30% TFE and without TFE using CD spectroscopy. The CD spectra of the cyclin A peptide did not show detectable changes as a function of pH both in the presence and absence of TFE, suggesting that the charged groups are not actively engaged in forming the secondary structure of the peptide (data not shown). Two dimensional NMR spectroscopy was used to precisely determine the secondary structure of the peptide induced by TFE at a concentration of 30% (*v*/*v*).

Secondary structure of the cyclin A peptide determined by NMR. The ^{1}H chemical shift of the cyclin A peptide in 30% TFE was well dispersed. The complete resonance assignment of the peptide was obtained by a combination of TOCSY and NOESY spectra, and the values are listed in Table 1. Figure 2A is the fingerprint region of the cyclin A peptide NOESY spectrum (300 ms mixing time) showing the sequence specific assignment of the peptide. The NH and NH region of the same NOESY spectrum is shown in Fig. 2B. A number of continuous, intense d_{NN} NOEs are present in Fig. 2B, indicating the existence of α-helical conformation in the peptide. In addition, a number of α-helix characteristic medium range NOEs were also observed for the peptide, and Fig. 2C represents part of the $C^\alpha H$ and $C^\beta H$ region of the peptide in a 70% D_2O/30% TFE-d_3 mixture at 25°C, pH 4.0. The NOEs observed for the cyclin A peptide are summarized in Fig. 3. In addition to the NOEs listed in Fig. 3, numerous side chain medium range NOEs within the helical segment of the peptide were also observed (e.g., NOEs between ring protons of Tyr185 to side chains of Ile182 and Glu188, 4H of His183 to side chain of Leu186). The pattern of the NOEs (intense d_{NN}, a continuous stretch of medium range $\alpha\beta(i, i + 3)$ NOE connectivities together with the medium range side chain NOEs) strongly suggests that the cyclin A peptide adopts an α-helical conformation from residues Asp181 to Cys193. We have also analyzed the conformation of the peptide in 30% TFE at different pH values (pH 5.0 and 6.0), and the results showed that the peptide adopts a same α-helical structure at higher pH values (data not shown).

Other than NOEs, chemical shift values have proven to be valuable in determining the secondary structure of peptides and proteins in solution (18). The $C^\alpha H$ and C^α chemical shift values are particularly informative in determining the secondary structure of a given peptide. To obtain C^α chemical shifts, a (^{1}H,^{13}C)-HMQC spectrum was acquired for the cyclin A peptide in 70% D_2O/30% TFE-d_3 mixture. The C^α chemical shift values are also listed in Table 1. Figure 4 shows the difference in the $C^\alpha H$ and C^α chemical shifts from the "random coiled" values for the cyclin A peptide. As expected, all $C^\alpha H$ resonances from residues Asp181 to Cys193 shift in the upfield direction; all C^α shifts within the same region are downfield. Therefore, both $C^\alpha H$ and C^α secondary chemical shift values reinforce the NOE-based conclusion that the cyclin A peptide adopts an α-helical conformation from Asp181 to Cys193. A Gaussian-shape distribution of the secondary chemical shift values seen in Fig. 4 indicates that the center of the α-helix may be ordered, and the two ends experience fraying. Further analysis of the helical part of the peptide using a helical wheel diagram shows that the α-helix of the cyclin A peptide is amphiphilic with the hydrophobic side clustered with Tyr185, Ile182, Leu186, and Met189 (Fig. 5).

DISCUSSION

The fact that deletion of the N-terminal α-helices of cyclins A and H abrogates their ability to bind to and activate CDK2 and CDK7 respectively supports the notion that the N-terminal α-helix, together with the cyclin fold, may confer CDK binding specificity of various members of the cyclin family (6, 11). Sequence analysis of cyclin G shows that it does not contain an N-terminal α-helix (19). The lack of the N-terminal α-helix could indicate that cyclin G may not function as a CDK activator, and indeed no CDK partner has been found for cyclin G to date. In this work, we have explored the possibility that the N-terminal α-helix of human cyclin A is a relatively independent structural unit in solution, by CD and NMR spectroscopic techniques. Although the peptide adopted an unordered structure in H_2O, a stable α-helical conformation could be induced by the helix-promoting solvent TFE. The secondary structure of the cyclin A peptide in 30% TFE was determined based on NOE information as well as on $C^\alpha H$ and C^α chemical shift data. The amino acid residues from Asp181 to Cys193 of the peptide were found to adopt an α-helical structure. The α-helix of the peptide detected in TFE/H_2O co-solvent is essentially identical to that found in intact cyclin A (3–5). In addition, the loop that links the N-terminal helix and the "helix 1" of the first cyclin fold remains as a flexible loop structure in the TFE/H_2O mixture. The results of this study further substantiate the notion that TFE promotes the formation of and/or stabilizes α-helical structures only when they are intrinsically encoded by the amino acid sequence [for recent examples, see (20–23)]. The crystal structure of cyclin H has also shown that it contains an N-terminal α-helix, although it packs with the cyclin folds rather differently than does the N-terminal α-helix of cyclin A. Given the overall structural and functional similarity between cyclin A

TABLE 1

Chemical Shift Values of the Cyclin A Peptide[a]

Residues	NH (ppm)	αH (ppm)	βH (ppm)	Others (ppm)	αC (ppm)
Asp-177	ND[b]	4.28	2.96, 2.93		ND[b]
Tyr-178	8.60	4.57	3.08, 3.02	2,6H 7.09	59.57
				3,5H 6.78	
His-179	8.09	4.40	3.22	2H 8.59	56.66
				4H 7.27	
Glu-180	8.19	4.30	2.17	γCH_2 2.47	53.51
Asp-181	8.32	4.64	2.91		55.38
Ile-182	8.36	4.08	1.94	γCH_3 0.89	64.00
				γCH_2 1.56, 1.29	
				δCH_3 0.92	
His-183	8.34	4.33	3.43	2H 8.65	56.66
				4H 7.35	
Thr-184	8.23	3.95	4.38	γCH_3 1.31	67.02
Tyr-185	8.01	4.28	3.18	2,6H 7.09	61.67
				3,5H 6.82	
Leu-186	8.46	4.02	1.94	γH 1.56	62.36
				δCH_3 0.93, 0.94	
Arg-187	7.95	4.11	2.02, 1.83	γCH_2 1.71	59.57
				δCH_2 3.29, 3.23	
				ϵNH 7.24	
Glu-188	8.18	4.06	2.28, 2.10	γCH_2 2.63, 2.42	58.40
Met-189	8.21	4.25	2.42, 2.29	γCH_2 2.84	57.94
				ϵCH_3 2.12	
Glu-190	8.15	4.07	2.31, 2.21	γCH_2 2.62, 2.41	59.22
Val-191	7.79	3.90	2.27	γCH_3 1.12, 1.02	65.39
Lys-192	8.01	4.24	2.01, 1.73	γCH_2 1.54	58.52
				δCH_2 1.78	
				ϵCH_2 3.01	
Cys-193	8.00	4.51	3.01		59.80
Lys-194	7.85	4.57	1.92	γCH_2 1.59	58.52
				δCH_2 1.78	
				ϵCH_2 3.07	
Pro-195		4.47	2.36, 1.93	γCH_2 2.07	63.88
				δCH_2 3.89, 3.66	
Lys-196	8.24	4.39	1.84, 1.73	γCH_2 1.56	56.42
				δCH_2 1.91	
				ϵCH_2 3.05	
Val-197	7.89	4.16	2.13	γCH_3 0.98	63.06
Gly-198	8.34	3.93			45.59
Tyr-199	7.85	4.57	3.09, 3.04	2,6H 7.16	55.73
				3,5H 6.88	
Met-200	8.04	4.42	2.09, 2.02	γCH_2 2.56, 2.50	57.47
				ϵCH_3 3.01	
Lys-201	8.16	4.34	1.98, 1.87	γCH_2 1.47	60.03
				δCH_2 1.76	
				ϵCH_2 3.04	
Lys-202	8.04	4.32	1.88	γCH_2 1.48	57.47
				δCH_2 1.76	
				ϵCH_2 3.04	
Gln-203	8.25	4.69	2.18, 2.03	γCH_2 2.44	53.98
Pro-204		4.45	2.33, 1.97	γCH_2 2.07	55.96
				ϵCH_2 3.83, 3.73	
Asp-205	8.37	4.73	2.89, 2.76		63.30
Ile-206	7.97	4.34	1.99	γCH_3 0.98	61.55
				γCH_2 1.48	
				δCH_3 0.92	
Thr-207	7.81	4.32	4.32	γCH_3 1.23	56.66
Asn-208	8.25	4.78	2.93, 2.81		60.62

[a] The chemical shift values of the cyclin A peptide were obtained in the present 30% TFE at 25°C, pH 4.0.
[b] Data not obtained.

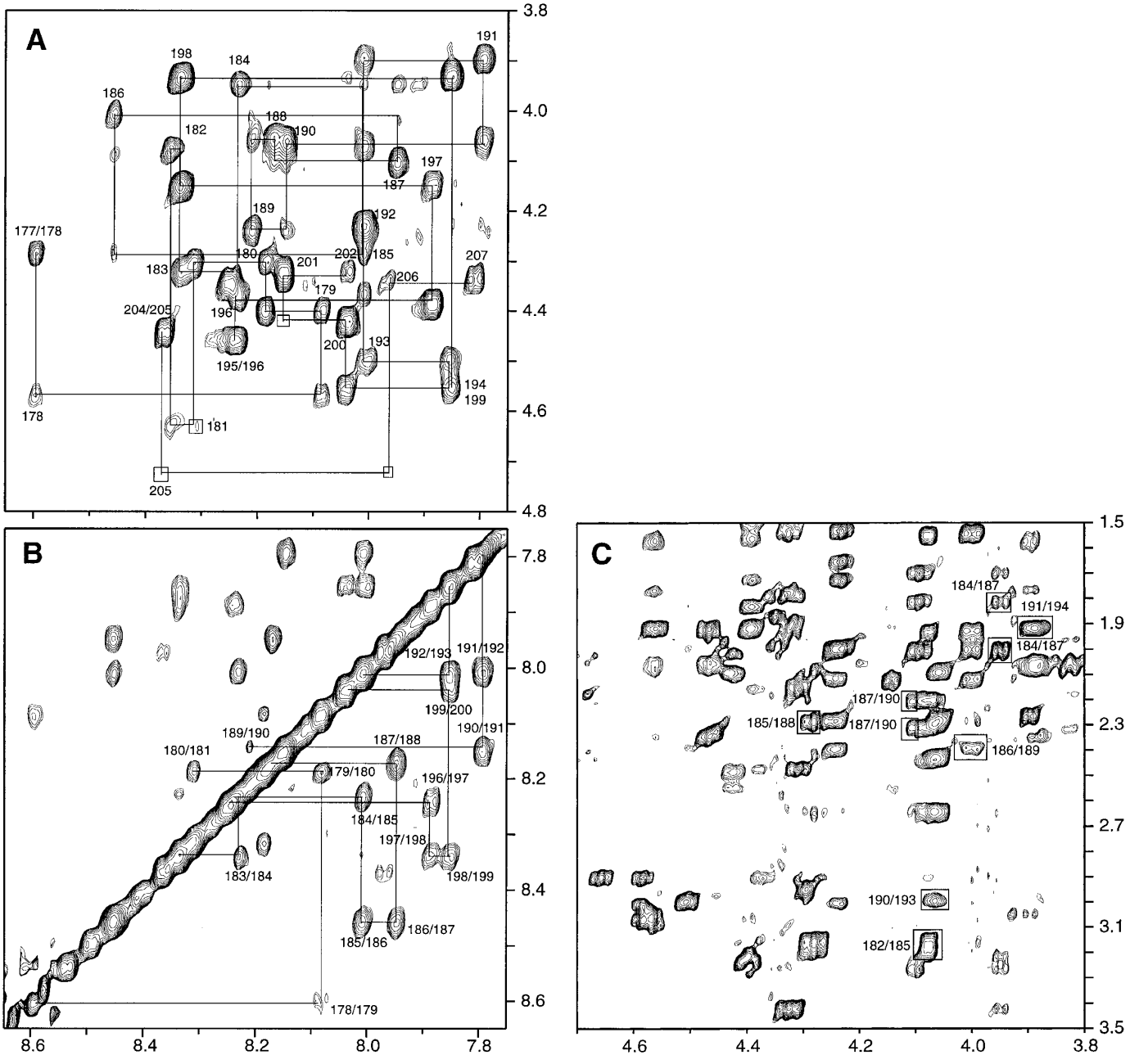

FIG. 2. Portions of a 500-MHz NOESY spectrum (300 ms mixing time) of the cyclin A peptide in 70% H_2O/30% TFE-d_3 at 25°C, pH 4.0, showing the NOE connectivities between (A) NH and $C^{\alpha}H$, (B) NH and NH, and (C) $C^{\alpha}H$ and $C^{\beta}H$ protons. The sequential backbone assignment is shown in A. Selected medium range, $\alpha\beta(i, i + 3)$ NOE connectivities are highlighted in C with open boxes.

and cyclin H, it was suggested that the N-terminal helix of cyclin H would change to a conformation similar to that of the N-terminal α-helix of cyclin A upon binding to CDK7 (7). Therefore, it is likely that the N-terminal helix peptide fragment in cyclin A and possibly also in other cyclins may function as a relatively independent structural unit compared to the tightly folded "cyclin-fold."

The N-terminal α-helix of the cyclin A peptide is amphiphilic in nature (Fig. 5). The crystal structure of cyclin A shows that the N-terminal peptide fits in the interface between the two cyclin folds. The packing of the N-terminal α-helix with the cyclin folds is mainly mediated by hydrophobic interactions between residues in the hydrophobic face of the peptide as shown in Fig. 5, and those in the interface of the cyclin repeats. The induction of an amphiphilic α-helix in the cyclin A peptide by TFE is reminiscent of α-helix induction in a number of calmodulin-binding peptides by TFE (20, 24, 25). In the absence of calmodulin, calmodulin-binding

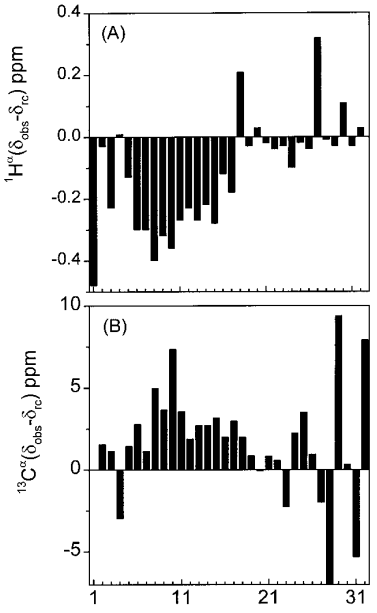

FIG. 3. Plot as a function of the residue number of the difference between the chemical shifts of (A) the $C^\alpha H$ and (B) C^α resonance and their corresponding "random coil" values. The random coil chemical shift values were obtained from (18).

peptides such as calmodulin binding domains of myosin light chain kinase, caldesmon, and nitric oxide synthase adopt unordered structures. The binding of calmodulin induces the formation of amphiphilic α-helical structures in these peptides. Interactions between the calmodulin-binding peptides and calmodulin are dominated by hydrophobic interactions between the hydrophobic face of the peptides and solvent-exposed hydrophobic surfaces of calmodulin [for a review, see (26)]. TFE/H_2O co-solvent induces, in calmodulin-binding peptides, α-helical structures identical to those induced by calmodulin binding (20, 24, 25). It is thus likely that TFE can mimic the hydrophobic interaction

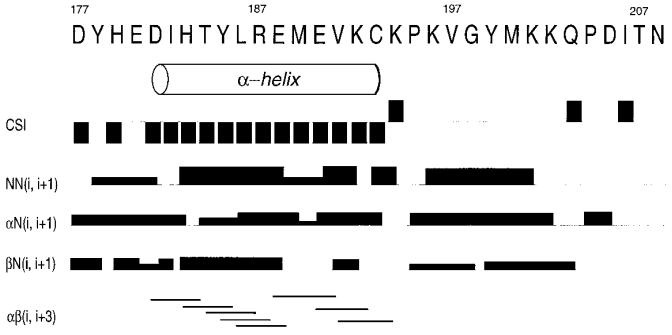

FIG. 4. Summary of NOE connectivities observed for the cyclin A peptide 70% H_2O/30% TFE-d_3 at 25°C, pH 4.0. The thickness of the lines represents the intensities of the NOE cross-peaks. The chemical shift index (CSI) derived from the $C^\alpha H$ and C^α values is also included in the figure.

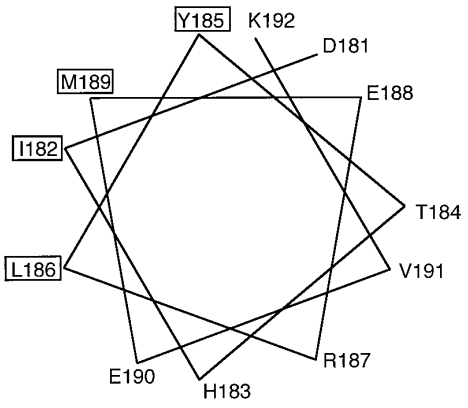

FIG. 5. Helical wheel presentation of the cyclin A peptide α-helix obtained from the NMR data. The hydrophobic amino acid residues on one side of the helix are highlighted with open boxes.

which induces α-helix formation in the N-terminal region of cyclin A.

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