The regions of securin and cyclin B proteins recognized by the ubiquitination machinery are natively unfolded

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Abstract The proteins securin and cyclin B are destroyed in mitosis by the ubiquitin/proteasome system. This destruction is important to mitotic progression. The N-terminal regions of these proteins contain the sequence features recognized by the ubiquitination system. We have demonstrated using circular dichroism and 1-D and 2-D nuclear magnetic resonance that these rather substantial regions are natively unfolded. Based on these findings, we propose a model that helps to explain previously enigmatic observations. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Natively unfolded; Mitosis; Circular dichroism; Nuclear magnetic resonance; Cyclin B; Securin

1. Introduction

The targeted destruction of proteins is critical in the regulation of many cellular processes, including antigen presentation [1,2], the inflammatory response [3–5], mis-folded protein turnover [6,7], and cell cycle control. Two dramatic examples in cell cycle control are the destruction of the proteins securin [8–10] and cyclin B [11,12]. The destruction of securin facilitates sister chromatid segregation by releasing the binding partner of securin, separase, which then cleaves the protein complexes that bind together the sister chromatids. The destruction of cyclin B is essential for mitotic exit, presumably because such destruction leads to the inactivation of its binding partner, the kinase cdc2.

The targeted destruction of proteins is accomplished by the ubiquitin/proteasome system (for reviews see [13–15]). In this system, the E1 (ubiquitin-activating enzyme) activates ubiquitin in an ATP-dependent manner and transfers ubiquitin to an E2 protein (ubiquitin-conjugating enzyme). The E2 alone or in concert with an E3 activity (ubiquitin ligase) transfers ubiquitin to the target protein. The C-terminal carboxyl group of ubiquitin is linked to the ε-amino group of a lysine in the target protein to form an isopeptide bond. Subsequently, a polyubiquitin chain is formed on the target protein in which one ubiquitin is linked covalently to the next. This polyubiquitin-target protein conjugate is recognized by the proteasome, which then hydrolyzes the target protein and releases free ubiquitin. The specificity of target protein ubiquitination is conferred by different cognate pairs of E2 and E3 activities. In the cases of mitotic-specific destruction, the ubiquitination of securin and cyclin B is performed by a specific mitotic E2 protein (E2-C in clam, UbcH10 in humans) and a specific E3 activity (the anaphase promoting complex/cyclosome or APC/C, a large multi-subunit protein complex). As might be expected for such important and irreversible events, the mitotic-specific destruction of securin and cyclin B is highly regulated by an intricate network of interactions that links APC/C activity and specificity with the readiness of the cell to proceed with the mitotic program.

The mechanism of ubiquitination has been studied extensively. However, little is known about the underlying structural requirements of the target protein. In the case of mitotic-specific destruction, the 'destruction box' serves as the major signal recognized by the ubiquitination apparatus. The destruction box is a sequence motif in the N-terminal region of proteins selectively destroyed in the mitotic program. The minimal consensus sequence for the destruction box is R-X-X-L. An additional destruction signal, the 'KEN box', is present in some mitotic proteins including cdc20 [16]. Securin from humans (PTTG), *Saccharomyces cerevisiae* securin (Pds1), and *Schizosaccharomyces pombe* (Cut2) have both a KEN box and a destruction box.

The regions of cyclin B and securin that contain the destruction box motif are independently functional elements. When expressed as a fusion with a non-mitotic protein, the fusion protein is degraded in a mitotic-specific fashion [12,17,18]. Moreover, the N-terminal fragments of cyclin B and fission yeast securin compete with the full-length protein for the destruction machinery [8,19]. Thus, these N-terminal regions of cyclin B and securin are themselves of considerable biological significance and therefore are appropriate choices for structural analysis.

Here we report that the regions of cyclin B and yeast securin Pds1 that comprise approximately the N-terminal 100 amino acid residues are natively unfolded. We demonstrate the unfolded nature of these rather large regions using the tech-

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Abbreviations: APC/C, anaphase promoting complex/cyclosome; CD, circular dichroism; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect

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niques of circular dichroism (CD) and nuclear magnetic resonance (NMR). These findings illuminate previous biochemical and biophysical results regarding mitotic-specific destruction and contribute to the growing realization of the biological importance of unstructured proteins.

2. Materials and methods

2.1. Cloning

Plasmids containing the coding sequence for full-length human cyclin B1 and yeast Pds1 were provided by Helen Piwinica-Worms (Washington University, St. Louis, MO, USA) and David Morgan (UCSF), respectively. The N-terminal 78 residues of cyclin B and the N-terminal 110 residues of Pds1 were subcloned into the pQE30 vector (Qiagen). These N-terminal protein constructs of human cyclin B and yeast Pds1 are designated as ND-cycB and ND-Pds1, respectively. Cloning into the pQE30 vector produces an additional N-terminal sequence of MRGSHHHHHHV. The proteins were overexpressed in *Escherichia coli*. Cultures were grown to a density of ~0.6 OD_{595}, induced with 1 mM IPTG, and further incubated for 3–4 h. Proteins were purified based on the His-tag provided by the vector using Talon Resin (Clontech) following manufacturer's protocols. The ND-cycB was purified to >98% by this method as assessed by Coomassie-stained SDS–PAGE. However, in the case of ND-Pds1, a contaminating protein remained. This protein was removed by cation exchange chromatography using a BioCad/SPRINT system.

2.2. Cell culture, elutriation and lysis

HeLa cells were grown in monolayer in Dulbecco's modified Eagle's media (DMEM; Gibco) supplemented with 10% fetal bovine serum (Gibco), at 37°C with 5% CO_2. Cells were harvested by trypsinization, centrifuged at 4000×*g*, 20 min, 4°C and resuspended in DMEM with 5% fetal bovine serum. G1 cells were isolated by elutriation (Beckman J-6M elutriator) and separation into G1 phase was verified by FACS analysis (data not shown). Elutriated cells were centrifuged at 4000×*g*, 20 min, 4°C. Extracts were prepared as described previously [20]. Briefly, cells were washed twice in ice-cold PBS and once in lysis buffer (50 mM Tris, 5 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT, pH 7.6 with 2 μl/ml protease inhibitor cocktail (Sigma)). Lysis buffer was removed and cells were resuspended in residual buffer, and lysed by tip sonication. Insoluble cellular debris was removed by centrifugation at 14 000×*g*, 20 min, 4°C. Cell extracts were used immediately in activity assays.

2.3. Activity assay

For each 22 μl reaction, 12 μl aliquots of HeLa cell extracts were supplemented with 4 μl ATP regenerating system (1.4 mM ATP, 40 mM phosphocreatine, 80 μg/μl phosphocreatine kinase, final concentration), 2 μl of 0.2 μg/μl recombinant UbcH10, 2 μl of 3.2 μg/μl biotinylated ubiquitin, and 6 μl of 1.5 μg/μl ND-cycB. In all reactions, the cyclin B fragment was added last. Reactions were incubated at 30°C. Time points were taken at 0, 5, 10, 20, 30, 60 and 90 min by removing 2 μl aliquots and freezing them in liquid nitrogen. Time point samples were thawed in the presence of an equal volume of SDS loading buffer, run on 20% SDS–PAGE followed by Western blot analysis using enhanced chemiluminescence reagents (Amersham-Pharmacia). Western blots were probed with anti-cyclin B antibody (Rockland) followed by anti-rabbit secondary antibody (Sigma) to detect cyclin B fragment, or with horseradish peroxidase–streptavidin (Pierce) to detect ubiquitinated products.

2.4. CD spectroscopy

CD spectroscopy was performed on an Aviv 202 spectrometer. Samples were prepared in 10 mM sodium phosphate, pH 7.0. The concentrations of the ND-Pds1 and ND-cycB both were approximately 0.05 mg/ml. Quartz cuvettes of 0.1 cm pathlength were used (Helma). Temperature control was accomplished by a Peltier element. Data were collected at 0.5 nm increments over the interval of 260–200 nm using a data acquisition time of 2 s for each data point. Two scans were taken at each temperature and the values were averaged.

2.5. 1-D NMR analysis

NMR spectra were acquired on a Varian INOVA 600 MHz spectrometer equipped with a triple resonance gradient probe. NMR samples were prepared by concentrating purified ND-cyclin B and ND-Pds1 polypeptides to ~1 mM in 140 mM NaCl, 12 mM NaP_i, 5% (v/v) D_2O and 50 μM NaN_3 at pH 7.2. All experiments were performed at 25°C with the proton carrier centered at the H_2O frequency (4.75 ppm) and the nitrogen carrier centered at 119 ppm.

1-D spectra were obtained using two alternative techniques for water suppression, WATERGATE [21] and presaturation [22,23]. For each experiment, a total of 32 scans was accumulated with a recycle delay of 1.5 s.

2.6. 2-D nuclear Overhauser effect (NOE) analysis

2-D ^1H,^{15}N-steady state NOE spectra [24] were recorded with (NOE) and without (no NOE) presaturation of the amide protons. Data were collected as 256×2048 complex points with spectral widths of 2400 and 6000 Hz for the ^{15}N and ^1H dimensions, respectively, with 40 scans per fid and a recycle time of 1.0 s. The steady state NOE values were calculated from the ratio of the intensities of the peaks with and without presaturation. The uncertainties in the steady state NOE values were calculated as described previously [25]. All NOE data were processed with nmrPipe [26], and visualized and analyzed with NMRDraw [26] and NMRView [27].

3. Results

3.1. Protein expression and activity

The N-terminal regions of human cyclin B1 and yeast Pds1 proteins (designated ND-cycB and ND-Pds1, respectively) were sub-cloned and expressed in *E. coli*. To demonstrate biological activity, we incubated the ND-cycB protein in extracts of HeLa cells synchronized in G1 phase. The mitotic ubiquitination machinery is active from mitosis until late G1 phase [28]. As shown in Fig. 2, the ND-cycB protein is active since it can be ubiquitinated and destroyed. Importantly, in our activity assay, we used protein samples that previously had been used in temperature-dependent CD experiments to ensure that our structural characterization was of bona fide active proteins. The N-terminal region of Pds1 is not recognized by the HeLa cell extract under these assay conditions (data not shown), which is consistent with previous observations [20]. Therefore, we have not demonstrated directly that ND-Pds1 is functional. However, given that (1) the ND-Pds1 construct is very similar to the ND-cycB construct, (2) ND-Pds1 was expressed and treated in much the same way as ND-cycB, (3) similar N-terminal constructs of fission yeast securin are functional [10,29], and (4) considerably shorter fragments containing the destruction box also are active [17], it is highly likely that ND-Pds1 is in its native state.

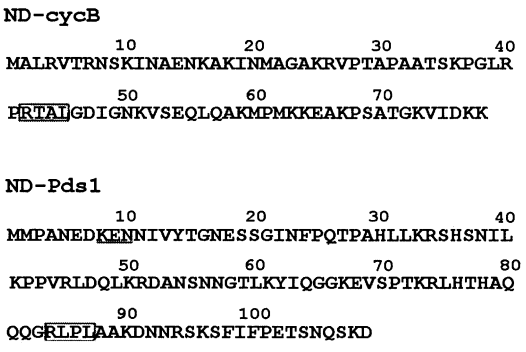

Fig. 1. The N-terminal sequences of human cyclin B and yeast Pds1. The boxed letters designate the destruction boxes. The underlined letters in the yeast Pds1 sequence represent the KEN box.

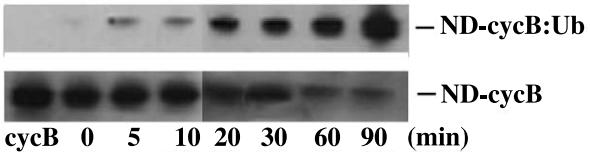

Fig. 2. Activity assay of ND-cycB. ND-cycB polypeptide was incubated for various times as indicated with extracts of HeLa cells synchronized in G1 phase supplemented with an ATP regenerating system and biotinylated ubiquitin as indicated in the text. Immunoblot analysis of the reaction mixtures indicates that the concentration of ND-cycB decreases with time while the concentration of ND-cycB conjugated with ubiquitin increases with time. Thus, the ND-cycB fragment is a competent substrate for the ubiquitin/proteasome system.

3.2. CD analysis

CD is a sensitive method for analyzing secondary structural content in proteins, and is particularly sensitive to α-helices. The CD spectra of ND-cycB and ND-Pds1 (Fig. 3) are devoid of the significant signatures of secondary structure, most notably negative bands at 222 nm and 208 nm for α-helix and a negative band at 217 nm for β-sheet. Instead, the predominant feature is a strong negative band at 200 nm, which is correlated with a predominantly unstructured polypeptide.

A more compelling analysis involves examination of the degree of cooperativity in the change of CD spectra with increasing temperature. Cooperativity seems to be an unfailing hallmark of the folded-to-unfolded transition. As can be seen from measurements at the two characteristic wavelengths of 200 nm and 222 nm (Fig. 3), the variation of the ellipticity values with temperature does not display cooperativity; instead, the values vary linearly. The ellipticity at 222 nm (sensitive to polypeptide helical content) decreases only slightly with increasing temperature. The cause of the decrease in ellipticity is not well understood but can not be due to increased helical content since the temperature is being increased. The ellipticity at 200 nm becomes less negative with increasing temperature, presumably due to the melting out of polyproline-like left-handed helical stretches that are thought to exist in extended conformations [30–32].

3.3. ^{1}H-NMR analysis

Like CD spectroscopy, ^{1}H-NMR spectroscopy provides information regarding protein secondary structure content. It is complementary to CD in that it is in some respects more sensitive to the presence of β-strands than α-helices. The ^{1}H-NMR spectra of the ND-cycB and ND-Pds1 in H_2O were recorded using two different solvent suppression schemes, namely the WATERGATE and presaturation methods. WATERGATE suppresses the H_2O signal by aligning the excess water proton spins with the $+z$ axis during acquisition. Hence, the water protons are suppressed when magnetization in the x–y plane is measured. In the presaturation method, the populations of the water protons with spin-up

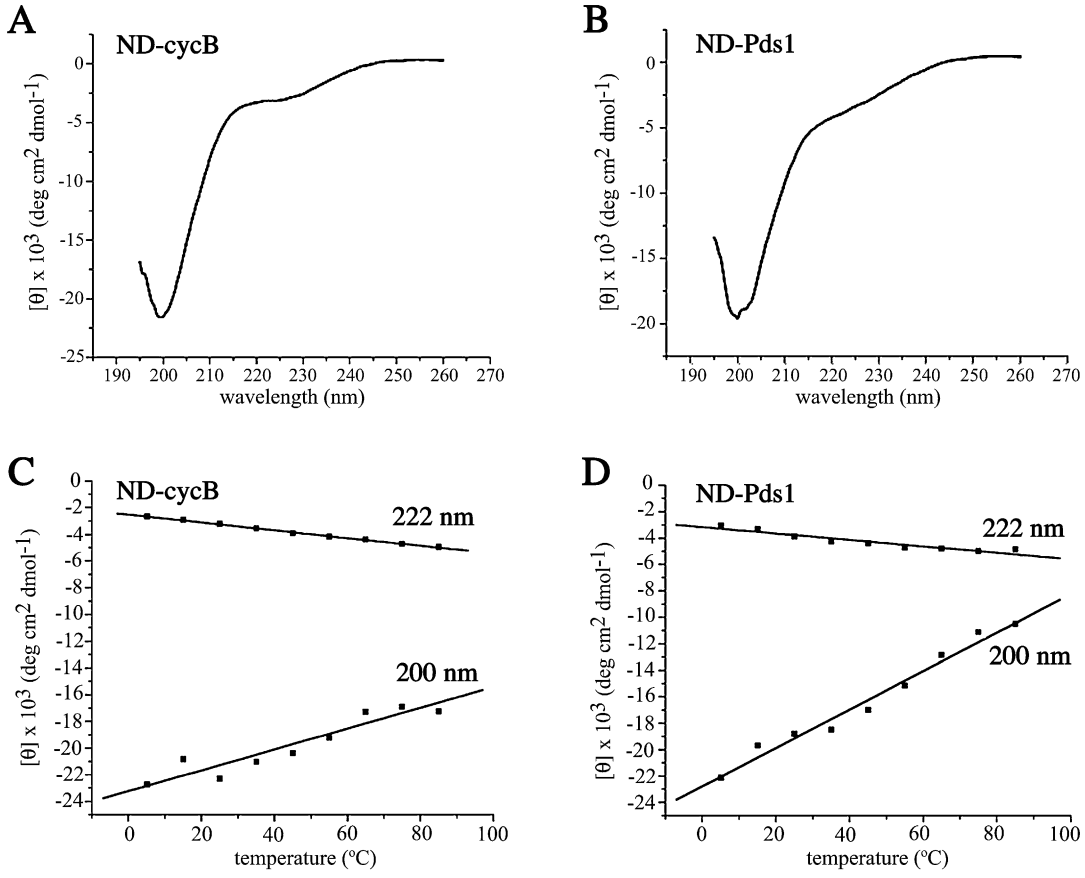

Fig. 3. CD analysis. The N-terminal fragments ND-cycB and ND-Pds1 were characterized by measuring ellipticity while scanning wavelength at various temperatures. For clarity, only CD spectra acquired at 15°C are shown for ND-cycB and ND-Pds1 in panels A and B, respectively. The primary feature of a strong negative band at 200 nm suggests that the fragments lack any significant secondary structure. The lack of cooperativity in the change of the CD signal at 200 nm or 222 nm provides further indication that these fragments are unstructured (C and D).

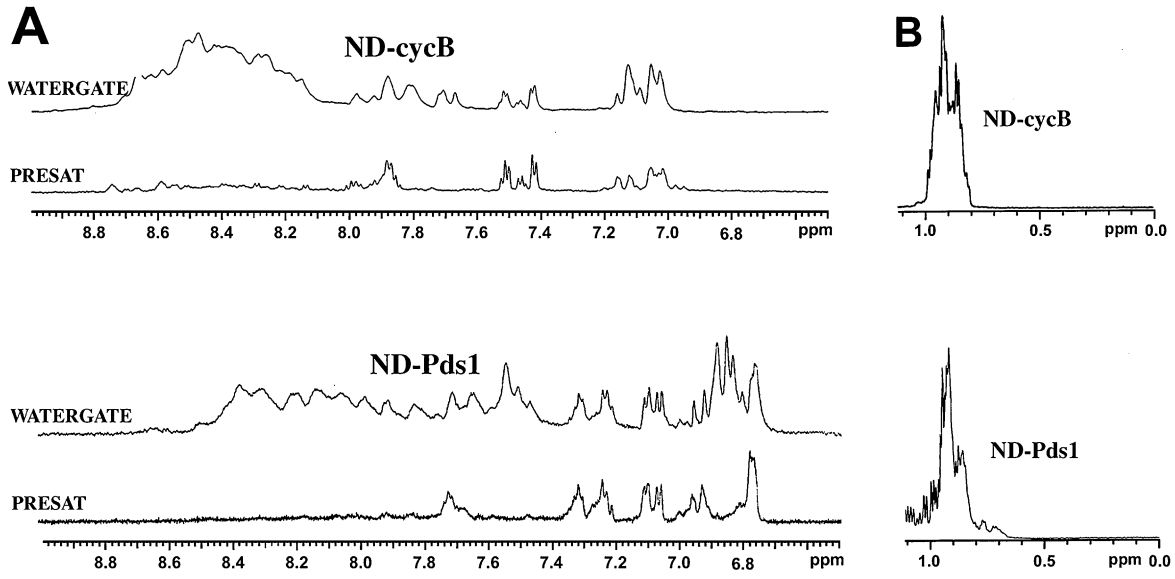

Fig. 4. 1-D NMR analysis. A: Comparison of ^{1}H-NMR spectra obtained by WATERGATE and presaturation (PRESAT) water suppression methods indicates as explained in the text that virtually all amide hydrogen atoms observed in the WATERGATE spectra can exchange with the bulk solvent. Therefore, there is little evidence of structure in the ND-cycB and ND-Pds1 fragments sufficient to prevent chemical exchange either via sequestration from solvent or via intra-molecular hydrogen bonding. B: ^{1}H-NMR spectra corresponding to the methyl proton region display limited dispersion and no up-field peaks indicative of methyl protons in a hydrophobic environment.

and spin-down are equalized by sustained irradiation at the H_2O frequency. Therefore, the net signal from water will be near zero during data acquisition. Importantly, presaturated water protons will exchange with unprotected amide hydrogen atoms, that is, with exchangeable hydrogen atoms that are not buried or participating in a stable hydrogen bond within the polypeptide. This exchange tends to equalize the spin-up and spin-down populations of the protons bonded to the exposed nitrogen atoms and substantially reduces or eliminates their contribution to the spectra. By comparing spectra obtained with the two solvent suppression techniques, the degree of protection from exchange of the polypeptide hydrogen atoms can be ascertained. As shown in Fig. 4A, virtually all of the peak intensity arising from amide protons (mostly 8 ppm and above) present in the WATERGATE spectra is absent or significantly diminished in the presaturation spectra. This indicates that almost all amide protons observed in the WATERGATE spectra are unprotected. The lack of dispersion seen for the amide chemical shifts in the WATERGATE spectra indicates a very similar environment for all amide hydrogen atoms in the proteins and therefore a random coil state for the polypeptide. Further indication of the unfolded state of both N-terminal fragments comes from examination of the methyl proton region of the spectra. As shown in Fig. 4B, the methyl protons display little dispersion. Furthermore, the methyl proton peaks are clustered in the region of ~1.0–0.8 ppm, whereas peaks from methyl protons in a hydrophobic environment typically are shifted upfield to ~0.5–0.0 ppm. Thus, ^{1}H-NMR studies strongly indicate a largely unfolded conformation for both N-terminal fragments.

3.4. $^{1}H,^{15}N$-NOE analysis

The value of ^{1}H,^{15}N steady state NOEs is a sensitive probe of the degree of internal motion of the associated H–N bond vector [33]. The number of observable peaks in the heteronuclear single quantum correlation (HSQC) spectra recorded with and without presaturation of amide protons is less than the number expected based on protein length (Fig. 5). In the case of ND-cycB, 66 of an expected 84 peaks are observed. Most of the missing peaks can be attributed to spectral overlap (data not shown). In the case of ND-Pds1, 64 peaks are missing out of an expected 114 peaks. The paucity of peaks can not be attributed solely to overlap. It is possible that the reduced number of peaks is due in part to conformational exchange on the microsecond time scale [33]. HSQC spectra taken of ND-Pds1 in the presence of 8 M urea resulted in approximately 15 additional distinct peaks (data not shown), suggesting that perhaps some limited residual structure exists in ND-Pds1. Of the observable NOEs, almost all have negative values, indicating fast tumbling and local flexibility. Thus, ^{1}H,^{15}N NOE analysis provides little indication of stable structure.

4. Discussion

The results of the activity assay, CD and NMR studies taken together, provide strong evidence that the N-terminal regions of cyclin B and securin, the same regions recognized by the ubiquitination apparatus, are natively unfolded. The appreciation of the importance of intrinsically unstructured proteins in diverse cellular processes is growing rapidly (reviewed in, for example, [34–38]). The unfolded state has been proposed to confer the ability to bind to multiple partners, bind with high specificity and low affinity, and aid in the assembly of macromolecular complexes. Approximately 15 000 proteins in the Swiss-Prot Database [39] are predicted to be either entirely unfolded or contain significant unfolded regions [40]. A thorough understanding of the nature and function of the unfolded state is of critical importance and has broad implications in structural genomics.

Given the importance of natively unfolded proteins, much recent work has been performed to identify the underlying

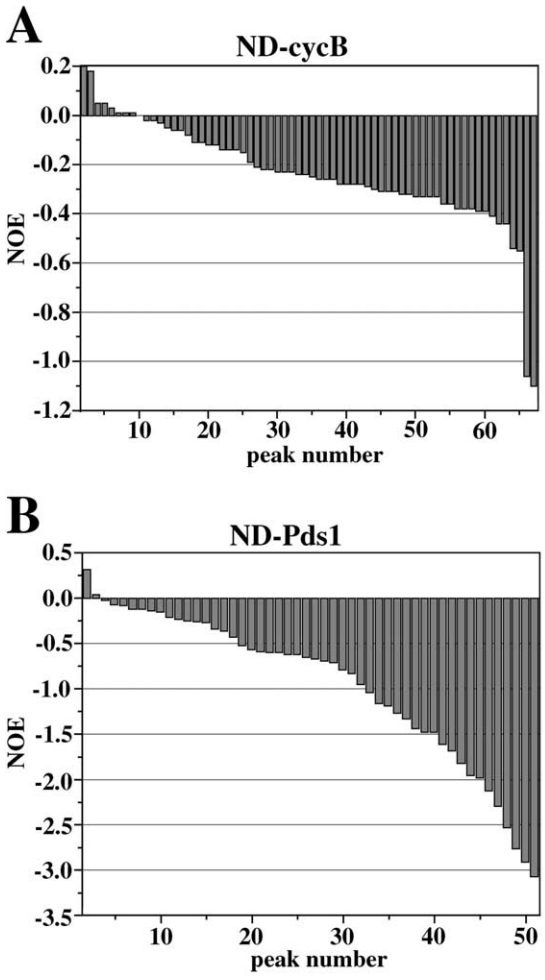

Fig. 5. $^{1}H,^{15}N$ NOE analysis. Peak number is assigned in order of decreasing NOE values. The almost uniformly negative NOE values observed for both ND-cycB and ND-Pds1 (panels A and B, respectively) indicate that the observed regions of cycB and Pds1 are highly flexible and possess little residual structure.

characteristics that give rise to lack of structure. At least four such predictors for discriminating folded vs. natively unfolded proteins have been found. First, in a comparison of known unstructured and small globular proteins, a combination of high net charge normalized by the number of residues and a low average hydrophobicity was found to be a strong predictor of an unstructured state [35]. In particular, in the 'phase space' of normalized net charge and average hydrophobicity, natively unfolded proteins were found to occupy a region defined by a normalized net charge of 0.12 ± 0.09 and average hydrophobicity of 0.39 ± 0.05; whereas folded proteins were found to occupy a region defined by a normalized net charge of 0.04 ± 0.04 and an average hydrophobicity of 0.48 ± 0.03. Using the same method (the ExPASy WWW server [41]) for calculating these parameters for the ND-cycB and ND-Pds1, gives normalized net charges of 0.17 and 0.06, respectively and average hydrophobicity of 0.42 and 0.38, respectively; placing both proteins in the hydrophobicity and net charge 'phase space' corresponding to unfolded proteins. Second, analysis of amino acid composition in natively unfolded proteins indicates a deficit of C, W, Y, I, and V residues and a surfeit of R, S, P, E, K residues [42]. The composition of the N-terminal regions of cyclin B and Pds1 is in overall agreement with this correlation (Fig. 1). Third, in crystal structures, the amino acid residue types with relatively large main chain temperature factors are also the amino acid residue types that tend to be on the surface of a protein. In order of increasing flexibility, the amino acid residue types are W, C, F, I, Y, V, L, H, M, A, T, R, G, Q, S, N, P, D, E, and K [43]. The N-terminal regions of both cyclin B and Pds1 possess a preponderance of residues that tend to be more flexible and hence more solvent exposed. Fourth, Romero and colleagues [42] have found using an information theoretic approach that sequences of self-folded proteins are information rich or complex, with complexity values as defined by Wooton [44] above 2.9. Sequences of intrinsically disordered regions have a broader range of complexity values, extending from approximately 1.0 to 4.1. The information theoretic approach, therefore, is more valuable for defining the lower complexity value limit for a folded protein than for defining an upper limit for an unfolded protein. As a case in point, the complexity values for ND-cycB and ND-Pds1 are 3.6 and 4.0, respectively, which are in a range occupied by both ordered and disordered proteins. Though a number of useful predictors based on sequence have been found for distinguishing folded from natively unfolded proteins, a detailed understanding at the physical chemistry level of why a particular sequence leads to a folded state or an unfolded state is still lacking.

Our finding that the N-terminal fragments of cyclin B and Pds1 are natively unfolded sheds light on intriguing earlier experimental observations. First, an extensive analysis by King and colleagues of the pattern of ubiquitination of the N-terminal fragment of cyclin B indicates that the ubiquitination occurs at several different lysine residues with no preference for a particular lysine residue [17]. A mechanism that allows multiple sites of ubiquitination has been difficult to conceptualize. This conceptualization is simplified if, as demonstrated here, the N-terminal region is disordered. In such a case, any of the various lysine residues could be brought into the active site by random motion of the polypeptide. Second, the mechanism of polyubiquination has remained a central mystery in the ubiquitin pathway. The basic set of proteins E1, E2, APC/C and ubiquitin are the only enzymes required to form polyubiquitin conjugates of mitotic proteins [45]. Again, an unfolded target polypeptide region, simply by its flexibility, could permit the terminal ubiquitin to come into the proper orientation to allow addition of a further ubiquitin molecule. Third, the APC/C structure determined by electron microscopy has been shown to contain a large cavity [45]. The necessity of such a large cavity could be explained if it serves to provide a volume sufficient to allow flexible motion of the substrate sample yet still prevents promiscuous ubiquitination of lysine residues from non-target proteins. Then, this cavity would be akin to the central cavity in the proteasome in which the unfolded polypeptide is cleaved by proteolytic activities present in the cavity wall and the proteolytic activities are safely sequestered from the cytoplasm. Thus, the unstructured state of these regions of critical mitotic proteins suggests a simple model that can be used to explain a variety of enigmatic observations.

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