Stability of the β-Sheet of the WW Domain: A Molecular Dynamics Simulation Study

Gulshat T. Ibragimova and Rebecca C. Wade
European Molecular Biology Laboratory, 69012 Heidelberg, Germany

ABSTRACT The WW domain consists of ~40 residues, has no disulfide bridges, and forms a three-stranded antiparallel β-sheet that is monomeric in solution. It thus provides a model system for studying β-sheet stability in native proteins. We performed molecular dynamics simulations of two WW domains, YAP65 and FBP28, with very different stability characteristics, in order to explore the initial unfolding of the β-sheet. The less stable YAP domain is much more sensitive to simulation conditions than the FBP domain. Under standard simulation conditions in water (with or without charge-balancing counterions) at 300 K, the β-sheet of the YAP WW domain disintegrated at early stages of the simulations. Disintegration commenced with the breakage of a hydrogen bond between the second and third strands of the β-sheet due to an anticorrelated transition of the Tyr-28 ψ and Phe-29 φ angles. Electrostatic interactions play a role in this event, and the YAP WW domain structure is more stable when simulated with a complete explicit model of the surrounding ionic strength. Other factors affecting stability of the β-sheet are side-chain packing, the conformational entropy of the flexible chain termini, and the binding of cognate peptide.

INTRODUCTION

Despite many studies of protein folding and much recent progress in understanding of the energy landscapes for protein folding (Dill and Chan, 1997), the factors governing protein folding and stability are not fully understood. The driving forces for protein folding include hydrophobic interactions, electrostatic, hydrogen bonding and van der Waals interactions between protein atoms, and the rearrangement of water molecules around the protein chain on folding (Creighton, 1993). Folding is opposed by the loss of configurational entropy of the protein chain with the magnitude of this effect being dependent on the flexibility of the native protein fold. The balance of the forces governing folding differs for different protein sequences resulting in different protein structures with differing stability. Determining the relative magnitudes of the factors governing the folding and stability of a given specific native protein sequence is thus not straightforward.

The formation and stabilization of β-folds is less well characterized than that of α-helical structures. One reason is that helix formation can rely solely on interactions local in sequence, whereas β-sheet formation is dependent, by definition, on formation of hydrogen bonds between residues more separated in sequence. It is possible to rationally design helical sequences and improve their stability by, for example, appropriate placement of helix-capping amino acid residues, insertion of helix-forming residues, and optimization of side-chain interactions along the helix (Munoz and Serrano, 1996). On the other hand, the ability to design β-fold structures is less advanced, although a recent breakthrough is the rational design of a three-stranded antiparallel β-sheet peptide (Kortemme et al., 1998).

Even when some of the "rules" are known for altering the stability of a protein fold by introducing single point mutations, understanding the factors that govern the stability of native proteins and their folding and unfolding funnels is complicated due to the presence of many small, often opposing, effects. In this study, our goal is to obtain insights into the stability and unfolding pathways of the β-sheet of the WW domain. The WW domain is composed of ~40 residues with two highly conserved tryptophans and a proline (Fig. 1), and its structure is one of the smallest native folds revealed to date. Due to its small size, lack of disulfide bridges, and the fact that it does not aggregate in dilute solution, it can be considered as a model for how nature achieves and modulates β-sheet stability.

The WW domain binds to proline-rich peptide sequences. The binding of ligands by the WW domain is thought to play a role in several human genetic disorders, such as Liddle's syndrome, muscular dystrophy, and Alzheimer's disease (Sudol, 1996) and in the budding process of human immunodeficiency virus and Rous sarcoma virus (Garnier et al., 1996). The first structure of a WW domain (YAP65), that from YAP (yes kinase-associated protein), was revealed by NMR (Macias et al., 1996) and consists of a twisted three-stranded antiparallel β-sheet (Fig. 2). A difficulty during the structure determination of this particular WW domain was obtaining a construct producing good quality NMR spectra. This problem was overcome by making a construct of 57 amino-acid residues containing 10 additional residues both N- and C-terminal to the domain boundaries (Fig. 1). No secondary structure was found for these N- and C-terminal sequence extensions. Although the N-terminal region is flexible and disordered, the structure determination revealed that it forms a "buckle" covering a hydrophobic part of the surface of the β-sheet. Also neces-

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Address reprint requests to Rebecca C. Wade, European Molecular Biology Laboratory, Meyerhofstrasse 1, 69012 Heidelberg, Germany. Tel.: 49-6221-387553; Fax: 49-6221-387517; E-mail: wade@embl-heidelberg.de.

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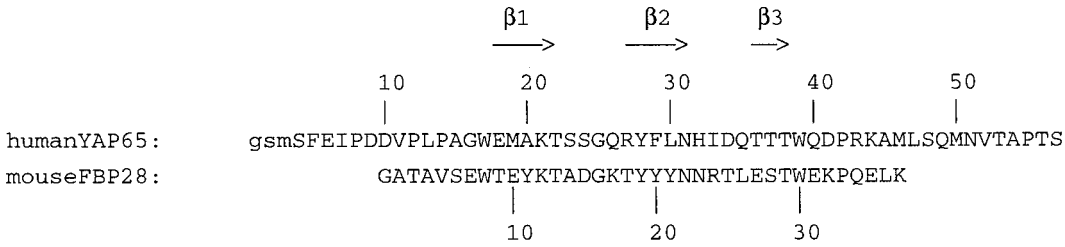

FIGURE 1 Alignment of the sequences of the YAP and FBP WW domains used for the NMR structure determinations. The highly conserved residues in the WW domain are W17, W39, and P42 in the YAP WW domain.

sary for obtaining good quality spectra was the addition of a proline-rich peptide with a PP*X*Y sequence motif which is bound by this type of WW domain.

Recently, the structure of a WW domain (FBP28) from the formin-binding protein (FBP) has been revealed by NMR (M. Macias and H. Oschkinat, unpublished data). It is known to bind proline-rich peptides with a PPLP sequence motif (Chan et al., 1996). This structure could be solved without the addition of extra N-terminal residues or the cognate peptide and at a higher temperature than the YAP WW domain (303 K rather than 285 K). Moreover, mutation studies show that its structure is very robust to the introduction of mutations (M. Macias, unpublished data). Despite its much greater stability, the structure of the FBP WW domain consists of the same twisted antiparallel β-fold revealed for the YAP WW domain (Fig. 2).

To investigate the reasons for the differences in stability of these two WW domains, we have applied molecular dynamics (MD) simulation techniques. In the present article, we describe the results of MD simulations of the YAP and FBP WW domains under different conditions. Analysis of the trajectories provides insights, at the atomic level, into how the YAP WW domain unfolds and the factors stabilizing the β-sheet in the WW domain. The results are compared to experimental observations.

MATERIALS AND METHODS

The initial coordinates for MD simulation of the YAP WW domain were taken from the lowest energy NMR structure of the WW domain in complex with a proline-rich peptide consisting of 10 residues (Macias et al., 1996). Because of the high flexibility of the N- and C-termini, only residues 1–50 of the sequence shown in Fig. 1 were defined in the NMR structure and, for residues 1–4, only the backbone atoms were assigned coordinates. Consequently, molecular dynamics simulations were performed for residues 1–50, with the first four residues modelled as glycine residues. As the experimental data indicated very little conformational change of the WW domain upon binding the peptide, the peptide was simply removed to set up MD runs without peptide. To investigate the role of the N-terminus for stability of the YAP WW domain, the first 10 amino-acid residues were removed for some of the simulations. The initial coordinates for MD simulation of the FBP WW domain were taken from the lowest energy NMR structure of the FBP WW domain (37 residues) (M. Macias and H. Oschkinat, unpublished data).

The charge distribution on the surface of the WW domains was modeled at the pHs at which the NMR structures were obtained (pH 6 for the YAP WW domain and pH 6.8 for the FBP WW domain). Arg and Lys residues were protonated and Asp and Glu residues were deprotonated. The single histidine present in the YAP WW domain, His-32, was singly protonated at the $N^{\delta 1}$ position, as this was the most favorable site according to the hydrogen placement algorithm (Hooft et al., 1996) used. This algorithm is based on optimization of the hydrogen bond network and is implemented in the WHATIF package (Vriend and Sander, 1993).

Initial setup, dynamics runs, and analysis were performed as described previously (Ibragimova and Wade, 1998). The AMBER 4.1 program (Pearlman et al., 1995) was used with the all-atom force field (Cornell et al., 1995) and the particle mesh Ewald (PME) method (Darden et al., 1993). After equilibrating the water, ions, and hydrogen atoms in the presence of the fixed non-hydrogen protein atoms, all of the systems simulated were subjected to a gradual heating from 0 to 300 K over 15 ps before continuing simulations at 300 K for data collection.

Three different procedures were used to model salt conditions. For "counterions only" MD simulations, the system generated consisted of the WW domain with charge-balancing counterions at the protein surface surrounded by water molecules. For "counterions + salt" MD simulations,

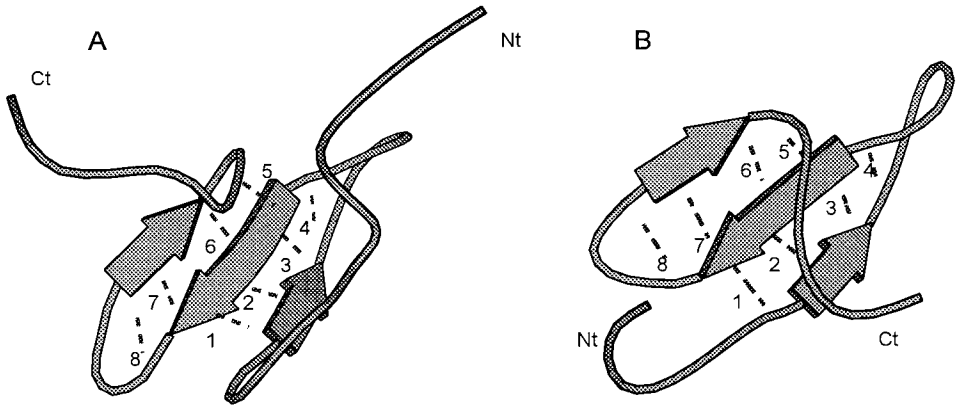

FIGURE 2 Overall fold of the YAP (*A*) and FBP (*B*) WW domains. Hydrogen bonds are shown by *dashed lines* and numbered according to Table 2. This figure was prepared using the MOLSCRIPT program (Kraulis, 1991).

the system consisted of the WW domain with charge-balancing counterions surrounded by 0.2 M ionic solution with water molecules and explicit Na^+ and Cl^- ions. This is thus a complete model of a moderate salt concentration comparable to the physiological ionic strength of 0.15 M and the ionic strength of 0.1 M used for structure determination. In addition, one simulation of the YAP WW domain with a peptide was carried out without any counterions. This simulation was performed using truncation for both van der Waals and electrostatic interactions beyond a cutoff distance of 9 Å rather than the PME method. The simulated systems consisted of 12,087–16,759 atoms, including 3,827–5,315 water molecules, in periodic boxes with sides of ~50–65 Å length.

The TRAJAN program (Worth et al., 1996) was used to identify motions leading to destruction of the structure of the WW domain. The RMSD cutoff for distinguishing conformational wells along the MD trajectory with the TRAJAN program was set to 1.8 Å.

RESULTS

The MD simulations performed are summarized and the main characteristics of the WW domains during the MD simulations are shown in Table 1. Simulations were performed with three models of the ionic strength: no explicit ions in the periodic box; "counterions only," in which only charge-balancing counterions (Na^+ and Cl^-) were simulated; and "counterions + salt," in which explicit ions were present throughout the periodic box at a concentration corresponding to 0.2 M ionic strength. The RMSD values of the backbone atoms of the β-sheet over the last 50 ps of the simulations with counterions only without peptide were more than twice as large for the YAP WW domain (simulation *A*) as for the FBP domain (simulation *H*) (1.77 vs. 0.78 Å). Increasing the salt concentration (simulation *B*) reduced the RMSD values of these atoms of the YAP WW domain to ~1 Å. Complexing the YAP WW domain with its cognate peptide (simulation *C*) and/or removing the 10 N-terminal residues (simulations *D*, *E*, *F*, *G*) (even with the "counterions only" model) also lowered RMSD values to ~1 Å.

The stability of the WW domain was monitored by the presence of secondary structure during the last 50 ps of each simulation. As shown in Table 1, no secondary structure was observed for the YAP WW domain at the end of the simulations both without counterions (*I*) and with counterions only (*A*) although the tertiary structure was not completely destroyed. In contrast, the FBP WW domain maintained its fold when simulated with counterions only (simulation *H*). Full modelling of the ionic strength by adding further explicit ions prevented unfolding of the YAP WW domain (simulations *B* and *C*). Removing the 10 N-terminal residues and complexing the domain with its cognate peptide also kept the secondary structure stable with the counterions + salt model (simulations *E* and *G*). With counterions only, removing the 10 N-terminal residues with or without complexing the peptide only partially stabilized the secondary structure (simulations *D* and *F*). The third β-strand was disrupted by the breakage of one hydrogen bond.

DISCUSSION

Disintegration of the β-sheet of the YAP WW domain

To explain the effects of different simulation conditions, the disintegration of the β-sheet of the YAP WW domain was considered in detail.

The sequences of the YAP and FBP WW domains are 24% identical (Fig. 1) and the domains have the same fold (Fig. 2). This consists of three antiparallel β-strands that are kept close together by eight hydrogen bonds. Throughout almost all of the MD simulation of the YAP WW domain with the "counterions only" model and without peptide (simulation *A*), two pairs of hydrogen bonds (numbered 3, 4, 5, and 6 in Table 2) were disrupted. When no counterions were present (simulation *I*), these hydrogen bonds were also mostly disrupted, although hydrogen bond 4 was a bit more stable (being present for 30 of the 40 ps of simulation compared to 16 of the 260 ps of simulation *A* with counter-

TABLE 1 Characteristics of MD simulations

MD simulation identifier	Origin of WW domain	Residues present	Presence of peptide	MD simulation time (ps)*	Salt conditions	Number of charge-balancing counterions		Total number of counterions		RMSD over the last 50 ps of MS (Å)#	Occurrence of β-strands over the last 50 ps of MD (ps)		
						Na^+	Cl^-	Na^+	Cl^-		Strand 1	Stand 2	Strand 3
A	YAP	1–50	No	260	Counterions only	6	4	6	4	1.77 ± 0.06	0	0	0
B	YAP	1–50	No	1000	Counterions + salt	6	4	28	26	1.15 ± 0.15	50	50	50
C	YAP	1–50	Yes	600	Counterions + salt	6	4	19	17	0.99 ± 0.06	50	50	50
D	YAP	11–50	No	260	Counterions only	3	4	3	4	1.13 ± 0.09	50	50	5
E	YAP	11–50	No	320	Counterions + salt	3	4	21	22	1.17 ± 0.07	50	50	50
F	YAP	11–50	Yes	260	Counterions only	3	4	3	4	0.91 ± 0.05	50	50	34
G	YAP	11–50	Yes	340	Counterions + salt	3	4	21	22	0.98 ± 0.07	50	50	50
H	FBP	1–37	No	320	Counterions only	5	4	5	4	0.78 ± 0.13	50	50	50
I	YAP	1–50	Yes	40	No counterions	0	0	0	0	1.49 ± 0.05§	0¶	1¶	0¶

*The 15 ps of heating for every MD run are not included.
#RMSD values were calculated for backbone atoms of the β-sheet.
§Over the last 30 ps of MD simulation time.
¶Only the data for the last structure are shown.

TABLE 2 Stability of hydrogen bonds in YAP and FBP WW domains during MD simulations*

No.	Hydrogen bonds in WW domain of		MD simulation identifiers#						
	YAP	FBP	*A*	*B*	*C*	*D*	*F*	*H*	*I*
1	E 18 N–H. . .O 30 L	T 9 N–H. . .O 21 Y	65	99	98	99	99	89	65
2	L 30 N–H. . .O 18 E	Y 21 N–H. . .O 9 T	100	100	100	100	100	98	99
3	A 20 N–H. . .O 28 Y	Y 11 N–H. . .O 19 Y	3	100	100	100	100	100	14
4	Y 28 N–H. . .O 20 A	Y 19 N–H. . .O 11 Y	6	100	99	99	100	78	75
5	F 29 N–H. . .O 38 T	Y 20 N–H. . .O 29 T	5	99	100	100	100	100	16
6	T 38 N–H. . .O 29 F	T 29 N–H. . .O 20 Y	18	99	100	100	99	82	9
7	N 31 N–H. . .O 36 T	N 22 N–H. . .O 27 E	68	94	100	65	98	64	92
8	T 36 N–H. . .O 31 N	E 27 N–H. . .O 22 N	92	54	46	23	64	98	96

*Numbers are % occupancies of hydrogen bonds during the whole simulation (after equilibration).
#MD simulation identifiers are defined in Table 1.

ions). When the N-terminus of the YAP WW domain was removed, a different hydrogen bond pair (7 and 8) was perturbed during the simulations with (*F*) and without peptide (*D*), but to a lesser extent. Hydrogen bond 8 was also the least stable in the simulations of the YAP WW domain with the counterions + salt model (*B* and *C*) and hydrogen bond 7 was the least stable in the simulation of the FBP domain (*H*). Thus it seems that perturbations to hydrogen bonds 7 and 8 are a general feature and, while allowing the β-sheet to twist, do not necessarily result in loss of the β-sheet structure. On the other hand, disruption of hydrogen bonds 3–6 has a drastic effect on the stability of the secondary structure of the YAP WW domain.

To investigate how hydrogen bonds 3–6 are disrupted in the simulation of the YAP WW domain with counterions only (simulation *A*), the TRAJAN program (Worth et al., 1996) was used to identify the most important atomic motions that accompany the disintegration of the β-sheet of the YAP WW domain. Surprisingly, the first motion leading to destruction of the β-sheet was the reorientation of the C═O bond of Tyr-28 and the N—H bond of Phe-29. These bonds on the second β-strand reoriented relative to the first and third strands (Fig. 3) and this resulted in the loss of hydrogen bonds 3 (Ala-20—Tyr-28) and 5 (Phe-29—Thr 38). This happened after only 12 ps of simulation time subsequent to the heating phase. Thereafter, stable hydrogen bonds were formed by the backbone O atom of Tyr-28 to the amide H atom of Thr-38, and the backbone amide H atom of Phe-29 to the backbone O atom of Arg-27. The original conformation was never restored. This reorientational event is characterized by anticorrelated transitions of the ψ angle of Tyr-28 and the ϕ angle of Phe-29 after 12 ps of simulation time (Fig. 4). It is worth mentioning that the same reorientation still occurred when heating was performed more gradually (over 50 ps instead of 15 ps, data not shown). In the simulation without counterions (*I*), the same bonds reoriented after 6 ps of simulation time after heating.

A further indication that the backbone interactions of Tyr-28 and Phe-29 are a point of structural vulnerability, sensitive to perturbation of the surroundings and important for the secondary structure of WW domains, comes from NMR data for the Yjq8 WW domain from yeast (M. Macias, V. Gervais, C. Civera, and H. Oschkinat, unpublished data). Compared to the FBP and YAP WW domains, the Yjq8 WW domain lacks three nuclear Overhauser effects between residues corresponding to Met-19, Ala-20, Phe-29, and Leu-30 in the YAP WW domain. This may be a reason why the structure of the Yjq8 WW domain is less stable than that of the FBP domain.

Role of side-chain packing for stability of the β-sheet fold

Destruction of hydrogen bonding at Tyr-28 of the β-sheet of the YAP WW domain allowed increased conformational

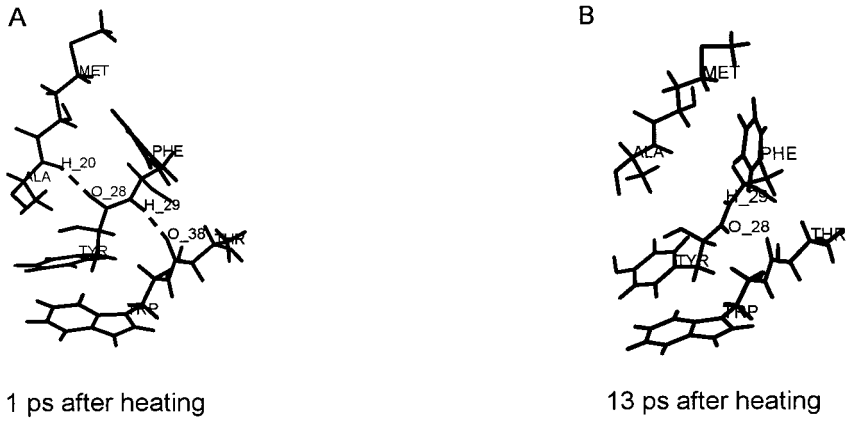

FIGURE 3 Change of orientation of the Phe-29 N—H and Tyr-28 C═O bonds relative to the first and third β-strands of the YAP WW domain after 12 ps of MD simulation with the "counterions only" model (simulation *A*).

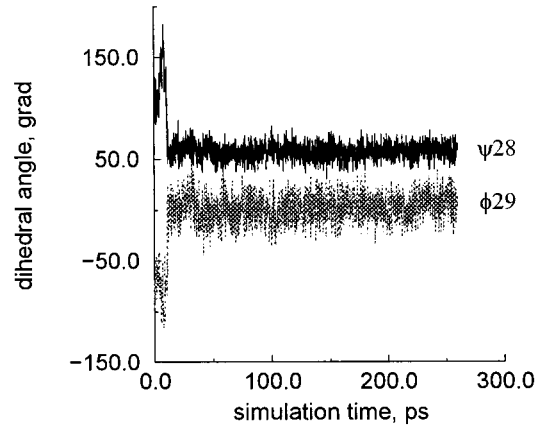

FIGURE 4 Evolution of the dihedral angles ψ of Tyr-28 and ϕ of Phe-29 of the YAP WW domain during MD simulation with the "counterions only" model (simulation *A*).

mobility of its aromatic ring compared to that of the corresponding Tyr-19 in the FBP WW domain (Fig. 5). In the FBP WW domain, there are two other aromatic residues close by, Tyr-11 and Tyr-21, instead of Ala-20 and Leu-30 of the YAP WW domain (Fig. 6). These additional aromatic rings may stabilize the initial conformation of the FBP WW domain. Such stabilization has, for example, been observed for pairs of aromatic residues in adjacent strands of the β-sheet of the B1 domain of streptococcal protein G (Smith and Regan, 1995). Analysis of a nonhomologous set of protein structures by Hutchinson et al. (1998) showed that interstrand interactions between two aromatic residues in anti-parallel β-sheets are favored when they are positioned so that two backbone hydrogen bonds are formed between these residues. This favorable arrangement is satisfied by Tyr-11 and Tyr-19 of the FBP WW domain (corresponding to Ala-20 and Tyr-28 in the YAP WW domain), which have two backbone hydrogen bonds between each other and their aromatic rings oriented in an approximately edge-to-face fashion.

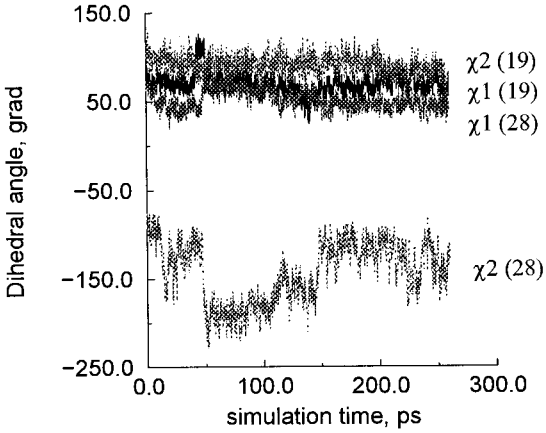

FIGURE 5 Evolution of the side-chain dihedral angles of Tyr-28 of the YAP WW domain and of Tyr-19 of the FBP WW domain during MD simulations with the "counterions only" model (simulations *A* and *H*).

Role of long-range electrostatic interactions for stability of the β-sheet fold

The $C_i{=}O_i$ and $N_{i+1}{-}H_{i+1}$ bonds forming the hydrogen bonding pattern of the β-sheet fold may be considered as dipoles. To stabilize their initial orientation, they should be surrounded by a charge distribution resulting in opposite polarity. For the YAP WW domain, the nonbonded interaction energies between the Phe-29 amide H and the Tyr-28 carbonyl O atoms and components of the simulated system were calculated (Fig. 7).

In the MD simulation of the YAP WW domain without any counterions (simulation *I*) in the periodic box, the destruction of the β-sheet through the reorientation of the Tyr-28 C═O and Phe-29 N—H bonds occurred after only 6 ps of simulation time. After a corresponding anticorrelated transition, the Tyr-28 ψ and Phe-29 ϕ dihedral angles had similar values to those for the MD simulation with the "counterions only" model (simulation *A*) (differing by less than 20°, data not shown). As shown in Fig. 7 *A*, the interaction energy with charged residues is approximately constant for the initial 6 ps of simulation, then decreases over the period of the simulation from 6–20 ps before starting to level out again. Thus, as the system equilibrates (the total energy of the protein drops during the first 10 ps of the simulation and then levels out), the interactions of the Phe-29 amide H and the Tyr-28 carbonyl O atoms become more favorable and, in particular, interactions with charged residues are improved by reorientation of the Tyr-28 C═O and Phe-29 N—H bonds, although this results in destruction of the secondary structure of the YAP WW domain.

In the simulation with counterions only (*A*), the interaction energy with charged residues becomes less favorable and the interaction energy with counterions becomes more favorable around the time that the Tyr-28 C═O and Phe-29 N—H bonds reorient (after 12 ps of simulation; Fig. 7 *B*). After reorientation, the interaction energies level out indicating that this part of the structure only then becomes equilibrated (although the total energy of the protein and counterions was approximately constant from the beginning of the simulation). While the reorientation results in less favorable interactions with all charged residues, the interactions with some nearby residues (e.g., Glu-18) improves greatly and counterion interactions also improve by ~6 kcal/mol.

For comparison, the corresponding nonbonded interactions of another pair of atoms (the carbonyl O of Leu-30 and the amide H of Asn-31) were calculated (Fig. 7 *C*). The carbonyl and amide bonds to these atoms did not change their initial orientation during the MD simulation with the "counterions only" model (*A*) and thus hydrogen bonds 1 and 7 were maintained throughout the simulation. The energy values calculated do not show any significant changes, indicating that this part of the system equilibrated before that around the Tyr-28 C═O and Phe-29 N—H bonds. On the other hand, the total interaction energy for charged residues is less favorable than for the H Phe-29 and O

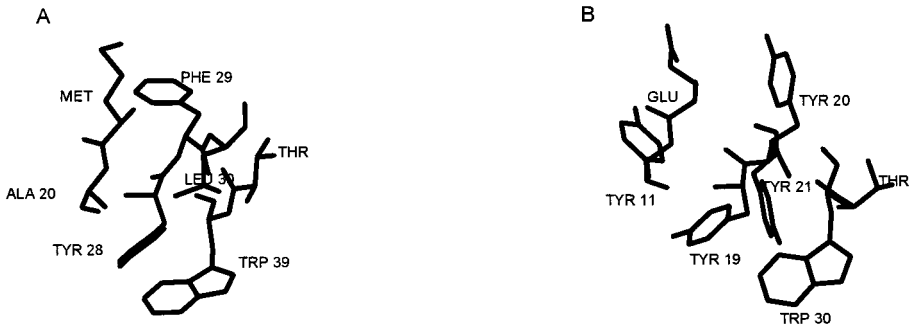

FIGURE 6 Local environment of Tyr-28 in the NMR structure of the YAP WW domain (*A*) and Tyr-19 in the NMR structure of the FBP WW domain (*B*) (M. Macias and H. Oschkinat, unpublished data). Only non-hydrogen atoms are shown.

Tyr-28 pair and that for counterions is more favorable. As the total interaction energy with the rest of the system is more favorable by ~5 kcal/mol, local packing interactions, especially with Trp-17, make a greater stabilizing contribution and may be important for maintaining the initial orientations of the Leu-30 carbonyl and Asn-31 amide bonds.

Stabilizing factors in the MD simulations

As mentioned above, the key event in destruction of the YAP WW domain is the reorientation of the Tyr-28 C═O and Phe-29 N—H bonds relative to the first and third strands of the β-sheet. This reorientation was not observed when 1) the 10 N-terminal residues were removed; 2) the "counterions + salt" model of ionic strength was used; or 3) the cognate peptide was added.

Role of the N-terminal residues

In MD simulation, in apparent contrast to the experimental observation (Macias et al., 1996), removing the 10 N-terminal residues stabilized the YAP WW domain. In fact, these residues are not part of the WW domain repeat. They were added to obtain a folded domain suitable for structure determination by NMR and were suggested to cover a hydrophobic patch on the domain surface with, in particular, Ile-7 interacting with Phe-29. They could affect the WW domain by increasing the stability of WW monomers or reducing dimerization and aggregation of WW monomers. In our simulations, the fold of the domain was still maintained after removing the N-terminal residues. In simulations with the N-terminal residues present, the initial structure in this region was not maintained. Ile-7 moved away from Phe-29 and other residues in the N-terminus came close to the domain surface, covering the hydrophobic regions.

The "cover hydrophobic-patch" interactions revealed in the NMR studies do not seem to be very important for stability of the YAP WW domain in MD simulations.The N-terminus may, however, also stabilize the WW domain fold through its entropic contribution. No secondary structure was detected experimentally in the N-terminal part of the YAP WW domain. This relative disorder allows a larger conformational space to be explored, in particular on the domain surface. Standard MD simulations of proteins do not permit full consideration of entropy because of the limited time scale accessible. As a result, the behavior of a small protein during a simulation might be strongly affected by the initial instantaneous structure of a segment of residues whose fold is unresolved. In the case of the YAP WW domain, there is a high concentration of negatively charged residues at the N-terminus (Glu-6, Asp-9, Asp-10). They keep one positively charged counterion very close to its initial position at the beginning of the MD simulation under low salt conditions. When the 10 N-terminal residues were removed, this counterion was able to move freely in the simulations under low salt conditions and approached the Tyr-28 carbonyl and Phe-29 amide bonds. In its new position, energy evaluation shows a stabilizing contribution of ~4 kcal/mol for this counterion with the carbonyl O of Tyr-28 and the amide H of Phe-29 (simulation *D*). Thus, it might prevent the reorientation of this bond pair and maintain the initial β-fold at the beginning of the MD simulation.

Role of salt concentration

The effect of performing simulations with the full "counterions + salt" model was discussed before (Ibragimova and Wade, 1998). We proposed that the additional salt ions (besides the charge-balancing counterions) may act to navigate the protein on the potential energy surface along those pathways that keep the secondary structure stable. Similar findings have been reported very recently for simulations of the PH domain by Pfeiffer et al. (1999). In the present work, we see that these additional ions screen subtle and sometimes random interactions caused by charged residues that lead to destruction of the WW domain fold at early stages of the MD simulations. It should be noted that specific long-residence ion-protein interactions are not observed during the simulations. The ions are constantly interchanging and moving to new positions, so while they may make transient strong interactions with the protein, on average the ions can be considered to act as a diffuse charge screening cloud.

Role of the cognate peptide

The role of peptide binding for stability of the WW domain may be assigned to packing interactions and the formation

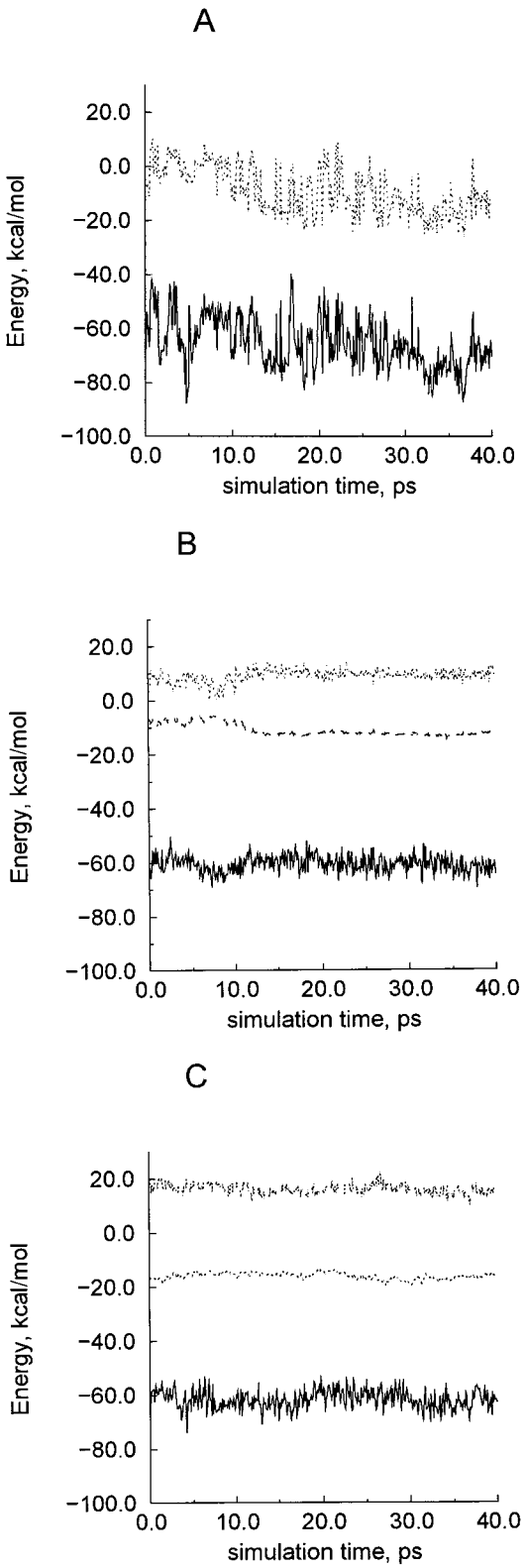

FIGURE 7 Sum of the nonbonded interaction energies with other atoms of the Phe-29 amide H and Tyr-28 carbonyl O atoms of the YAP WW domain during MD simulation without counterions (*A*) (simulation *I*) and with the "counterions only" model (*B*) (simulation *A*). (*C*) Sum of the nonbonded interaction energies with other atoms of the Asn-31 amide H and Leu-30 carbonyl O atoms of the YAP WW domain during MD simulation with the "counterions only" model (simulation *A*). ——, with all other protein atoms and counterions; - - - -, with counterions only; ·····, with charged residues only. Larger fluctuations of energy values in *A* compared to *B* and *C* are due to the computation of the electrostatic interactions with a cutoff.

of hydrogen bonds between the domain and the peptide. In the simulations of the WW domain without the N-terminal 10 residues and with counterions only (*F* and *G*), there are three hydrogen bonds and their occupancies are as given in parentheses: Tyr-28 OH···O Pro-6′ (~100%), His-32 ND1···OH Tyr-7′ (~50–60%), and Thr-37 OH···O Pro-5′ (~30%). The hydrogen bonding between the domain and the peptide helps to keep the second and third strands of the β-sheet close together, hindering destruction of the β-sheet. However, as the MD simulation of the YAP WW domain with the peptide without any counterions (*I*) shows, the effect of the peptide on secondary structure stability in the simulations is smaller than that of the salt model.

CONCLUSIONS

The YAP WW domain provides a good system for examining the initial events in protein unfolding and a stringent test of MD simulation protocols. Simulations of the much more stable FBP WW domain provide a benchmark against which to evaluate the results of the YAP WW domain simulations.

After the work was completed, Koepf et al. (1999) published a study of the unfolding of the YAP WW domain. Addition of guanidinium hydrochloride and urea denaturants caused the WW domain to unfold to a denatured state that still appeared to retain some structure in the form of a hydrophobic cluster. In contrast, thermal denaturation appeared to be more complete. Although the T_m is 49°C, the shapes of the thermal unfolding curves obtained by circular dichroism spectroscopy indicate that factors such as dimerization may affect the structure and that unfolding of the YAP WW domain monomer may start at significantly lower temperatures. Our simulation data relate more closely to thermal than chaotropic denaturation and provide atomic-level insights into what the first events in unfolding may be.

Unfolding of the β-sheet of the YAP WW domain is found to commence with the breakage of two hydrogen bonds between the strands of the β-sheet due to an anticorrelated transition of the Tyr-28 ψ and Phe-29 ϕ angles. This leads to further disruption of the cooperative hydrogen bond network that forms the β-sheet. Electrostatic interactions influence this reorientation event in the second strand which does not take place when the full "counterions + salt" model of the ionic strength (0.2 M) is used in the simulations. Such a complete model is not often used in MD simulations of proteins which are not highly charged (the net charge of the simulated YAP WW is −2e and of the FBP WW is −1e), in which it is commonly considered sufficient either to neglect ions altogether or to model only charge-balancing counterions. Comparison of the YAP WW domain with the FBP WW domain indicates that aromatic

side-chain packing may also influence the susceptibility of the hydrogen bonds made by the carbonyl O of Tyr-28 and the amide H of Phe-29 to disruption. Indeed, based on these simulation data, we proposed that the double mutant A20Y,L30Y would be more stable, and after this work was completed, this mutant was found by circular dichroism spectroscopy (M. Macias, unpublished data) to have a T_m 12°C higher than the wild-type protein.

As observed experimentally, peptide binding stabilized the protein fold in the simulations. The hydrogen bonds it forms to the protein tend to extend the hydrogen bond network of the β-sheet and thus stabilize it. On the other hand, the role of the N-terminal flexible region of the YAP WW domain for its stability was difficult to identify in the MD simulations and the 10 N-terminal disordered residues tended to destabilize the simulated YAP WW domain. This indicates that the mechanism by which this region stabilizes the protein may be largely entropic. This property could not be investigated thoroughly in the standard simulations performed due to insufficient sampling of the conformations of the disordered regions of the protein. On the other hand, this region may act by affecting formation of dimers and further oligomerization.

Gradual relaxation of the protein structure and equilibration of the surrounding ionic solution can be expected to occur on the nanosecond time scale (Pfeiffer et al., 1999). Nevertheless, the initial unfolding events observed in this study occurred very early in the simulations and thus the stability of the WW domain could be studied with relatively short simulations ranging in length from 40 ps to 1 ns. From the *in silico* perspective, stabilization of the YAP WW domain could be achieved by removal of the highly disordered peptide chain ends and using a full model of the surrounding ionic solution. These factors may also be important for simulation of other proteins. Our simulations also point to factors that are likely to affect WW domain stability in vitro or in vivo. Although we have not done a quantitative analysis of the ionic strength dependence of stability over a range of ionic strengths, our results indicate the importance of electrostatic intraprotein and protein-solvent interactions, as well as side-chain packing and the entropic contributions of flexible regions, for the stability of the β-sheet.

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