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An Exceptionally Stable Helix from the Ribosomal Protein L9: Implications for Protein Folding and Stability

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The ribosomal protein L9 has an unusual structure comprising two compact globular domains connected by a 34 residue α-helix. The middle 17 residues of the helix are exposed to solvent while the first seven pack against and form part of the N-terminal domain, and the last ten form part of the C-terminal domain. Here we report results which show that a peptide corresponding to the central helix of L9 is monomeric in aqueous solution and >85% helical at 1°C and 68(±7)% helical at 25°C. This is considerably more helical than any other protein fragment studied to date. Another peptide corresponding to the middle 17 residues of the helix is monomeric and is 41(±4)% helical at 1°C. Because the central helix has high intrinsic stability the globular N and C-terminal domains will likely be stabilized by their interactions with the helix. Therefore, the stability of the two terminal domains should not be completely independent because both domains gain stability from a shared structural element, the central helix. Also, the ability of the central helix to form native-like structure in isolation highlights a potential role for the helix in the early stages of the folding process.

Keywords: L9; protein folding; α-helix; helix-coil transition; protein fragments

It has been proposed that interactions between residues close in primary sequence play a critical role in the early stages of protein folding. These interactions stabilize local structures which can interact with each other and initiate the formation of larger and more complicated structures (Anfinsen & Scheraga, 1975; Kim & Baldwin, 1990; Munoz & Serrano, 1996). One approach used to discover possible initiation sites of protein folding is to determine the conformational preferences of protein fragments (Dyson & Wright, 1993). Like an unfolded protein, small protein fragments do not have tertiary structure, and they offer the advantage that they can be studied in conditions where the full-length protein is folded. Any structures stable in a small protein fragment could be stable in the early stages of protein folding.

The first protein fragment discovered to have significant helical structure in aqueous solution was the S-peptide from ribonuclease A, which was shown to be partially helical and monomeric at 0°C (Brown & Klee, 1971). Since then many protein fragments have been analyzed, primarily with circular dichroism (CD) and nuclear magnetic resonance (NMR) (see for example Munoz & Serrano, 1994). In several cases the entire sequence of a protein has been split into short pieces (Segawa *et al.*, 1991; Dyson *et al.*, 1992a,b; Jimenez *et al.*, 1993; Kemmink & Creighton, 1993; Blanco & Serrano, 1995; Itzhaki *et al.*, 1995; Viguera *et al.*, 1995; Yang *et al.*, 1995). Some of the most notable results involve fragments which are partially helical. A 27 residue peptide corresponding to the sequence of

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Abbreviations used: 2D NMR, two-dimensional nuclear magnetic resonance; CD, circular dichroism; Fmoc, [[(9-fluorenylmethyl)oxy]carbonyl]; ROESY, rotating frame nucluear Overhauser experiment; TBTU, 2-(1H-benzotriazol-1-yl)-1,1,3,3,-tetramethyluronium tetrafluoroborate; TOCSY, total correlated spectroscopy.

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the H-helix in myoglobin is 30% helical at 0°C (Waltho *et al*., 1993). Most protein fragments do not, however, adopt considerable native-like structure in aqueous solution. Often the helix inducing solvent, trifluoroethanol (TFE), is required to observe significant helical structure. We report here that the central helix of the ribosomal protein L9 is over 85% helical in aqueous solution at 1°C.

L9 from the bacterium *Bacillus stearothermophilus* contains two small globular domains separated by a 34 residue α-helix, residues 41 to 74 (Hoffman *et al*., 1994, 1996). A ribbon diagram of L9 is shown in Figure 1. The central 17 residues of the helix are largely solvent exposed, while the first seven form part of the N-terminal domain and the last ten form part of the C-terminal domain. The N-terminal domain consists of a three-stranded anti-parallel β-sheet sandwiched between a small helix and the N-terminal end of the central helix. Two leucine residues from the central helix, Leu44 and Leu47, pack against the β-sheet and form part of the hydrophobic core of the N-terminal domain. Also, there is a hydrogen bond between the hydroxyl of Thr40 and the amide of Asn43. Asn43 is in the first turn of the central helix and therefore would normally not have a hydrogen bond partner. The C-terminal domain contains two long loops, an α-helix, and a three-stranded β-sheet which packs against the C-terminal end of the central helix. Three residues from the central helix, Ala65, Leu68, and Leu72, form part of the hydrophobic core of the C-terminal domain while Leu62 caps the hydrophobic core and is partially buried from solvent.

In sequences of L9 from different species, the length of the long helix is invariant and its central residues are predominantly hydrophilic, but the exact sequence of the helix varies considerably (Hoffman *et al*., 1994, 1996). The exceptions are the residues that pack against the two domains, which are conserved among the different species. These results suggest that the long central helix acts as a rigid spacer which properly orientates the position of the N and C-terminal domains (Hoffman *et al*., 1994). Both domains contain binding sites for 23 S RNA (Branlant *et al*., 1976; Tumminia *et al*., 1994; Adamski *et al*., 1996), and the relative orientation of the domains is likely important for binding RNA. Recent mutagenesis experiments have shown that adding a residue to the helix weakens RNA binding (Adamski *et al*., 1996).

We have synthesized two peptides with sequences derived from the central helix of L9 from the bacterium *B. stearothermophilus*. One denoted as L9:41-74 contains all 34 residues, 41 to 74, of the helix while the other, denoted as L9:48-64, consists of the central 17 residues, 48 to 64, which are exposed to solvent in the protein. The sequence of L9:41-74 is listed here with the sequence of L9:48-64 in bold:

41 51 61 71
Ac-PANLKAL**EAQ KQKEQRQAAE ELAN**AKKLKE QLEK-NH_2

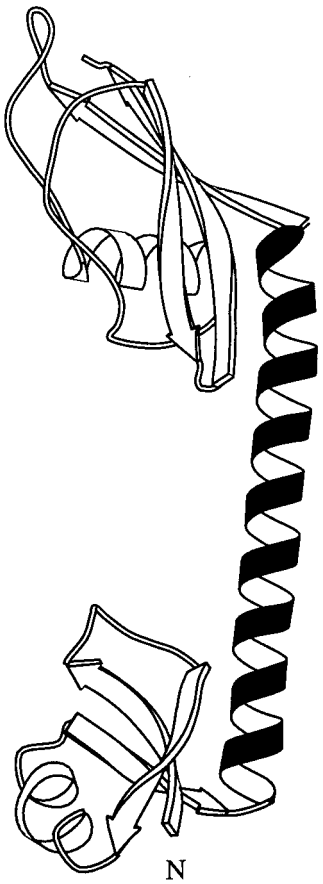

Figure 1. Ribbon diagram of L9 (drawing made using the program MOLSCRIPT; Kraulis, 1991). Two peptides were synthesized. L9:41-74 consists of the whole central helix, residues 41 to 74. L9:48-64 contains only the solvent exposed region of the helix, residues 48 to 64. The peptides were synthesized using FMOC protected amino acids and TBTU mediated amide coupling on a Millipore 9050 plus automated peptide synthesizer. The N and C termini of the peptides were blocked with acetyl and carboxamide, respectively. The peptides were purified by reverse-phase, high performance liquid chromatography (HPLC). Fast atomic bombardment (FAB) mass spectrometry and amino acid analysis confirmed the identity of pure products.

The N terminus is acetylated and the C terminus is amidated in both peptides.

Sedimentation equilibrium experiments and concentration dependent circular dichroism (CD) experiments indicate that L9:41-74 is monomeric in aqueous solution (Figure 2). The apparent relative molecular mass derived from a global fit to the centrifugation data is 4030(±560) and is in agreement with the expected monomer relative molecular mass, 3917. The residuals show no systematic trend. The CD signal for L9:41-74 is independent of concentration between 3 μM and 1 mM at 2°C suggesting that it is monomeric over this range of concentrations. L9:41-74 was also examined by gel filtration performed at room temperature. Interestingly the apparent relative molecular mass as

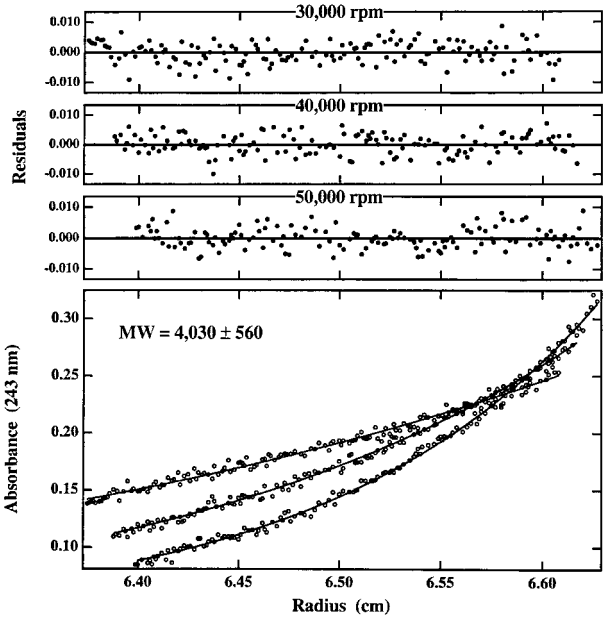

Figure 2. Analytical ultracentrifugation of L9:41-74 shows that it is monomeric. The absorbance at 243 nm is plotted *versus* radial distance for three different rotor speeds, 30,000, 40,000, and 50,000 rpm. The residuals are shown for each rotor speed. The data were globally fit to a single species allowing the relative molecular mass to float. The apparent relative molecular mass, 4030(±560) is in agreement with the true relative molecular mass, 3917. Experiments were performed at 25°C with a Beckman XL-A analytical ultracentrifuge, 12 mm pathlength, six-channel, charcoal-filled, Epon cells with quartz windows were used. Ten scans were averaged. Partial specific volumes were calculated from the weighted average of the partial specific volumes of the individual amino acids. The HID program from the Analytical Ultracentrifugation Facility at the University of Connecticut was used. The sample was dialyzed against 10 mM Mops (pH 7.0). The peptide concentration was 50 μM. The circular dichroism (CD) signals of L9:41-74 and L9:48-64 were examined at 2°C as a function of peptide concentration using an Aviv 62A DS spectrometer. The mean residue ellipticity at 222 nm was measured for peptide concentrations between 3 μM and 1 mM in 10 mM Mops (pH 7.0), and found to be independent of concentration. Gel filtration was also performed on L9:41-74. A Sephadex G50 column (relative molecular mass range: 1500 to 30,000) was standardized with the insulin B chain, aprotinin, ubiquitin, cytochrome *c*, α-lactalbumin, myoglobin, carbonic anhydrase, and superoxide dismutase. The buffer contained 50 mM NaCl, 2 mM sodium phosphate, 2 mM sodium borate, and 2 mM sodium citrate. Peptide (1 mg) was added to the column at room temperature.

determined by gel filtration is 2.4 times its true relative mass. It probably elutes with this apparent relative molecular mass because it is helical (evidence to follow) and therefore has a greater Stokes radius than a globular protein of the same relative molecular mass. A globular protein with the relative molecular mass of L9:41-74, 3919, has a Stokes radius of approximately 12 Å assuming that there is 0.35 g of bound water per gram of protein (Tinoco *et al.*, 1978; Cantor & Schimmel, 1980). A 34 residue helix can be approximated by an ellipsoid 50 Å long and 8 Å wide, and will have a Stokes radius of approximately 15 Å (Tanford, 1961; Tinoco *et al.*, 1978). A globular protein with a Stokes radius of 15 Å has a relative molecular mass of about 9000. The apparent relative molecular mass of L9:41-74 derived from gel filtration is 9300.

At 1°C in 10 mM Mops (pH 7.4), L9:41-74 is over 85% helical as judged by circular dichroism (Figure 3(a)). The mean residue ellipticity at 222 nm is $-36{,}600(\pm3600)$ deg cm^2 dmol^{-1} which corresponds to 98(±10)% helix. It is important to point out that the rotational strength of the transitions that give rise to the helical band at 222 nm depends upon the local backbone conformation and is sensitive to small changes in backbone dihedral angles (Manning *et al.*, 1988; Manning & Woody, 1991). As the temperature is raised the amount of helicity decreases, but at room temperature the peptide is still highly helical. The mean residue ellipticity at 222 nm is $-23{,}500(\pm2400)$ deg cm^2 dmol^{-1} at 25°C corresponding to 68(±7)% helix. There is an isodichroic point at 202 nm suggesting that each residue in the peptide is only sampling a helical or coil conformation. The mean residue ellipticity at 222 nm *versus* temperature is shown in Figure 3(c). At 85°C the mean residue ellipticity at 222 nm is $-8400(\pm800)$ deg cm^2 dmol^{-1} corresponding to 18(±2)% helix.

Concentration dependent CD experiments on L9:48-64 indicate that it is also monomeric at 2°C in aqueous solution. The mean residue ellipticity at 222 nm is independent of concentration between 3 μM and 1 mM.

At 1°C in 10 mM Mops (pH 7.4), L9:48-64 shows significant helicity (Figure 3(b)). The mean residue ellipticity at 222 nm is $-13{,}800(\pm1400)$ deg cm^2 dmol^{-1} corresponding to 41(±4)% helix. As the temperature is raised the CD signal decreases until 40°C where it levels off at -4000 deg cm^2 dmol^{-1} (Figure 3(c)). L9:48-64 was also examined by 2D NMR at 2°C. Because of resonance overlap it was not possible to make sequential assignments for all of the residues in the peptide, but it was possible to assign peaks to a particular spin type and therefore determine chemical shift indices for all of the residues (Wishart *et al.*, 1992). All the residues have a chemical shift index of -1 providing some evidence that the peptide is uniformly helical, although there may be fraying at the end of the helices undetectable by this crude measure of helicity. Even though this peptide is less helical than L9:41-74, it is still considerably more helical than most other protein fragments that have been studied. Simple helix-coil transition theory of homopolymers predicts that longer peptides should display a higher percentage of helical structure (Poland & Scheraga, 1970) and this effect has been observed experimentally (Scholtz *et al.*, 1991a). It is likely that length effects contribute to the higher helical content of L9:41-74 compared to L9:48-64.

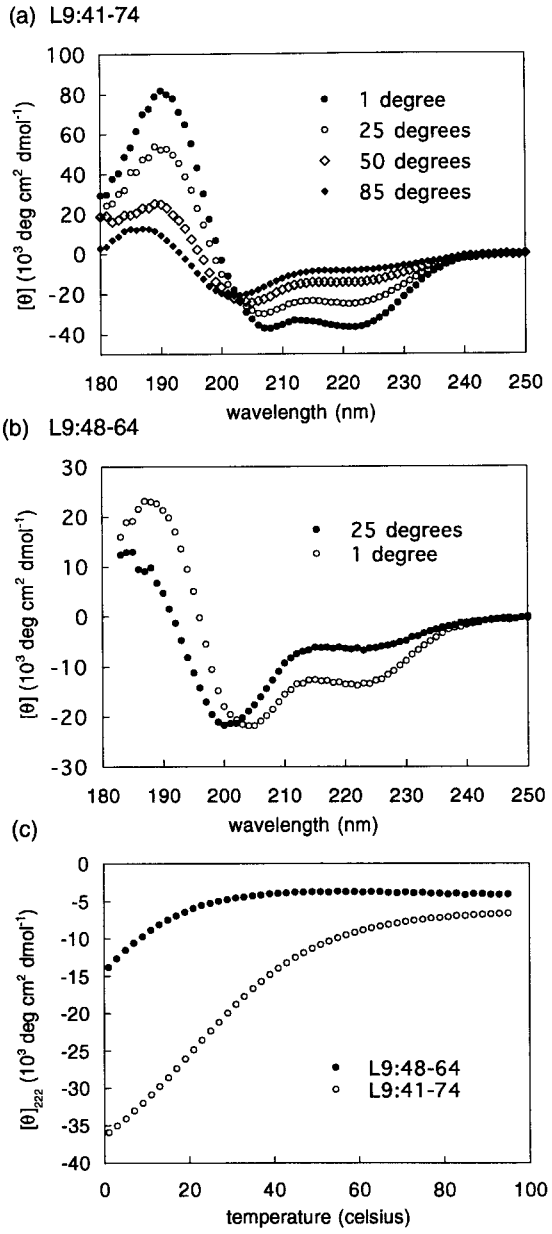

Figure 3. Far-UV CD spectra of (a) L9:41-74 and (b) L9:48-64 at different temperatures. Solutions contained 10 mM Mops (pH 7.0). Peptide concentrations were 286 μM for L9:41-74 and 430 μM for L9:48-64. Spectra were obtained on an Aviv 62A DS spectrometer with a 0.01 cm cuvet and a 1 nm bandwidth. Spectra are the average of three scans with an averaging time of three seconds per data point. Quantitative amino acid analysis (Commonwealth Biolabs, Richmond VA.) was used in triplicate on three separate samples of L9:41-74 and two separate samples of L9:48-64 to determine peptide concentrations. (c) Mean residue ellipticity of L9:41-74 and L9:48-64 at 222 nm as a function of temperature. Data were collected with a 1 cm pathlength cuvet and a 1 nm bandwidth. Each data point is the result of 60 seconds of averaging. Solutions contained 10 mM Mops (pH 7.0). Fraction helix was determined from the measured mean residue ellipticity at 222 nm, $[\theta]_{obs}$, using:

$$f_h = ([\theta]_{obs} - [\theta]_C)/([\theta]_H - [\theta]_C) \quad (1)$$

There are a number of possible explanations for the high helical content of these peptides. There are no β-branched amino acids in the peptides. β-Branched amino acids are particularly unfavorable in a helix because the β-branch collides with the peptide backbone in two of the three χ1 rotamers. The first residue of L9:41-74 is a proline, which is known to be favorable for helix formation (Richardson & Richardson, 1988). Both peptides contain primarily Ala, Glu, Lys, Gln, Arg and Leu (31 of 34 residues in L9:41-74, 16 of 17 residues in L9:48-64), which are in the top half of most scales of intrinsic helix propensities (O'Neil & DeGrado, 1990; Wojcik *et al.*, 1990; Padmanabhan & Baldwin, 1991; Chakrabartty *et al.*, 1994; Luque *et al.*, 1996). However, only alanine and arginine have helix propagation factors greater than one, and therefore are the only amino acids which prefer a helical conformation to a random coil conformation in the absence of side-chain interactions (Chakrabartty *et al.*, 1994). Using a program developed by Doig and co-workers which is based on Lifson-Roig helix-coil theory and only utilizes helix propagation factors (values from Rohl *et al.*, 1996 were used) and N-cap propensities it is predicted that L9:41-74 should be 13% helical and L9:48-64 4% helical at 0°C (Lifson & Roig, 1961; Doig *et al.*, 1994). These percentages are much lower than what was observed experimentally indicating that side-chain–side-chain interactions and side-chain–helix macrodipole interactions must make important contributions to the stability of both peptides. Support for this hypothesis is provided by calculations with a program developed by Serrano and co-workers, AGADIR1s, which explicitly includes energies for side-chain–side-chain interactions and side-chain–helix macrodipole interactions (Munoz & Serrano, 1994, 1995, 1997). AGADIR1s predicts that L9:41-74 will be 61% helical at 1°C (pH 7.0) and 16% helical at 25°C (pH 7.0). It also predicts that L9:48-64 will be 8% helical at 1°C (pH 7). These calculations all underestimate the actual helicity, but they do come closer to the experimental values than the Lifson-Roig model without side-chain interactions. These results indicate that the experimental data presented here will be useful for the further refinement of helix prediction programs.

where $[\theta]_H$ corresponds to 100% helix and $[\theta]_C$ corresponds to complete coil:

$$[\theta]_H = -40,000 \times (1 - 2.5/n) + 100 \times T \quad (2)$$

$$[\theta]_C = 640 - 45 \times T \quad (3)$$

and where n is the number of residues in the peptide, T is temperature in °C

L9:48-64 was also examined by 2D NMR on a Bruker Instruments AMX 600 spectrometer at 2°C. TOCSY (mixing time = 75 ms) and ROESY (mixing time = 225 ms) experiments were run. The peptide concentration was 6 mM in 90% H_2O/10% ^2H_2O at pH 4.7.

Both peptides contain a large number of potentially stabilizing side-chain–side-chain interactions, as well as potentially favorable electrostatic interactions with the helix macrodipole (Shoemaker *et al.*, 1987; Fairman *et al.*, 1989). Lys·Glu ($i, i+4$) and ($i, i+3$) interactions are known to be helix stabilizing as well as Gln·Glu ($i, i+4$) interactions (Scholtz *et al.*, 1993). In L9:41-74 possible favorable interactions include seven Lys·Glu ($i, i+4$) or ($i, i+3$), one Arg·Glu ($i, i+4$), and two Gln·Glu ($i, i+4$). Electrostatic interactions can also destabilize a helix. A Lys·Lys ($i, i+3$) or Glu·Glu ($i, i+3$) interaction is destabilizing as well as a Lys·Glu ($i, i+1$) interaction (Scholtz *et al.*, 1993). There are several possible destabilizing interactions in L9:41-74 including three Lys·Glu ($i, i+1$), one Lys·Lys ($i, i+3$), one Glu·Glu ($i, i+3$), and one Arg·Glu ($i, i+2$). L9:48-64 is potentially stabilized by two Lys·Glu ($i, i+3$), one Arg·Glu ($i, i+4$), and two Gln·Glu ($i, i+4$) and potentially destabilized by one Lys·Glu ($i, i+1$).

To examine the role of electrostatic interactions in stabilizing these peptides, the mean residue ellipticity at 222 nm was measured as a function of ionic strength and pH (Figure 4). If charge–charge interactions make a net favorable contribution to helix stability then the peptide's helicity should be reduced at higher ionic strength. For example, a peptide studied by Lyu *et al.* with the sequence $YS(E_4K_4)_2$ contains numerous ($i, i+4$) salt bridges and is 67% helical at 0 M NaCl and 22% helical at 2.5 M NaCl (Lyu *et al.*, 1992). At pH 7.0 L9:41-74 shows a decrease in ellipticity between 0 M NaCl and 2.5 M NaCl and is 68(±7)% helical at 0 M NaCl, 25°C and 41(±4)% helical at 2.5 M NaCl, 25°C. Interpretation of this result is complicated by the fact that NaCl at high concentrations can destabilize a helix because of Hoffmeister effects (Scholtz *et al.*, 1991b). Even with this caveat it is apparent that electrostatic interactions are stabilizing L9:41-74 since the helicity of the peptide decreases at low amounts of added salt (below 500 mM) where the Hoffmeister effects should be minimized. L9:48-64 shows a small increase in helicity with the addition of 400 mM NaCl, 41(±4)% helical at 0 M NaCl and 43(±4)% helical at 400 mM NaCl. At higher concentrations of salt the helix is destabilized, presumably in part by Hoffmeister effects.

pH titrations also highlight the important role of side-chain–side-chain interactions and side-chain–helix macrodipole interactions. The helicity of both peptides is dependent on pH (Figure 4). L9:41-74 decreases from 98(±10)% helical to 85(±9)% helical at 1°C when the lysine residues are deprotonated by raising the pH above 11. The helicity does not change when the glutamic acid residues are protonated by lowering the pH. However, if the experiment is repeated at 25°C, L9:41-74 is 68(±7)% helical at pH 7, 74(±7)% helical at pH 2, and 51(±5)% helical at pH 12. This indicates that lowering the pH stabilizes a region of L9:41-74 which is already completely helical at 1°C. It is interesting that removing the charges on the lysine residues is destabilizing, while doing so on the glutamic acid residues is not. In part this likely reflects the fact that a $Lys^{+1}\cdot Glu^{0}$ hydrogen bond is stronger than a $Lys^{0}\cdot Glu^{-1}$ hydrogen bond (Lyu *et al.*, 1992; Scholtz *et al.*, 1993). As a result there are strong Lys·Glu ($i, i+4$) and ($i, i+3$) interactions at pH 2 but not at pH 12. In addition, for L9:41-74 charge–helix macrodipole interactions should be destabilizing at high pH and stabilizing at low pH. In L9:41-74 there are two lysine residues at the C terminus which should interact favorably with the helix dipole when protonated, and two glutamic acid residues which should be destabilizing when unprotonated.

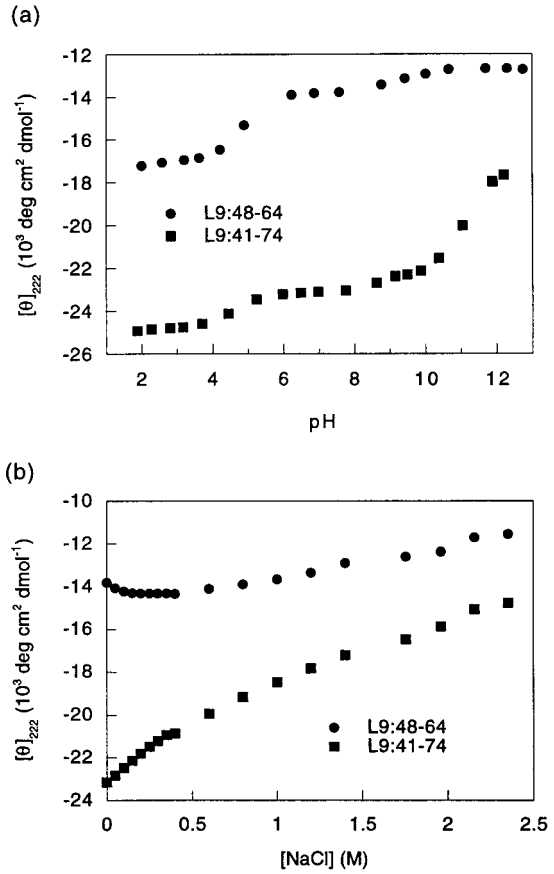

Figure 4. (a) Mean residue ellipticity of L9:41-74 and L9:48-64 at 222 nm as a function of pH. Samples were made in 2 mM sodium phosphate, 2 mM sodium borate, and 2 mM sodium citrate. The initial solution was made at high pH and titrated to lower pHs with small amounts of concentrated HCl. A stir bar within the cuvet was used to mix the solution. A total of 50 μl HCl was added to 3.5 ml of sample. The data were collected with peptide concentrations of 10 μM in a 1 cm pathlength cuvet. (b) Mean residue ellipticity at 222 nm as a function of NaCl concentration. Samples were made in 2 mM sodium phosphate, 2 mM sodium borate, 2 mM sodium citrate (pH 7.0). The peptide concentrations were 15 μM.

The helicity of L9:48-64 is also dependent on the protonation state of the lysine and the glutamic acid residues. At 1°C it is 38% helical at pH 11, 41% helical at pH 7, and 51% helical at pH 2. As with L9:41-74, the peptide is most helical at low pH. One factor in this increased helicity is the higher intrinsic propensity of a glutamic acid to be in a helix at low pH. In a scale measured by Baldwin and co-workers the propagation factor for Glu^0 is 0.70 and for Glu^{-1} it is 0.54 (Rohl *et al*., 1996). Using the value for Glu^0 in Doig's Lifson-Roig algorithm (Doig *et al*., 1994) changes the predicted helicity of L9:48-64 from 4% helix to 11% helix indicating that pH dependent changes in intrinsic propensities can have a significant affect on helicity. This effect is likely important to the increased stability of L9:48-64 at low pH.

The long helix of L9 appears to play an important structural role by acting as a rigid spacer, orientating the compact N and C-terminal domains so that they are properly positioned to bind to RNA. The central portion of this helix is fully exposed to solvent and presumably the protein has evolved to stabilize the helix in the absence of extensive tertiary interactions with the remainder of the protein. Thus it is not surprising that peptides corresponding to or derived from the long central helix of L9 show an unusually high propensity to fold in isolation. Many other studies of protein fragments have examined small to mid-sized peptides derived from regions of intact proteins which are helical in the native state, but which pack against and are intimately associated with the rest of the protein. In most of these cases the peptides are unstructured in isolation. There are several other proteins which contain long solvent exposed α-helices in their crystal structure. The most famous examples are probably calmodulin and troponin C. Recent NMR studies have, however, convincingly demonstrated that the central helical region of these molecules is flexible or disordered in solution (Barbato *et al*., 1992; Slupsky & Sykes, 1995). The critical difference must be that the central helix of L9 plays an essential structural role while the long connecting helices in troponin C and calmodulin do not. It is interesting that short peptides have been designed to have more structure than is seen in any protein fragments besides L9:41-74 (Scholtz & Baldwin, 1992). Like L9:41-74, these peptides have been designed to be stable in water without tertiary interactions.

Studies by Hoffman and co-workers on full-length L9 show that as the temperature is raised the central helix remains intact until the protein starts to unfold above 60°C. The isolated helix is largely unstructured at 60°C indicating that the helix must be stabilized by its interactions with the N and C-terminal domains. It is also likely that the helix is important to the stability of the terminal domains since the helix packs against and contributes residues to the hydrophobic cores of both domains. Therefore, the stability of the two terminal domains should not be completely independent because both domains gain stability from a shared structural element, the central helix. We have shown that the helix has a high intrinsic stability and can form even without interactions with the rest of the protein. The ability of the central helix to form native-like structure in isolation highlights a potential role for the helix in the early stages of the folding process. It has been shown that individual helices can fold with time constants on the order of nanoseconds (Thompson, 1996; Williams *et al*., 1996). Once formed the helix may help initiate the folding of the two terminal domains. If the helix does act to initiate folding then the folding of the two domains will not be entirely independent.

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