Human S100b protein: Formation of a tetramer from synthetic calcium-binding site peptides

CRAIG DONALDSON,¹ KATHRYN R. BARBER,¹ CYRIL M. KAY,² AND GARY S. SHAW¹

¹ Department of Biochemistry and McLaughlin Macromolecular Structure Facility, The University of Western Ontario, London, Ontario N6A 5C1, Canada

² Department of Biochemistry and MRC Group in Protein Structure and Function, University of Alberta, Edmonton, Alberta T6G 2H7, Canada

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Abstract

Human brain S100b protein is a unique calcium-binding protein comprised of two identical 91-amino acid polypeptide chains that each contain two proposed helix-loop-helix (EF-hand) calcium-binding sites. In order to probe the assembly of the four calcium-binding sites in S100b, a peptide comprised of the N-terminal 46 residues of S100b protein was synthesized and studied by CD and ¹H NMR spectroscopies as a function of concentration and temperature. At relatively high peptide concentrations and in the absence of calcium, the peptide exhibited a significant proportion of α -helix (45%). Decreasing the peptide concentration led to a loss of α -helix as monitored by CD spectroscopy and coincident changes in the ¹H NMR spectrum. These changes were also observed by ¹H NMR spectroscopy as a function of temperature where it was observed that the T_m of the peptide was lowered approximately 14 °C with a 17-fold decrease in peptide concentration. Sedimentation equilibrium studies were used to determine that the peptide formed a tetramer in solution in the absence of calcium. It is proposed that this tetrameric fold also occurs in S100b and is a result of the interaction of portions of all four calcium-binding sites.

Keywords: calcium-binding; peptides; S100b; tetramer

Calcium-binding proteins form a large class of regulatory proteins that are important as second messenger molecules in such diverse functions as vision, muscle contraction, enzyme activation, and neurotransmitter release. In particular many of these calcium binding proteins are members of the helix-loop-helix or EF-hand family of proteins (Kretsinger, 1987; Sekharudu & Sundaralingam, 1988; Marsden et al., 1989; Strynadka & James, 1989). These proteins contain several contiguous calcium-binding sites, each comprised of a central 12-residue loop, where calcium is coordinated, and two flanking helices. X-ray crystallographic studies and multiple sequence analyses of these proteins has further established that these sites occur in pairs, with between two and eight sites per protein. For example, parvalbumin, oncomodulin, and calbindin D_{9k} all contain two functional calcium-binding sites and troponin-C and calmodulin each have four sites that form two independent two-site domains. Detailed structural information from the X-ray structures has revealed that the calcium-binding domains of these proteins are

each comprised of two pseudosymmetric helix-loop-helix calcium-binding sites (Strynadka & James, 1989).

One subset of the EF-hand family of proteins, for which there is little three-dimensional structural information available, are the S100 proteins. These proteins are a group of small acidic proteins, first discovered by Moore, that are a major constituent of brain protein and are primarily found in the cytoplasm of glial cells (Moore, 1965). Early sequencing studies (Isobe & Okuyama, 1978, 1981; Isobe et al., 1981) demonstrated two different forms of the protein, S100 α (93 residues) and S100 β (91 residues) and established that the protein existed as the dimeric isoforms, S100a₀ ($\alpha\alpha$), S100a ($\alpha\beta$), and S100b ($\beta\beta$). This observation is unique for the S100 proteins because they appear to be the only EF-hand proteins that exist as homo- and heterodimers. A second unique feature of the S100 proteins is their weak affinity for calcium ions. The closest relative of the S100 protein family, calbindin D_{9k}, bears about 50% sequence similarity to S100b and exhibits a K_d of about 0.01 μ M (Linse et al., 1987), whereas S100b binds calcium approximately 1,000-fold weaker (Baudier & Gerard, 1983, 1986; Mani et al., 1983; Baudier et al., 1986b). Both of these features suggest that S100b, and likely other proteins of the S100 protein family, have some unique structural features that warrant investigation.

Reprint requests to: Gary S. Shaw, Department of Biochemistry and McLaughlin Macromolecular Structure Facility, The University of Western Ontario, London, Ontario N6A 5C1, Canada; e-mail: shaw@merlin.biochem.uwo.ca.

Fig. 2. Far-UV CD spectra of the 200 μ M hs1bI peptide in 50 mM MOPS, 50 mM KCl at pH 7.19 (—) and in 50% TFE (---) at 25 $^{\circ}$ C in the absence of calcium. Molar ellipticities at 222 nm (θ_{222}) were $-12,340$ and $-17,380$ $\text{deg cm}^2 \text{dmol}^{-1}$, respectively.

in hs1bI, over this concentration range would not be expected if hs1bI existed in a monomeric state.

Temperature dependence studies

To parallel the dilution experiments of Figure 3, the effect of temperature on hs1bI was investigated at three different peptide concentrations (0.1–1.7 mM) that were monitored by ^1H NMR spectroscopy. The goals of these experiments were to determine if hs1bI could be thermally unfolded and to determine whether this thermal unfolding was concentration dependent. Similar experiments have been used previously to study the unfolding of the arc repressor DNA binding protein (Bowie & Sauer, 1989) and the dimerization domain of transcription factor LFB1 (De Francesco et al., 1991) as a function of temperature and concentration. In both of these latter cases, the peptides underwent a thermal transition from α -helical folded dimer to unfolded monomer. We were interested to see if a similar transition for hs1bI could be observed, which may indicate that the α -helical structure of hs1bI resulted from an associated state similar to

Fig. 3. Concentration dependence of hs1bI measured by NMR spectroscopy. Aromatic (A) and methyl (B) regions of the 500-MHz ^1H NMR spectra of hs1bI in D_2O , 50 mM KCl, 40 μ M DSS, pH 7.28, at 25 $^{\circ}$ C are shown as a function of peptide concentration. Peptide concentrations are indicated to the right of the spectra and spectra were scaled by adjusting the vertical scale of each spectrum such that the $(\text{CH}_3)_3\text{Si}$ -peak (from DSS internal standard) was the same integral in all spectra. Several peaks that undergo either a shift in frequency or significant changes in peak height as a function of peptide concentration are shaded. Resonances marked (x) arise from $-\text{CH}_2-$ protons from DSS.

that observed in other calcium-binding peptides (Kay et al., 1991; Monera et al., 1992; Linse et al., 1993; Shaw et al., 1994). In Figure 4A the aromatic region of 0.1 mM hs1b1 is shown as a function of temperatures ranging from 10 to 75 °C. At the lower temperatures, the peaks were all broad and featureless, similar to those shown for the initial spectra in the concentration-dependence studies (Fig. 3). As the temperature was increased however, the aromatic region of the spectrum underwent dramatic changes, including the shifting and sharpening of several resonances. Further, near 75 °C, the spectrum of hs1b1 became more similar to that obtained for the 3 μ M sample at 25 °C (Fig. 3), suggesting that the observed species is similar in both cases. We suggest that the 75 °C spectrum of 0.1 mM hs1b1 is of the unfolded monomer and that the same species is formed by dilution in Figure 3.

Because 0.1 mM hs1b1 can be unfolded at high temperature, we assessed the concentration dependence of this unfolding of hs1b1 using several higher hs1b1 concentrations and ^1H NMR spectroscopy. In Figure 4A several resonances of 0.1 mM hs1b1 were measured as a function of temperature and plotted in Figure 4B. Corresponding data from the 1.7 mM hs1b1 sample are included in Figure 4B. From this graph it is clear that the change in resonance frequencies for the 0.1 mM hs1b1 sample (open symbols) each follow a smooth sigmoidal curve over the temperature range studied. This suggests that the transition from folded multimer to unfolded monomer was complete for the 0.1 mM sample. A $T_m = 45^\circ\text{C}$ is estimated from this data for 0.1 mM hs1b1. In contrast to the 0.1 mM hs1b1 sample, the data for 1.7 mM showed incomplete melting profiles. As a result, the initial plateau for the 1.7 mM sample was maintained to higher temperatures and the midpoint from the sigmoidal transition was shifted to a higher temperature compared to 0.1 mM hs1b1. A $T_m = 59.4^\circ\text{C}$ is estimated from this data for 1.7 mM hs1b1, although this may be a conservative estimate because no upper plateau in the melting profile was reached. Nevertheless, this represents a T_m of $\approx 14^\circ\text{C}$ higher than for the 0.1 mM sample. As with the previous ^1H NMR results, this increase in T_m with increasing peptide concentration is indicative of a transition from a folded multimeric species to an unstructured monomer.

Folding and unfolding of hs1b1

The dissociation of hs1b1 from a higher molecular weight species to an unfolded monomer can be assessed using the equilibrium shown in Equation 1

where U is the unfolded monomer, F is the folded species, and n is the degree of association. Because CD experiments have shown that hs1b1 is α -helical at higher concentrations (>0.1 mM) and ^1H NMR experiments suggest that hs1b1 may be unfolded at lower concentrations (<6 μ M) or higher temperatures, we studied the unfolding of hs1b1 by monitoring the helix-coil transition at θ_{222} as a function of peptide concentration. This has been done previously for a number of other α -helical peptides (Ho & DeGrado, 1987; Monera et al., 1992; Shaw et al., 1994).

In Figure 5, the magnitude of θ_{222} is plotted versus the concentration of hs1b1 over the concentration range of 1–200 μ M. It is clear that the magnitude of θ_{222} decreases significantly as the concentration of hs1b1 decreases, suggesting that the α -helix content of the peptide is decreasing as a function of concentra-

Fig. 4. Temperature dependence of hs1b1. **A:** Aromatic regions of the 500-MHz ^1H NMR spectra of 100 μ M hs1b1 in D_2O , 50 mM KCl, 40 μ M DSS, pH 7.28, are shown as a function of temperature between 10 and 75 °C. The temperatures at which each spectrum was acquired are indicated to the right of the plots. Several peaks that underwent a significant chemical shift change as a function of temperature are indicated with the dotted line and corresponding symbol. **B:** Plot of temperature dependence of hs1b1-derived peak frequencies in (A) for 100 μ M hs1b1 (open symbols) and 1.7 mM hs1b1 (closed symbols). Symbols used in (B) are shown above the corresponding peaks in (A).

Fig. 5. Loss of structure in hs1b1 as a function of concentration as measured by CD spectroscopy. The graph shows the molar ellipticity (as $-\theta_{222}$) (●) determined at a variety of peptide concentrations ranging between 1 and 200 μM . Data have been fit using Equation 2 (described in the text) for $n = 4$ (—), although $n = 3$ gives a similar fit.

tion. The lowest concentration studied (1 μM) had $\theta_{222} = -2,698$ degrees, a value very close to that obtained for chemically denatured helix-loop-helix calcium binding peptides from TnC (Monera et al., 1992; Shaw et al., 1994). This observation shows that the α -helix in hs1b1 is a result of a specific association of the peptide.

The degree of association (n) for the α -helical hs1b1 multimer was determined from the data shown in Figure 5 and Equation 2, where θ_{max} is θ_{222} for the multimer, θ_{min} is θ_{222} for the monomer, $[P_t]$ is total peptide concentration, and K_d is the dissociation constant for the multimer-to-monomer equilibrium.

$$[P_t] = \left\{ \frac{(\theta_{\text{obs}} - \theta_{\text{min}})K_d}{n(\theta_{\text{max}} - \theta_{\text{min}}) \left[1 - \frac{(\theta_{\text{obs}} - \theta_{\text{min}})}{(\theta_{\text{max}} - \theta_{\text{min}})} \right]^n} \right\}^{1/(n-1)} \quad (2)$$

This method has been used previously in order to determine the degree of association (n) of other synthetic α -helical proteins, including helix-loop-helix peptides (Ho & DeGrado, 1987; Monera et al., 1992). We used values between 2 and 8 to fit these data. In Figure 5, a curve for $n = 4$ is shown, although values of $n = 3$ and $n = 4$ yielded equally good fits. This suggested that the α -helical multimer is a complex of three or four peptide monomers. This result is largely a function of θ_{222} at the lowest concentrations studied, where for $n = 4$, $\theta_{\text{min}} = -2,678$ degrees is calculated, and for $n = 3$, $\theta_{\text{min}} = -2,602$ degrees is calculated. An analysis of the data in Figure 5 shows that θ_{obs} never reaches θ_{min} , causing an ambiguous value of n to be determined. Fitting the data for $n = 2$ yielded impractical values for θ_{min} having large positive ellipticities.

Sedimentation equilibrium studies

Because fitting of the CD data did not allow an accurate value for n to be determined, we used sedimentation equilibrium to obtain this. We subjected hs1b1 to several sedimentation equilibrium experiments consisting of different initial peptide concentrations and different rotor speeds. Figure 6A shows three

independent results where the observed molecular weight (obtained from the slope of $\ln y$ versus r^2) was plotted versus the peptide concentration at that point in the cell. The significant curvatures of the data suggest that the peptide does not exist as a single molecular weight species, in agreement with NMR and CD spectroscopic results. In addition, the similarity of the three runs suggests that the association of the peptide is similar in all cases. These data were then analyzed and iteratively fit using Equation 3, which is similar to Equation 2, but has been adapted for the observed and expected molecular weights.

$$[P_t] = \left\{ \frac{(\text{MW}_{\text{obs}} - \text{MW}_{\text{mon}})K_d}{n(\text{MW}_n - \text{MW}_{\text{mon}}) \left[1 - \frac{(\text{MW}_{\text{obs}} - \text{MW}_{\text{mon}})}{(\text{MW}_n - \text{MW}_{\text{mon}})} \right]^n} \right\}^{1/(n-1)} \quad (3)$$

Fig. 6. Plot of point average calculated molecular weight (MW) versus concentration obtained from sedimentation equilibrium experiments of hs1b1. Data were collected and plotted as the natural logarithm of the optical activity ($\ln y$) versus the distance moved through the centrifugation cell (r^2) and fitted with a second order polynomial equation. Point average molecular weights were calculated from the slope of this curve at each point. **A:** Data are shown for samples with initial hs1b1 concentrations of 4.08 mg/mL at a rotor speed of 20,000 rpm (▲) and 5.96 mg/mL at rotor speeds of 17,000 rpm (□) and 20,000 rpm (○). **B:** Data for the 5.96-mg/mL sample at a rotor speed of 17,000 rpm (□) are shown, which have been iteratively fit using Equation 3 (described in the text) for $n = 3$ (---), $n = 4$ (—), and $n = 5$ (---) using a monomer molecular weight $\text{MW}_{\text{mon}} = 5,426$ g/mol.

These data were fit in two different manners. In the first, values for n were fixed at integer values, ranging between 2 and 6, a value for monomer molecular weight $MW_{\text{mon}} = 5,426$ g/mol was used, and values for K_d and MW_n were determined in an iterative fashion. From this method, it was found that the best-fit curves were obtained for $n = 4$. In a second method, MW_n was set to $n \times (MW_{\text{mon}})$ and values for n and K_d were determined. This method yielded a value of $n = 3.75 \pm 0.14$. These values for the degree of association of hs1bI, in combination with the CD data, are consistent with formation of a tetramer at higher concentrations, which is mostly α -helical in nature.

Discussion

X-ray crystallographic studies of calcium-binding proteins such as troponin-C (Herzberg & James, 1988; Satyshur et al., 1988), calmodulin (Babu et al., 1988), calbindin D_{9k} (Szebenyi & Moffat, 1986), and parvalbumin (Kretsinger & Nockolds, 1973; Swain et al., 1989) have shown that the helix-loop-helix calcium-binding sites in these proteins are paired. It has further been determined that synthetic helix-loop-helix peptides can assemble to form two-site homo- and hetero-site domains similar in structure to the native domain found in proteins such as troponin-C and calbindin D_{9k} (Shaw et al., 1990, 1992a, 1992b; Kay et al., 1991; Linse et al., 1993). In the present studies we have used this peptide approach to determine if the calcium-binding sites in the dimeric calcium-binding protein S100b are organized in a similar pairwise fashion to those in troponin-C, calmodulin, or calbindin D_{9k} , or if the sites in S100b adopt a unique arrangement.

Folding and stoichiometry of hs1bI

Human brain S100b is a dimeric protein having two proposed helix-loop-helix calcium-binding motifs (Fig. 1) in each monomer much like the sequence of its monomeric relative calbindin D_{9k} . It has been suggested that the pseudo helix-loop-helix site I in S100b has a low affinity for calcium at pH 7.5 ($K_d < 1$ mM), whereas the classical helix-loop-helix site II has a somewhat higher affinity for the metal ion ($K_d \approx 0.1$ mM) (Baudier & Gerard, 1983, 1986; Mani & Kay, 1983; Mani et al., 1983; Baudier et al., 1986b). In agreement with this, the peptide used in this work, hs1bI, representing the sequence of site I of human hs1bI showed little or no affinity for calcium at pH 7.3 as determined by ^1H NMR spectra in the presence and absence of either EDTA or calcium (data not shown). This feature of calcium binding for hs1bI and S100b is unique to these proteins and is in contrast to that found for calbindin D_{9k} , where site I peptides have a significantly higher affinity for calcium ($K_d \approx 1\text{--}40$ μM) (Tsuji & Kaiser, 1991; Finn et al., 1992) and site I in the native protein, which exhibits still tighter binding ($K_d \approx 0.01$ μM) (Linse et al., 1987).

CD measurements show that hs1bI is mostly α -helical (21 residues) in the absence of calcium, much like that found for S100b protein (Mani et al., 1983). This is also similar to a calbindin D_{9k} homodimer, comprised of residues 1–43 and linked by a disulfide bridge (Cys F1₂), which has been found to contain about 80% of the α -helical content in the calcium-free state compared to the calcium-bound state (Linse et al., 1993). However, these observations are in contrast to that of a 37-residue site I peptide from calbindin D_{9k} , which shows little α -helical character in the absence of calcium and requires calcium binding to

induce α -helix (Tsuji & Kaiser, 1991). It is also likely that this latter calbindin D_{9k} peptide is a dimer in the presence of calcium based on observations by Linse et al. (1993). These cases, as well as previous work with troponin C peptides (Shaw et al., 1990; Monera et al., 1992) show that the association of these calcium-binding peptides is the necessary requirement to induce α -helical structure. This would also suggest that the significant α -helix character in hs1bI in the absence of calcium is a result of the peptide existing in an associated form.

The state of association of the hs1bI peptide was determined by ^1H NMR, CD, and sedimentation measurements. Dilution of hs1bI led to a significant decrease in α -helical structure as determined by CD spectroscopy such that, at low concentrations of hs1bI, little α -helix is present. This is reflected in NMR experiments, which show small changes in chemical shift and line narrowing as peptide concentration is decreased or the temperature is increased. Together these observations suggest a specific association of hs1bI as has been previously observed for other calcium-binding peptides (Monera et al., 1992; Shaw et al., 1994) and 4-helix bundle peptides (Ho & DeGrado, 1987; Kaumaya et al., 1990). We also considered that a more general aggregation of hs1bI may be occurring. However, the observation that CD and sedimentation data could be best fit for values near 4 rather than higher integer values opposes this idea. In addition, it has been previously observed in studies with other α -helical peptides (Kaumaya et al., 1990; Killian et al., 1994), that general aggregation leads to the formation of higher order aggregates that have much lower α -helix content (<20%) compared to oligomers of defined stoichiometry (40–50%). In this work, the high α -helical content observed for hs1bI (45%) agrees well with that expected for a specifically associated oligomer.

The stoichiometry of association of hs1bI was determined by CD experiments to be either 3 or 4 and by sedimentation equilibria to be close to 4, suggesting a tetrameric complex. These values present an interesting result compared to other calcium-binding helix-loop-helix peptides that have values near $n = 2$ (Monera et al., 1992) when studied by similar methods. However, it is also clear from the present results that the tetramer molecular weight is not observed. This likely arises from the propensity of hs1bI to form a tetramer, but probably indicates that the association is weak. From sedimentation equilibrium and CD experiments, we could only estimate a $K_{\text{diss}} > 3$ mM. In this manner hs1bI may be similar to other calcium peptide domains from troponin C and calbindin D_{9k} , where each protein exhibits one peptide domain that is significantly less stable than the other. For example in troponin-C, site III peptides dimerize more easily ($K_{\text{diss}} \approx 10$ μM) than the corresponding site IV peptide ($K_{\text{diss}} \approx 2,000$ μM) (Kay et al., 1991; Shaw et al., 1994). From this analysis, it is possible that site II in S100b may form a more stable tetrameric complex, reflective of its stronger intermonomer interactions in the protein.

Model for S100b structure

The objective of this study was to assess the organization of the calcium-binding sites in S100b with respect to each other using a synthetic peptide model. It is informative to make some comparisons between the ability of hs1bI to form a tetrameric complex and the dimeric complexes formed from calbindin D_{9k} (Linse et al., 1993) and troponin C (Shaw et al., 1990, 1992a, 1992b; Kay et al., 1991) peptides. By sequence analysis, cal-

bindin D_{9k} is the most similar calcium-binding protein to S100b for which a three-dimensional structure is known. The pseudo calcium-binding site in calbindin D_{9k} contains a 14-residue loop as does S100b. The two most noticeable differences between these two proteins are that S100b is a native dimer, whereas calbindin D_{9k} is a monomer, and the calcium affinity of calbindin D_{9k} is 1,000-fold greater than observed in S100b. These differences in calcium affinity and secondary structure of these proteins may point to significant structural differences in S100b compared to calbindin D_{9k} particularly in the arrangement of calcium-binding sites. Coincident with these observations, 43-residue peptides from calbindin D_{9k} bind calcium tightly and form dimers similar in fold to the parent protein. In the case of hs1bI, calcium binding is weak as in S100b, and the protein forms a tetramer. We believe this tetramer is representative of the arrangement of the calcium-binding sites in S100b, suggesting that all four calcium sites interact. It may also be possible that the pseudo calcium-binding site in S100b is structurally unlike that in calbindin D_{9k}, which may partially account for its lower affinity than calbindin D_{9k}. This rationale has been proposed by Rosevear and co-workers (Brito et al., 1991, 1993; Krudy et al., 1992) for the inactive calcium-binding site I in cardiac troponin-C, which includes an extra residue in the calcium-binding loop. However, site I is still proposed to be paired with site II in cardiac troponin-C. In the case of S100b this could suggest that its pseudo calcium-binding site I has a modified structure as suggested in cardiac troponin-C but rather than only two sites interacting two pairs of sites in S100b are brought together to form a tetrameric arrangement of calcium-binding sites. This is perhaps not surprising because the major contributors for the association of calcium-binding sites are the significant hydrophobic interactions that are found at the interface of the calcium-binding sites and are concentrated in the α -helices rather than the calcium-binding loops.

Because hs1bI can form a tetramer, this would suggest that in S100b a novel arrangement of calcium-binding sites may be occurring that involves interactions between regions of all four sites rather than only two sites, as occurs in two-site domains such as troponin C and calmodulin. It is also interesting that in peptide studies of troponin-C no evidence for a peptide tetramer has been found (Kay et al., 1991; Monera et al., 1992), again suggesting a difference between the arrangement of the calcium-binding sites in troponin-C and S100b even though both have four calcium-binding sites. This proposal of four interacting sites in S100b may also account for previous calcium-binding experiments with S100b (Baudier & Gerard, 1983, 1986; Mani & Kay, 1983; Mani et al., 1983; Baudier et al., 1986b) that yield dissociation constants of between 0.1 and 1 mM for both sites. It has been shown that at relatively low S100b protein concentrations, where a significant monomeric population may be present, the calcium affinity is increased by up to 10-fold (Baudier, 1988; Baudier et al., 1986a). These observations may suggest that in the dimeric form of S100b the interaction of all four calcium-binding sites perturbs these sites and effectively decreases calcium affinity.

Materials and methods

Peptide synthesis and purification

A peptide comprising residues 1–46 from human S100b (hs1bI) protein was synthesized by Chiron Mimotopes using F-moc

chemistry and purified by reversed-phase HPLC. Composition of the peptide was confirmed using amino acid analysis and ion-desorption mass spectrometry, expected mass 5,425.8, found 5,425.0

NMR spectroscopy

¹H NMR spectra were collected using a Varian Unity 500 spectrometer at 25 °C. Typically a spectral width of 6,000 Hz was used with the transmitter set on the H₂O resonance. Water suppression was accomplished during a 2.0-s relaxation delay between transients using a weak presaturation pulse. All spectra were referenced to the trimethylsilyl resonance of sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) (internal) at 0.00 ppm, zero filled to 64 K, and processed using line broadening of 0.5 or 1.0 Hz. For the dilution experiments, a 110 μ M peptide sample was prepared in 500 μ L D₂O containing 50 mM KCl at pH 7.28 (uncorrected meter reading) and 40 μ M DSS. After collection of each data set, a measured volume of the sample was removed from the NMR tube and replaced by an equal amount of D₂O containing 50 mM KCl at pH 7.28. The sample was thoroughly mixed (vortexed) in the NMR tube and centrifuged to ensure all the liquid was at the bottom of the tube. As each spectrum was acquired, both the number of transients and the receiver gain had to be increased in order to obtain a reasonable signal to noise. Scaling of spectra was accomplished by adjusting the vertical scale of each spectrum such that the (CH₃)₃Si-peak of DSS, which underwent the equivalent dilution as the peptide did, was the same height in all spectra.

Thermal denaturations were done over a temperature range of 10–75 °C at 5 °C intervals. Three peptide samples were utilized; each comprised of 1.7, 0.5, and 0.1 mM hs1bI in 500 μ L D₂O containing 50 mM KCl at pH 7.19. At each temperature, the sample was allowed to equilibrate for at least 30 min before a spectrum was acquired.

CD spectroscopy

A stock solution of 200 μ M hs1bI was prepared in 1.0 mL of 10 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS) containing 50 mM KCl at pH 7.19. Dilution was accomplished using the same 10 mM MOPS, 50 mM KCl solution as the original stock sample was prepared in. CD spectra were measured using a JASCO J-720 spectropolarimeter interfaced with an Epson Equity computer and controlled by JASCO software. Spectra were recorded using calibrated 0.2- and 0.02-cm-pathlength cells at 25 °C using a thermostatted cell holder and circulating water bath.

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