Relative stabilities of synthetic peptide homo- and heterodimeric troponin-C domains

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Abstract

It has previously been shown that synthetic peptides corresponding to calcium-binding sites III (SCIII) and IV (SCIV) from troponin-C can undergo a calcium-induced dimerization to form the respective homodimers (Shaw GS, Hodges RS, Sykes BD, 1990, *Science* 249:280–283; Shaw GS et al., 1992a, *J Am Chem Soc* 114:6258–6259). In addition, an equimolar mixture of SCIII and SCIV has been shown to form preferentially the SCIII/SCIV heterodimer (Shaw GS et al., 1992a, *J Am Chem Soc* 114:6258–6259). The stabilities of these dimers have been investigated by using ¹H-NMR and circular dichroism spectroscopies to follow temperature- and guanidine hydrochloride (GuHCl)-induced denaturations. It has been found that the most stable species, the SCIII/SCIV heterodimer ($\Delta G_u^{H_2O} = -64.8$ kJ/mol), is about 13 kJ/mol more stable than the least stable species, the SCIV homodimer, while the SCIII homodimer is of intermediate stability. This trend of free energies agrees well with the trend of ΔG^0 values derived from the products of the dissociation constants for calcium binding and peptide association determined from earlier calcium-titration studies. These observations provide evidence that calcium affinity and the association of 2-calcium binding sites are tightly linked. However, it was noted that in all cases ΔG^0 was considerably more negative than $\Delta G_u^{H_2O}$ determined from GuHCl experiments. This difference increased as the stability of the peptide complex increased, providing evidence that linear extrapolation of GuHCl data for very stable proteins may significantly underestimate the value for ΔG^0 .

Keywords: calcium-binding sites; denaturation; synthetic peptides; troponin-C

A common structural motif among many calcium-binding proteins, including troponin-C (TnC), parvalbumin, recoverin, and calmodulin, is the “EF-hand” or helix-loop-helix motif where a contiguous stretch of about 30 amino acids forms each calcium-binding site. As the name implies, the helix-loop-helix calcium-binding site is comprised of a central 12-residue loop, where calcium is coordinated, and 2 flanking helices (Kretsinger & Nockolds, 1973). One observation from sequence analyses of helix-loop-helix calcium-binding proteins (Marsden et al., 1989) is that these sites usually occur in pairs, and up to 8 copies of this motif may exist in a particular protein. This has been confirmed from X-ray crystallographic studies of several of the helix-loop-helix calcium-binding proteins, which have shown that the 2 sites in parvalbumin (Kretsinger & Nockolds, 1973;

Declercq et al., 1988; Swain et al., 1989), oncomodulin (Ahmed et al., 1990), and calbindin D_{9k} (Szebenyi et al., 1986) each form a 2-site domain, whereas the 4 calcium-binding sites in TnC (Herzberg & James, 1988; Satyshur et al., 1988) and calmodulin (Babu et al., 1988) form 2 independent 2-site domains. An analysis of these X-ray structures reveals several common structural components (Strynadka & James, 1989). Firstly, the 2 calcium-binding sites are pseudosymmetric, having an approximate 2-fold rotational relationship between helix-loop-helix motifs. Secondly, a short 3-residue antiparallel β -sheet is formed between the amino acids at positions 7–9 of each calcium-binding loop. Finally, 2 distinct hydrophobic “pockets” are formed at the interface of the 2-site domain. For example, in the C-terminal domain of TnC, the first hydrophobic center is formed at the interface of the incoming helix (helix E) of site III and the outgoing helix (helix H) of site IV. The second is formed at the interface of the outgoing helix (helix F) of site III and the incoming helix (helix G) of site IV.

These common structural features undoubtedly result in the considerable stabilities of these 2-site domains. For example, parvalbumin (Williams et al., 1986; Cox et al., 1990) and the C-terminal domain of TnC (Tsalkova & Privalov, 1980, 1985) are all stable at temperatures in excess of 90 °C in the calcium-

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bound form. Conversely, in the apo states these proteins are significantly less stable and are unfolded completely at much lower temperatures. A corollary of this observation is that while the folded forms of these proteins are all similar in the presence of calcium, the structures of the apo forms can be markedly different. For example, the C-terminal domain of TnC has little or no regular structure in the absence of calcium (Drabikowski et al., 1982), whereas both apo-parvalbumin (Williams et al., 1986) and apo-calbindin D_{9k} (Kördel et al., 1989; Skelton et al., 1990) appear to have a similar fold compared to their respective calcium-bound forms.

A novel technique to assess the calcium-binding properties of the helix-loop-helix calcium-binding proteins is to use synthetic peptides that represent a single metal-ion site (Reid et al., 1981; Gariépy et al., 1983, 1985). Recently, we have determined that this approach is particularly applicable for 2-site calcium-binding domains, which can be assembled from a pair of synthetic peptides each comprising a single helix-loop-helix sequence (Shaw et al., 1990). In this manner, it has been possible to form the site III (Shaw et al., 1990, 1991a) and site IV (Kay et al., 1991) homodimers of TnC in the presence of calcium. The 3-dimensional structures for these 2 TnC homodimers have now been determined using NMR spectroscopy (Kay et al., 1991; Shaw et al., 1992b). In each case the 2 peptides comprising the dimer are symmetrically arranged in a head-to-tail fashion, similar in structure to that observed for sites III and IV in the X-ray structures of TnC (Herzberg & James, 1988; Satyshur et al., 1988). It has also been shown by Monera et al. (1992) that hydrophobic interactions between the α -helices are not only responsible for dimerization but also have a strong influence on the calcium affinity of the 2-site domain. More recently it has been observed that a site III/site IV heterodimer from TnC is preferentially formed compared to the individual homodimers (Shaw et al., 1992a, 1992c). These observations have prompted the present work where we have used the site III and site IV homodimers and the site III/site IV heterodimer to investigate the basis for the stability of the C-terminal domain of TnC in the presence of calcium. This has been accomplished by monitoring the chemical unfolding equilibria of each dimer by NMR and circular dichroism spectroscopies.

Results

Peptides SCIII and SCIV each represent a single helix-loop-helix calcium-binding site from chicken skeletal TnC (Fig. 1). In the absence of calcium these peptides or ones very similar in length possess little regular secondary structure as assessed by either CD or ¹H NMR spectroscopy (Reid et al., 1981; Gariépy et al., 1983, 1985; Shaw et al., 1990, 1991a; Monera et al., 1992). In addition, an equimolar mixture of the SCIII and SCIV peptides shows no evidence of any interaction between the 2 peptides. However, in the presence of calcium, the SCIII, SCIV, and SCIII/SCIV peptides bind the metal ion and form a well-folded species (Shaw et al., 1992a). From early studies using CD, it was determined that similar folded peptides contained a high degree of α -helix structure and that this could be used to monitor the degree that the peptide was folded based on the ellipticity minimum at 222 nm (Reid et al., 1981; Drabikowski et al., 1982; Monera et al., 1992).

Although CD spectroscopy provides a useful method to monitor the helix-coil transition in calcium-binding peptides, it yields

Fig. 1. Ribbon diagram of the calcium-saturated form of CTnC adapted from the X-ray structure of TnC (Herzberg & James, 1988) and generated using the program MOLSCRIPT (Kraulis, 1991). The figure shows residues 89–162, which comprise CTnC, residues 93–126, which contain calcium-binding site III and comprise peptide SCIII (shaded), and residues 129–162, which contain calcium-binding site IV and comprise peptide SCIV (shaded).

little information about the 3-dimensional structure of the folded polypeptide. High-resolution NMR spectroscopy has been used to determine that single-site peptides from the C-terminal of TnC form dimers in solution only in the presence of calcium (Shaw et al., 1990, 1991a, 1992a; Kay et al., 1991; Monera et al., 1992). In the case of SCIII, an analysis of calcium titration data and NOE spectra showed that this peptide existed as a symmetric homodimer in solution (Shaw et al., 1990, 1991a). By analogy SCIV was also shown to form a symmetric homodimer although at much higher peptide and calcium concentrations (Shaw et al., 1992a). A site IV peptide fragment from rabbit TnC was also shown to be a homodimer using sedimentation equilibrium and NMR spectroscopy (Kay et al., 1991). In addition, the preferential formation of an SCIII/SCIV heterodimer was determined using NMR spectroscopy, calcium titration experiments, and a comparison with the ¹H-NMR spectrum of a C-terminal domain tryptic fragment (Shaw et al., 1991b, 1992a, 1992b, 1992c).

Mechanism of folding and unfolding

A mechanism for the formation of folded dimers, which involves calcium binding and peptide association, has been proposed based on calcium titration data (Shaw et al., 1991a, 1992c) and is shown below. In the unfolding pathway the calcium-saturated dimer F₂Ca₂ loses a calcium (K₂) to form a 1-calcium dimer, F₂Ca. Dissociation of this dimer (K_{dimer}) can form an

unfolded apo-peptide, U, and the folded 1-calcium monomer, FCa, which is in equilibrium (K_1) with the unfolded apo-peptide and calcium.

Ideally, this folding pathway is most easily studied if it is simplified to a 2-state model, existing between folded dimer (F_2Ca_2) and unfolded monomer (U), as has been done in many other studies (Ho & DeGrado, 1987; Bowie & Sauer, 1989; Hodges et al., 1990; De Francesco et al., 1991; Zhu et al., 1992) using CD spectroscopy to follow the helix-to-coil transition. The complicating factor in the present pathway compared to other simple 2-state pathways is the presence of bound calcium ions. In the above mechanism, originally proposed for SCIII, at least 2 intermediates may exist, FCa and F_2Ca (Shaw et al., 1991a, 1992c). It has previously been found that the unfolding reaction is dependent on peptide concentration (Monera et al., 1992), which rules out the unimolecular reaction in Equation 3 as the one monitored by CD spectroscopy. Also, we have calculated that the calcium dissociation constants for the SCIII, SCIV, and SCIII/SCIV dimers range between 0.002 and 2 mM (Shaw et al., 1991a, 1991b, 1992c). Based on these observations, we used an excess of calcium (20 mM) for CD experiments to minimize the effects that calcium would have on the denaturation experiments and to ensure that the F_2Ca_2 species was populated through the unfolding transition.² This allows the proposed pathway to be simplified to a 2-state mechanism (Equation 4) where only the 2-calcium folded dimer, F_2Ca_2 , and the unfolded monomer, U, are present in significant amounts in the transition zone so that

where

$$K_u = \frac{[U]^2[Ca]^2}{[F_2Ca_2]} = 2P_t[(1 - f_f)^2/f_f][Ca_t - P_t(f_f)]^2 \quad (5)$$

and f_f is the molar fraction of folded peptide, P_t is the total peptide concentration, and K_u is the dissociation constant for the equilibrium. From this generalized form a more specific case can be obtained when $Ca_t \gg P_t$ (Equation 6) as in the CD experiments where Ca_t is at least 100-fold greater than the peptide concentration (P_t). This allows a pseudo-dissociation constant K_{obs} to be calculated (Equation 7) from the molar fraction of folded peptide based on θ_{222} .

$$K_u = 2P_t[(1 - f_f)^2/f_f][Ca_t]^2, \quad (6)$$

² This series of equilibria can also be expressed in a manner that may be easier to envision as a 2-state model between unfolded monomers and folded monomers and dimers by rewriting the above expressions as

For these equilibria, K_1 is identical to that in Equation 3 and $K'_{dimer} = (K_2/K_1)K_{dimer}$. However, this analysis cannot account for the apparent stoichiometry 0.5 Ca:1.0 peptide, which is observed in calcium titrations (Shaw et al., 1991a).

where

$$K_{obs} = \frac{K_u}{[Ca_t]^2} = 2P_t[(1 - f_f)^2/f_f]. \quad (7)$$

In ¹H-NMR experiments of the SCIII/SCIV heterodimer where $Ca_t \gg P_t \gg K_1, K_2, K_{dimer}$, Equation 5 was used to calculate K_u . Equations 5, 6, and 7 are identical to those used previously for simple 2-state unfolding transitions (Ho & DeGrado, 1987; Bowie & Sauer, 1989; Hodges et al., 1990; De Francesco et al., 1991; Zhu et al., 1992) with the exception of terms to account for calcium in the present experiments.

Thermal stability

Unfolding of the calcium-saturated SCIII/SCIV heterodimer was monitored using ¹H-NMR spectroscopy as a function of temperature. In Figure 2, a series of spectra are shown for the thermal unfolding of the SCIII/SCIV heterodimer (169 μ M) in the presence of 448 μ M Ca^{2+} between 5 and 70 °C. This figure shows that at the lower temperatures (5–40 °C) the spectra are all very similar in terms of the positions and intensities of the resonances. However, as the temperature of the sample is increased above 40 °C, resonances characteristic of the folded form of the protein broaden, shift, and lose intensity until eventually most of the resonances from the heterodimer are nearly gone. Because the ¹H resonances marked on Figure 2 correspond to residues throughout the heterodimer, it is clear that this transition is a result of global unfolding of the protein rather than a localized effect. In the final spectrum at 70 °C, most of the resonances, although very broad, are found near the frequencies observed for the apo-peptide mixture at 30 °C (Shaw et al., 1992a).

The unfolding of the SCIII/SCIV heterodimer in the presence of calcium is shown graphically in Figure 3A, where the integrated intensity for the resonances noted in Figure 2 are plotted versus the temperature. Only resonances for the SCIII/SCIV heterodimer, which exhibit a unique frequency from those in the apo-peptide mixture, were used. The molar fraction of folded dimer, f_f , was calculated from

$$f_f = \frac{I_{obs}}{I_f}, \quad (8)$$

where I_f is the integral of a particular ¹H resonance when the peptide is fully dimerized and I_{obs} is the observed integral at a given temperature. Figure 3A shows that little thermal unfolding of the SCIII/SCIV heterodimer occurs between 5 and 40 °C where the fraction of folded dimer is between 90 and 100%. Above 40 °C however, the proportion of dimer steadily decreases until, at 70 °C, only about 28% of the folded dimer is present. These data were fit using a least-squares sigmoidal function that had a midpoint, $T_m = 60$ °C, corresponding to the melting temperature of the SCIII/SCIV heterodimer at 169 μ M. Using the data in the transition range between 30 and 70 °C, a van't Hoff plot of thermal denaturation for the SCIII/SCIV heterodimer was constructed (Fig. 3B) based on the 2-state unfolding model. Over this temperature range the data yielded a linear correlation between $\ln K_u$ and $1/T$. From this graph the slope was used to determine the enthalpy of unfolding. A value of

Fig. 2. Temperature dependence of 2 regions of the 500-MHz ^1H -NMR spectrum of 169 μM SCIII/SCIV heterodimer in D_2O , 50 mM KCl, 30 mM imidazole d_4 , and 448 μM CaCl_2 at pH 7.2. **A:** The aromatic and some of the αCH protons. **B:** The up-field methyl protons in the heterodimer, each plotted as a function of temperature. Assigned resonances for the SCIII/SCIV heterodimer are indicated as: F102 δCH ($\bullet$), F151 δCH (Δ), F154 ϵCH ($\blacktriangledown$), Ile 149 αCH ($\blacksquare$), Y112 αCH ($\blacksquare$), I104 γCH_3 ($\blacktriangle$) and I113 δCH_3 (∇).

$\Delta H_u = 183 \text{ kJ/mol}$ was found, which is in the same range as that obtained for the unfolding of the calcium-saturated TR_2C fragment of TnC (218 kJ/mol; Tsalkova & Privalov, 1985) and calcium-saturated rat α -parvalbumin (183 kJ/mol; Williams et al., 1986).

CD spectra

Representative CD spectra for the SCIII, SCIV, and SCIII/SCIV dimers and the C-terminal domain (residues 89–162) from chicken skeletal troponin-C (CTnC) are shown in Figure 4, each in the presence of excess calcium. The spectra are typical of peptides containing a high proportion of α -helix having minima near 208 nm and 222 nm (Greenfield & Fasman, 1969). A compar-

ison of the ellipticities at 222 nm for the peptide dimers shows that the SCIII homodimer has the greatest minimum ($-14,870$ degrees), the SCIV homodimer has the least ($-11,630$ degrees), and the SCIII/SCIV heterodimer is intermediate ($-12,600$ degrees). This suggests that the SCIII dimer may have more α -helical content than does SCIV. These observations are in agreement with NMR studies, which have determined that 18–20 residues are in an α -helix conformation in each monomer of the SCIII dimer (Shaw et al., 1992b), whereas about 17 residues are in an α -helix conformation in each monomer of the site IV homodimer (Kay et al., 1991). The ellipticity for CTnC was $-15,220$ degrees, slightly larger than that obtained for any of the dimeric peptides, but in good agreement with that measured previously on a tryptic fragment from rabbit skeletal TnC com-

Fig. 3. **A:** Thermal denaturation of the SCIII/SCIV heterodimer derived from the integrals of the ^1H -NMR resonances indicated in Figure 2. The fraction of folded dimer (f_f) is plotted as a function of temperature and was calculated as described in the Materials and methods. The first 3 data points are not shown. The data were iteratively fit to determine $T_m = 60^\circ\text{C}$. **B:** van't Hoff plot of $\ln K_u$ vs. $1/T$ generated from the data between 35 and 70°C in A. Linear regression was used to fit the data over this range ($r^2 = 0.997$) to yield $\Delta H = 183 \text{ kJ mol}^{-1}$. From this value of ΔH and the value of $\Delta G = -68 \text{ kJ mol}^{-1}$ at $T_m = 60^\circ\text{C}$, a value of $\Delta S = 346 \text{ J K}^{-1} \text{ mol}^{-1}$ was calculated.

Fig. 4. CD spectra of the SCIII, SCIV, and SCIII/SCIV dimers and CTnC in 10 mM MOPS, 50 mM KCl, and 20 mM CaCl_2 in the absence of GuHCl (—) and in the presence of GuHCl (---) at 25 °C and either pH 7.20 (dimers) or pH 7.15 (CTnC). The spectra shown are (a) 189 μM SCIII, (b) 188 μM SCIV, (c) 190 μM SCIII/SCIV, (d) 82 μM CTnC, (e) 189 μM SCIII + 5 M GuHCl, (f) 188 μM SCIV + 5 M GuHCl, (g) 190 μM SCIII/SCIV + 7.33 M GuHCl, and (h) 82 μM CTnC + 6.6 M GuHCl + 5 mM EGTA.

prising residues 89–159 (Leavis et al., 1978). This expected result arises from the α -helical nature of several residues in CTnC that have little or no regular secondary structure in the peptide dimers. This is especially true for the C-terminal end of site III (toward Gly 126) and N-terminal end of site IV (toward Val 129, Fig. 1), which are connected via a short linker in CTnC absent in the peptide dimers. Based on the X-ray structure of TnC (Herzberg & James, 1988; Satyshur et al., 1988), this region contains a significant amount of α -helix (about 4 of 11 residues between residues 122 and 132), whereas the peptide dimers are relatively unstructured in this region (Kay et al., 1991; Shaw et al., 1992b).

In the presence of the denaturing agent guanidine hydrochloride (GuHCl), the CD spectra (Fig. 4) of the peptides and CTnC show a significant decrease in magnitude in θ_{222} , suggesting that a significant loss in α -helix structure has occurred. At high concentrations of GuHCl, θ_{222} reaches a maximum (avg. $-2,430 \pm 440$ degrees) for the 3 peptides. This value is significantly smaller than that measured by Monera et al. (1992) for a site III peptide in benign conditions and in the absence of calcium ($-6,300$ degrees), which has been suggested to be mostly random-coil structure and is not a dimer. Thus, it appears that GuHCl is an effective agent in dissociating the peptide dimers and fully denaturing them. In contrast, denaturation of CTnC in the presence of calcium was markedly different, and full denaturation could not be obtained in the maximum concentration of GuHCl used (7.8 M). However, in the presence of 5 mM EGTA, CTnC could be denatured at GuHCl concentrations greater than 6 M. A similar result has been noted in calorimetric studies of TnC where it was found that the T_m for TnC or the C-terminal domain shifted to lower temperatures as the EGTA:Ca ratio was increased. In Figure 4 the CD spectrum of denatured CTnC is shown as determined in the presence of 6.6 M GuHCl and 5 mM EGTA. As with the peptide dimers, denatured CTnC shows a significant decrease in negative ellipticity at 222 nm, suggesting a loss in α -helix structure.

Dimer unfolding by GuHCl

Denaturation curves for the calcium-saturated SCIII, SCIV, and SCIII/SCIV dimers and CTnC are shown in Figure 5 as the fraction of folded peptide or protein, f_f , at a given GuHCl concentration. Two concentrations of each peptide were used: approximately 190 μM and 19 μM for the SCIII and SCIV homodimers and 190 μM and 9.6 μM for the SCIII/SCIV heterodimer. Each pair of curves for each dimer shows a smooth sigmoidal transition (monophasic) characteristic of a 2-state unfolding process and a strong dependence on peptide concentration, characteristic of dimerization. This observation agrees well with the ^1H -NMR data in this work and earlier studies (Shaw et al., 1991a, 1991b, 1992c), which showed that these peptides were dimers at similar concentrations. In the cases of the SCIII and SCIII/SCIV dimers, a plateau near unity for fraction folded (f_f) is obtained in the absence of GuHCl and at the lowest peptide concentrations studied (18.9 and 9.6 μM , respectively). These observations indicate that only folded dimers are present at the outset of the denaturations. In contrast to the denaturation curves for the SCIII homodimer and SCIII/SCIV heterodimer, the fraction folded for the SCIV homodimer does not reach unity nor have a plateau at the outset of the denaturations. This suggests that SCIV was not completely dimerized at either concentration studied. From extrapolation of the 2 curves it was calculated that SCIV was about 93% dimer at the highest concentration studied (188 μM) and only 80% dimer at the lower peptide concentration (18.8 μM) with no GuHCl added. These observations are in agreement with sedimentation equilibrium studies of Kay et al. (1991), where it was found that a similar site IV peptide from TnC was only partly dimerized below concentrations of about 700 μM .

In the case of calcium-saturated CTnC, GuHCl alone could not fully denature the protein at the highest concentration studied (7.8 M), although it is clear from Figure 5D that the protein was beginning to unfold at GuHCl concentrations greater than 7 M. The curves in Figure 5 for the peptide dimers show a strong concentration dependence for each peptide at constant calcium concentration suggesting that the unfolding reaction is bimolecular as proposed with the 2-state model, involving a monomer-dimer equilibrium. One method to assess this dependence is to determine the difference in concentrations of GuHCl for 50% denaturation ($\text{GuHCl}_{1/2}$). In all 3 dimers, the $\text{GuHCl}_{1/2}$ is between 0.7 and 4.8 M GuHCl (Fig. 5). Although the values for the SCIV homodimer are low, the results for the SCIII homodimer and the SCIII/SCIV heterodimer are comparable to the $\text{GuHCl}_{1/2}$ values for other dimeric proteins at similar concentrations (Hodges et al., 1990; De Francesco et al., 1991; Zhu et al., 1992). Values for $\text{GuHCl}_{1/2}$ can also be used to give an idea of the relative stabilities of the dimers. In particular, a comparison of the $\text{GuHCl}_{1/2}$ values of the highest concentration samples reveals a $\text{GuHCl}_{1/2} = 4.8$ M is obtained for the SCIII/SCIV heterodimer, whereas significantly smaller values for $\text{GuHCl}_{1/2}$ are found for the SCIII (3.1 M) and SCIV (1.3 M) homodimers. Interestingly, decreasing the concentration of the SCIII/SCIV heterodimer by 120-fold resulted in a decrease of $\text{GuHCl}_{1/2}$ to 3.3 M, which is still a greater amount of GuHCl than was required to 50% denature the highest concentration of SCIII assessed. A further observation is that the $\text{GuHCl}_{1/2}$ values for the SCIII and SCIV homodimers studied at 18.8 μM are very similar to those reported for homodimers from cal-

Fig. 5. Guanidine denaturation curves for the SCIII, SCIV, and SCIII/SCIV dimers and CTnC in 10 mM MOPS, 50 mM KCl, and 20 mM CaCl₂. Each curve is plotted as the fraction of folded (f_f) vs. [GuHCl] for (A) 188 μ M SCIV (○) and 18.8 μ M SCIV (●), (B) 189 μ M SCIII (○) and 18.9 μ M SCIII (●), (C) 190 μ M (SCIII + SCIV) (○) and 9.6 μ M (SCIII + SCIV) (●), and (D) 82 μ M CTnC. The amount of folded dimer was determined from the magnitude of θ_{222} using $f_f = (\theta_{\text{obs}} - \theta_{\text{min}})/(\theta_{\text{max}} - \theta_{\text{min}})$. Each curve was iteratively fit using a sigmoidal function to yield the best fit lines shown. This process also calculated the [GuHCl]_{1/2} values for the dimers at high and low peptide concentrations, respectively, which are (A) 1.3 and 0.7 M for SCIV, (B) 3.1 and 2.4 M for SCIII, (C) 4.8 and 3.3 M for SCIII/SCIV, and (D) 8.4 M for CTnC.

bindin D_{9k} (F1 and F2, 3 M and 1.5 M, respectively) when studied at approximately 15 μ M (Linse et al., 1993). Qualitatively, this suggests that in the presence of calcium the SCIII homodimer from TnC is similar in stability to the F1 homodimer from calbindin D_{9k} and the SCIV and F2 homodimers are of similar stabilities. However, the dimers all have significantly lower values for GuHCl_{1/2} (at the concentrations measured) than CTnC (GuHCl_{1/2} = 8.4 M, estimated from curve-fitting of the available data, Fig. 5D).

The observations made in Figure 5 indicate that each of the dimers have different stabilities toward GuHCl denaturation with the heterodimer SCIII/SCIV being the most stable, the homodimer SCIV the least stable, and the homodimer SCIII of intermediate stability. This is shown in a more quantitative manner in Figure 6, where the free energy of unfolding ΔG_u (where $\Delta G_u = -RT \ln K_u = -RT \ln K_{\text{obs}} - RT \ln [\text{Ca}_i]^2$) is plotted against the GuHCl concentration for the data points, which were in the transition zone ($0.20 \leq f_f \leq 0.80$) in Figure 5. These data were least-squares fit according to the equation:

$$\Delta G_u = \Delta G_u^{\text{H}_2\text{O}} + m[\text{GuHCl}], \quad (9)$$

where $\Delta G_u^{\text{H}_2\text{O}}$ is the unfolding free energy in the absence of denaturant (Pace, 1975), which is extrapolated from Figure 6 (Table 1). The graphs show that for each peptide dimer the denaturation data for the 2 concentrations of each peptide lie on the same curve (within experimental error). Again, this observation is consistent with the initial 2-state proposal. The

slopes of the lines (Table 1), which can in some cases be used to assess the degree of cooperativity of unfolding, are also similar having a range of -5 to -7.9 kJ/mol M. These values are very similar to the slopes obtained for the GuHCl denaturation of several coiled-coil dimers (De Francesco et al., 1991; Zhu et al., 1992) and tetramers (Ho & DeGrado, 1987). Interestingly,

Table 1. Free energies of unfolding for dimers and C-terminal domain of TnC

Dimer	Dissociation constant ($\times 10^6$ M)			ΔG^0 ^a (kJ/mol)	$-\Delta G_u^{\text{H}_2\text{O}}$ ^b (kJ/mol)	m
	K_1	K_2	K_{dimer}			
SCIV ₂	260	260	2,000	-56.3	-51.8	-7.9
SCIII ₂	3	2,000	10	-75.4	-61.5	-6.5
SCIII/SCIV	3	2	10	-92.5	-64.8	-5.0
CTnC ^c	0.02	0.02	—	-87.8	—	—

^a ΔG^0 was calculated from $\Delta G^0 = -RT \ln K_{\text{tot}}$, where K_{tot} is the product of the individual dissociation constants. For each of the dimers, $K_{\text{tot}} = K_1 K_2 K_{\text{dimer}}$; for CTnC, $K_{\text{tot}} = K_1 K_2$.

^b $\Delta G_u^{\text{H}_2\text{O}}$ is the free energy based on the equilibrium constant K_u according to Equation 6, where $[\text{Ca}_i] = 20$ mM. Values are quoted as $-\Delta G_u^{\text{H}_2\text{O}}$ to clarify comparison to ΔG^0 from calcium-binding studies.

^c Calcium dissociation constants are from Leavis et al. (1978) for the C-terminal domain of rabbit TnC.

Fig. 6. Free energies of unfolding (ΔG_u) for the (A) SCIV, (B) SCIII, and (C) SCIII/SCIV dimers. Symbols refer to concentrations described in Figure 5. Values for ΔG_u were calculated from the fraction folded (f_f) shown in Figure 5 using Equation 6. The slopes for each curve are (A) -7.9 kJ/mol M for SCIV, (B) -6.5 kJ/mol M for SCIII, and (C) -5.0 kJ/mol M for SCIII/SCIV.

the slopes of the curves from Figure 6 are greater for the homodimers SCIII and SCIV than for the SCIII/SCIV heterodimer. This observation seems to be consistent with the slopes found for the denaturation of several synthetic peptide dimers where the heterodimer usually has a smaller value for the slope than the average of the 2 possible homodimers (Zhu et al., 1992).

Discussion

Synthetic peptides have been used previously to study calcium-induced folding of the helix-loop-helix motif and isolated loop motifs in calcium-binding proteins (Reid et al., 1981; Gariépy et al., 1983, 1985; Marsden et al., 1988; Shaw et al., 1990, 1991a; Monera et al., 1992). These studies have been successful in monitoring conformational changes that occur during calcium binding and in determining the effects that specific residues have on metal ion affinity. However, 1 important area where information is lacking is in the dependency that 1 helix-loop-helix has on its partner in a 2-site domain and how this contributes to the stability of the domain. The determination that helix-loop-helix peptides form a calcium-induced 2-site domain has now opened up this avenue of investigation (Shaw et al., 1991b; Monera et al., 1992). Recently, we have studied the effects that specific hydrophobic residues have on both the formation and the calcium affinity of the 2-site domain formed from 2 site III (SCIII) peptides (Monera et al., 1992). In the present study, we have extended this work to study the stabilities of the SCIII and SCIV homodimers and SCIII/SCIV heterodimer and compared these to the stability for the C-terminal domain of TnC.

Thermodynamics of heterodimer formation

Previously, we have shown that calcium titration studies of a mixture of SCIII and SCIV results in the formation of only the SCIII/SCIV heterodimer (Shaw et al., 1992a). The GuHCl denaturation studies in this work provide a rationale for this. A summary of the free energies of unfolding from these studies is shown in Table 1. From these data the SCIII/SCIV heterodimer exhibits a free energy of dimerization of about -65 kJ/mol, making it about 3 kJ/mol more stable than the SCIII homodimer and 13 kJ/mol more stable than the SCIV homo-

dimer. These differences in energy between dimers could result from differences between the energies of the folded states, the unfolded states, or both. Because the unfolded states of all 3 dimers are random coil, whereas the folded dimers appear to have varying degrees of α -helix (from CD spectroscopy), it is probable that the differences in energy arise mostly from differences in the folded states. This is similar to that suggested for synthetic Zn-binding 4-helix bundle proteins (Handel et al., 1993). Further, all 3 dimers can be formed at 25°C (the temperature of present experiments) and their formation is reversible. This analysis suggests that, on the basis of the $\Delta G_u^{\text{H}_2\text{O}}$ values calculated here, one might expect nearly identical amounts of the SCIII/SCIV heterodimer and SCIII homodimer to be formed and a lesser amount of the SCIV homodimer be formed from an equimolar mixture of SCIII and SCIV peptides under thermodynamic control. However, the formation of the SCIII dimer also necessitates the formation of the SCIV dimer according to the thermodynamic equilibrium

so that the SCIII/SCIV heterodimer will be preferentially formed when $\Delta G_u^{\text{H}_2\text{O}}$ (SCIII/SCIV) $< 1/2[\Delta G_u^{\text{H}_2\text{O}}$ (SCIII₂) + $\Delta G_u^{\text{H}_2\text{O}}$ (SCIV₂)] + $RT \ln 2$. From the data in Table 1, it is clear that the free energy of folding for the SCIII/SCIV heterodimer (-65 kJ/mol) is significantly larger than $1/2[\Delta G_u^{\text{H}_2\text{O}}$ (SCIII₂) + $\Delta G_u^{\text{H}_2\text{O}}$ (SCIV₂)] + $RT \ln 2$ (-58 kJ/mol). This difference offers a thermodynamic rationale for the presence of heterogenic 2-site domains in calcium-binding proteins in favor of the homogeneous sites from which they no doubt evolved (Kretsinger, 1987). It is also noteworthy that this observation is similar to those found for preferential formation of $\alpha\beta$ tropomyosin (Lehrer et al., 1989; Lehrer & Qian, 1990), the Fos-p1N-Jun-p1N heterodimers (O'Shea et al., 1988, 1989), and in synthetic coiled-coil proteins (Zhu et al., 1992).

One interesting comparison that can be made from this work is that between the stability of the SCIII/SCIV heterodimer and CTnC. From Table 1, the ΔG^0 values for these 2 complexes are comparable, suggesting that on this basis the 2 proteins are also of comparable stability. Because of the inaccuracies of the $\Delta G_u^{\text{H}_2\text{O}}$ value for CTnC (and SCIII/SCIV, see below), we have

chosen to compare the $\text{GuHCl}_{1/2}$ values for these 2 complexes. This approach is not straightforward because $\text{GuHCl}_{1/2}$ increases as the concentration of SCIII/SCIV increases, whereas $\text{GuHCl}_{1/2}$ for CTnC is concentration independent. However, a comparison can be made if a GuHCl denaturation curve for the SCIII/SCIV heterodimer is calculated using the ΔG_u values from the curves shown in Figure 6C for the SCIII/SCIV heterodimer at a total peptide concentration of 1 M (standard-state conditions). When this was done for the SCIII/SCIV heterodimer, it was calculated that the peptide dimer would still be 93% folded even at 8 M GuHCl , making it comparable in stability to CTnC.

Comparison of $\Delta G_u^{\text{H}_2\text{O}}$ with ΔG^0 from calcium dissociation constants

The free energies of folding for the 3 dimers ($\Delta G_u^{\text{H}_2\text{O}}$) were compared to the free energies calculated from the product of the 3 dissociation constants for the proposed formation of the peptide dimers (ΔG^0 , Equations 1–3) and also to published dissociation constants for the C-terminal domain of TnC. In the case of the C-terminal domain of TnC, ΔG^0 has also been measured calorimetrically for the binding of 2 calcium ions (Potter & Gergely, 1975; Yamada, 1978; Yamada & Kometani, 1982; Imaizumi et al., 1987). Although studies for rabbit TnC are not so clear, a $\Delta G^0 \approx -85$ kJ/mol was obtained for bullfrog TnC (Imaizumi et al., 1987), which is in excellent agreement with the ΔG^0 derived from dissociation constants (Table 1). Similar observations are also available for several other calcium-binding proteins (for example see Cox et al., 1990).

Denaturation studies that monitor the stability of calcium-binding proteins in the calcium form are rare. Urea denaturations have been reported for both TnC (McCubbin et al., 1982) and calmodulin (Martin & Bayley, 1986), although complete unfolding of the proteins was not accomplished. Several studies have also been reported for calbindin D_{9k} , but in the calcium-free state. This lack of data likely stems from the tremendous stability of these proteins in the calcium form. As shown in Table 1, it is clear from the large negative ΔG^0 values, from calcium-binding experiments, that all 3 dimers and CTnC fit this trend. However, in all cases $\Delta G_u^{\text{H}_2\text{O}}$ is significantly smaller than ΔG^0 . It is also apparent from these data that the difference between $\Delta G_u^{\text{H}_2\text{O}}$ and ΔG^0 is proportional to the stability of the complex studied. Thus, for the least stable dimeric species, the SCIV homodimer, this difference is -4.5 kJ/mol versus -27.7 kJ/mol for the SCIII/SCIV heterodimer. It is also likely that a similar situation exists for CTnC, although a good estimate of $\Delta G_u^{\text{H}_2\text{O}}$ could not be obtained from GuHCl experiments. One possible source for these observations is the curve-fitting method used to estimate $\Delta G_u^{\text{H}_2\text{O}}$, of which linear extrapolation is the most common and was the choice in this work. However, other methods, such as a quadratic dependence on GuHCl (Wendt et al., 1988) and the GuHCl -binding model (Pace, 1975), are available and generally yield a higher value for $\Delta G_u^{\text{H}_2\text{O}}$. Using either of these models resulted in $\Delta G_u^{\text{H}_2\text{O}} = -52.5$ kJ/mol for the SCIV homodimer, which is essentially the same value obtained from linear extrapolation. However, for the SCIII/SCIV heterodimer, this is not the case and a value of $\Delta G_u^{\text{H}_2\text{O}} = -73.0$ kJ/mol is obtained from hyperbolic fitting and -81.6 kJ/mol is obtained from the GuHCl -binding model. These values are considerably more negative than from linear

extrapolation (-64.8 kJ/mol) and are now approaching the value obtained of $\Delta G^0 = -92.5$ kJ/mol from calcium-binding experiments. It has been suggested that the linear extrapolation method probably yields a $\Delta G_u^{\text{H}_2\text{O}}$, which is too low and a method such as the GuHCl -binding model yields values of $\Delta G_u^{\text{H}_2\text{O}}$, which are too high (Pace, 1975). However, the results in this work may suggest that, for very stable proteins and peptide complexes, a nonlinear curve-fitting routine may be more appropriate to obtain a better estimate of ΔG^0 .

Cooperativity of folding and calcium binding

From earlier calcium-binding studies it was found that all 3 peptides bound calcium with a range of dissociation constants and formed dimers. It has also been suggested that these 2 processes are tightly linked (Shaw et al., 1991b, 1992c; Monera et al., 1992). From the guanidine denaturation studies here, sedimentation-equilibrium studies (Kay et al., 1991), and calcium-binding studies, it is clear that SCIV not only has the highest calcium dissociation constant (K_1) but also forms a dimer at significantly higher concentrations than either the SCIII homodimer or SCIII/SCIV heterodimer. However, in the cases of the SCIII and SCIII/SCIV dimers, both of these species formed dimers in the presence of calcium at the lowest concentrations tested here ($19 \mu\text{M}$ and $9.6 \mu\text{M}$, respectively) suggesting that the dimer dissociation constant, K_d , must at least be in this range. This finding is in agreement with the calculated values from calcium-titration data (Table 1). When linking these dissociation constants to the calcium-dissociation constants for the SCIII homodimer and the SCIII/SCIV heterodimer, one finds that K_1 (binding of calcium to SCIII) is 3.0×10^{-6} M and reflects relatively tight calcium binding. This value is about 1,000-fold tighter than binding to SCIV and shows that calcium binding to the SCIII/SCIV heterodimer and to the C-terminal of TnC must be sequential and that the initial step must be calcium binding to site III only. However, it is the second calcium binding event (K_2) upon which dimer formation has the greatest impact. From Table 1, it is noted that in the SCIII homodimer $K_2 \gg K_1$, whereas in the SCIII/SCIV heterodimer $K_2 \approx K_1$. This suggests that in SCIII, dimer formation perturbs binding of a second molecule of calcium, whereas in the SCIII/SCIV heterodimer, calcium, binding is enhanced so that calcium binding to the SCIV portion of the heterodimer is increased about 100-fold compared to that in the SCIV homodimer.

Materials and methods

Peptide synthesis and purification

Peptides comprising site III (residues 93–126; SCIII) and site IV (residues 129–162; SCIV) from chicken skeletal TnC were synthesized according to the procedures of Shaw et al. (1991b) and Monera et al. (1992) using standard t-boc chemistry on an Applied Biosystems model 430A peptide synthesizer. Crude peptides were purified using reversed-phase HPLC, and the amino acid composition of the purified peptides was confirmed using amino acid analysis and mass spectrometry.

NMR spectroscopy

^1H -NMR spectra were collected using a Varian VXR-500 spectrometer. Peptide samples were prepared in $500 \mu\text{L}$ D_2O buffer

containing 30 mM imidazole- d_4 and 50 mM KCl at pH 7.30 (uncorrected meter reading). The SCIII/SCIV peptide solution was prepared from individual SCIII and SCIV peptide solutions to give a sample that contained $179 \pm 8 \mu\text{M}$ SCIII and $159 \pm 13 \mu\text{M}$ SCIV as measured by quantitative amino acid analysis (see below). Because these concentrations are the same within experimental error, an average peptide concentration of $169 \mu\text{M}$ was used.

Thermal denaturations were done over a temperature range of 5–70 °C at 5 °C intervals. At each temperature the sample was allowed to equilibrate for at least 30 min before a spectrum was acquired.

CD spectroscopy

Individual stock solutions of 1.13 mM SCIII and 1.18 mM SCIV were prepared in 1.0 mL of 10 mM 3-(*N*-morpholino)propane-sulfonic acid (MOPS) containing 50 mM KCl and 20 mM CaCl_2 at pH 7.20. A stock solution of 0.49 mM recombinant C-terminal domain (residues 89–162) from chicken skeletal troponin-C was prepared in 0.5 mL of the same buffer including 5 mM dithiothreitol (DTT) at pH 7.15. The concentrations of these peptide and protein solutions were then determined from triplicate amino acid analyses using a Beckman Model 6300 amino acid analyzer equipped with a 25-cm ion-exchange column and post-column ninhydrin detection. For SCIII and CTnC, quantitative measurement was made by comparing the peak volumes for alanine and leucine to those of a 10-nmol standard sample containing these amino acids or an internal 3-nmol norleucine standard. For SCIV, a similar procedure was used for leucine residues only because SCIV contains no alanine residues. For the SCIII/SCIV solution, the appropriate volumes from 1.30 mM SCIII and 0.93 mM SCIV solutions were combined to yield an equimolar SCIII/SCIV stock solution containing 1.15 mM total peptide.

The CD spectra of SCIII, SCIV, and SCIII/SCIV were each determined at 2 different concentrations by diluting each of the stock solutions by 6-fold and 60-fold for SCIII and SCIV and 6-fold and 120-fold for SCIII/SCIV. CD spectra for the CTnC sample were acquired using a 6-fold dilution of the stock sample. Dilution was accomplished using the same 10 mM MOPS, 50 mM KCl, and 20 mM CaCl_2 buffer as the original stock samples were 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 path length cells at 25 °C using a thermostatted cell holder and circulating water bath.

Denaturation of the peptide dimers was done by incubating aliquots of the peptide solutions at several concentrations of GuHCl. Stock 6 M and 8 M GuHCl solutions were prepared in 10 mM MOPS, 50 mM KCl, and 20 mM CaCl_2 buffer and used for all additions. All incubations were left to stand overnight to ensure the samples had equilibrated prior to the acquisition of the individual CD spectra.

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