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Role of interchain α-helical hydrophobic interactions in Ca^{2+} affinity, formation, and stability of a two-site domain in troponin C

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Abstract

We have previously shown that a 34-residue synthetic peptide representing the calcium-binding site III of troponin C formed a symmetric two-site dimer consisting of two helix-loop-helix motifs arranged in a head-to-tail fashion (Shaw, G.S., Hodges, R.S., & Sykes, B.D., 1990, *Science 249*, 280-283). In this study the hydrophobicities of the α-helices were altered by replacing L-98 and F-102 in the N-terminal region and/or I-121 and L-122 in the C-terminal region with alanine residues. Our results showed that substitution of hydrophobic residues either in the N- or C-terminal region have little effect on α-helix formation but resulted in a 100- and 300-fold decrease in Ca^{2+} affinity, respectively. Simultaneous substitution of both hydrophobes in the N- and C-terminal region resulted in a 1,000-fold decrease in Ca^{2+} affinity. Data from guanidine hydrochloride denaturation studies suggested that intermolecular interactions occur and that the less hydrophobic analogs had a lower overall conformational stability. These data support the contention that the hydrophobic residues are important in the formation of the two-site domain in troponin C, and this hydrophobic association stabilizes Ca^{2+} affinity.

Keywords: α-helices; calcium affinity; calcium-binding proteins; dimerization; helix-loop-helix; hydrophobic interactions

A group of calcium-binding proteins including troponin C (TnC), calmodulin, parvalbumin, and more than 170 others known to date (Marsden et al., 1990; Heizmann & Hunziker, 1991) exhibit a common structural motif known as an "EF hand." The EF hand has a very high selectivity and affinity for calcium, and in the calcium-bound form consists of a 12-residue Ca^{2+}-binding loop flanked by two α-helices. There may be up to eight copies of this helix-loop-helix structure in a particular protein.

The calcium affinity of the helix-loop-helix structure or EF hand in TnC is affected by several factors that can be grouped under two broad categories. The first is the direct effect of the amino acid sequence in the Ca^{2+}-binding loop. Studies of intact proteins (Beckingham, 1991; Haiech et al., 1991; reviews by Reid & Hodges, 1980; Marsden et al., 1990; daSilva & Reinach, 1991) and synthetic peptides representing single Ca^{2+}-binding sites (Marsden et al., 1988) showed drastic differences in Ca^{2+} affinity when a single amino acid residue in the loop was replaced, especially those that are involved in coordinating the Ca^{2+} ion. The effect of the amino acid sequence in the loop is also reflected in the differences in calcium affinities of analogous Ca^{2+}-binding proteins from different species (Marsden et al., 1990).

Secondly, there are long-range interactions between sites and between domains that increase or stabilize the affinity of the Ca^{2+}-binding site. In native proteins, the EF hands are usually arranged such that two Ca^{2+}-binding sites are in contact with each other forming two-site domains (Sekharudu & Sundaralingam, 1988). Examples are sites I and II and sites III and IV in TnC (Kinemage 1; Herzberg & James, 1985; Satyshur et al., 1988) and calmodulin (Babu et al., 1985, 1988; Kretsinger et al., 1986) as well as the two calcium-binding sites in parvalbumin (Kretsinger & Nockolds, 1973). The association

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between two adjacent sites can be so strong that site-directed mutation of site III of calmodulin, for example, almost totally prevented Ca^{2+} binding in site IV, and vice versa (Beckingham, 1991). Even structural changes seemingly far removed from the individual Ca^{2+}-binding sites can have a direct effect on Ca^{2+} binding. The Ca^{2+}-binding ability of a mutant calmodulin was restored in the presence of a calmodulin-binding peptide (Haiech et al., 1991). Calcium binding of skeletal troponin C was reduced when either Glu-57 or Glu-88, which are both away from the Ca^{2+}-binding loop, were substituted with lysine (Fujimori et al., 1990). Also, the regulatory activity of sTnC was altered by introduction of a disulfide bridge between cysteine residues located in the segment connecting helix B with C and the central helix, respectively, in the N-terminal domain (Grabarek et al., 1990) or by mutations in the central helix (Dobrowolski et al., 1991; Sheng et al., 1991). These long-range conformational changes are believed to be involved in regulation of Ca^{2+} binding to TnC.

To study the details of the interactions between two adjacent sites in intact proteins is not an easy task. As an alternative Hodges and coworkers were the first to demonstrate the usefulness of synthetic peptides, representing a single Ca^{2+}-binding site (site III of TnC), in understanding the specificity and affinity of the Ca^{2+}-binding loop as it relates to the calcium-binding mechanism in intact proteins. For example, they showed that a synthetic 34-residue single Ca^{2+}-binding site had high Ca^{2+} affinity ($K_{Ca} \cong 10^5\ M^{-1}$) (Reid et al., 1981) but analogs with shorter N-terminal α-helices had significantly lower Ca^{2+} affinity (Reid et al., 1980, 1981). They noted that whereas the Ca^{2+}-inducible region is in the N-terminal α-helix, the C-terminal α-helix makes a significant contribution to the overall stabilization of the Ca^{2+}-binding loop (Reid et al., 1981; Gariépy et al., 1982). They also demonstrated that the structure of the synthetic Ca^{2+}-binding loop is similar to that of the native protein (Marsden et al., 1989), and its selectivity is a function of not only the amino acid sequence and net charge of the loop (Marsden et al., 1988) but also the nonliganding residues (Shaw et al., 1991b) and the size and charge of the metal ion (Gariépy et al., 1983, 1985).

Recently, a synthetic 34-residue peptide representing the calcium-binding site III of TnC was shown to form a symmetric two-site dimer in a head-to-tail arrangement in the presence of Ca^{2+} (Shaw et al., 1990, 1991a). As well, the structure of a 39-amino acid residue proteolytic fragment of rabbit skeletal TnC containing calcium-binding site IV has been determined by NMR methods to consist of a dimer of helix–loop–helix motifs related by a twofold axis of symmetry (Kay et al., 1991). When a mixture of synthetic 34-residue peptides representing calcium-binding site III and site IV of TnC was titrated with Ca^{2+}, a heterodimer was preferentially formed in a similar fashion (Shaw et al., 1992a), where it was suggested that the first Ca^{2+} ion bound to the site III peptide (Shaw et al., 1992b). In these dimers, two-dimensional ^1H NMR data showed extensive hydrophobic interactions between complementary α-helices in the N- and C-terminal regions. These interactions are believed to be very similar, if not identical, to the hydrophobic interactions between the α-helices of Ca^{2+}-binding sites III and IV in the carboxy-terminal of TnC. We have used this synthetic peptide approach to alter the hydrophobic residues of the N- and C-terminal α-helices of Ca^{2+}-binding site III to demonstrate the effects of hydrophobic residues in the α-helices on Ca^{2+} affinity and on the formation and stability of the two-site domain.

Results and discussion

The use of synthetic peptide analogs to mimic the helix–loop–helix Ca^{2+}-binding unit of skeletal TnC (Reid et al., 1980, 1981) has made significant contributions to our understanding of Ca^{2+}-induced protein folding. This same technique is used in this study to understand further how protein folding and association promote the stability of a Ca^{2+}-binding site. We have synthesized four peptide analogs of Ca^{2+}-binding site III of chicken skeletal TnC with altered hydrophobicities in the α-helices on either side of the Ca^{2+}-binding loop in order to study the effect of hydrophobic interactions on dimer formation and on Ca^{2+} affinity of the Ca^{2+}-binding site (Table 1). The specific pairs of hydrophobic amino acid residues that were selected for substitution were those that were involved in intra- and intermolecular hydrophobic interactions in the dimer (L-98 and F-102 in the N-terminal α-helix and I-121 and L-122 in the C-terminal α-helix) (Shaw et al., 1990) but far enough away from the Ca^{2+}-binding loop not to directly affect the coordination of calcium.

CD spectra

Figure 1 shows that the TnC analogs have different CD spectra, especially in the presence of either Ca^{2+} or trifluoroethanol (TFE). The mimima around 208 and 222 nm are typical of structures containing α-helices (Greenfield & Fasman, 1969) and $[\Theta]_{222}$ was used to calculate the percentage of α-helical structures (Chen et al., 1974; Nagy et al., 1978; Reid et al., 1981). In the absence of Ca^{2+}, there was only a small amount of α-helical structure in all the analogs (Table 2), and the minimum near 200 nm (Greenfield & Fasman, 1969) suggests that the majority of the structure is random coil, as previously observed from ^1H NMR data (Shaw et al., 1990). Removal of the hydrophobes in the N-terminal helix by substitution of both L-98 and F-102 with alanine (AAIL) did not have any effect on the original α-helicity in the absence or presence of Ca^{2+}. However, in the presence of 50% TFE there was a slight increase (18% or three residues) in the α-helicity of AAIL relative to that of the native sequence

Table 1. *Sequence of peptide analogs used in this study compared to chicken skeletal muscle troponin C site III*[a]

Name	Sequence
	Ca^{2+} binding loop
	Helix — Helix
	92 98 102 110 121 125
Site (III)	-G-K-S-E-E-E-L-A-N-C-F-R-I-F-Ⓓ-K-Ⓝ-A-Ⓓ-G-Ⓕ-I-Ⓓ-I-E-Ⓔ-L-G-E-I-L-R-A-T-G-
LFIL	Ac-C-K-S-E-E-E-L-A-N-A-F-R-I-F-D-K-N-A-D-G-Y-I-D-I-E-E-L-G-E-I-L-R-A-C-amide
AAIL	Ac-C-K-S-E-E-E-*A-A-N-A-*A-R-I-F-D-K-N-A-D-G-Y-I-D-I-E-E-L-G-E-I-L-R-A-C-amide
LFAA	Ac-C-K-S-E-E-E-L-A-N-A-F-R-I-F-D-K-N-A-D-G-Y-I-D-I-E-E-L-G-E-*A-*A-R-A-C-amide
AAAA	Ac-C-K-S-E-E-E-*A-A-N-A-*A-R-I-F-D-K-N-A-D-G-Y-I-D-I-E-E-L-G-E-*A-*A-R-A-C-amide

[a] In all peptide analogs Phe-112 was substituted with Tyr, Cys-101 by Ala (Shaw et al., 1990), and Gly-92 and Thr-125 by Cys residues. These substitutions have been shown to have no effect on Ca^{2+}-binding affinity compared to the native sequence and were made for other studies. The nomenclature used for each peptide refers to the hydrophobic residues at positions 98, 102, 121, and 122 in the N- and C-terminal helices. For example, LFIL refers to the native sequence of hydrophobic residues, and AAIL refers to two Ala residues substituted in the N-terminal helix at positions 98 and 102. The asterisks (*) denote positions of Ala substitutions in the four analogs. The calcium coordinating positions are circled.

(LFIL). This is in agreement with previous observations that Ala is a slightly better α-helix former than Leu or Phe (Lyu et al., 1990; O'Neil & DeGrado, 1990).

Removal of the hydrophobes in the C-terminal region by substitution of both I-121 and L-122 with alanine (LFAA) resulted in 50% reduction in the original helicity in the absence of Ca^{2+} (about three residues; Table 2). These three residues appear to be permanently uncoiled because this reduction in molar ellipticity was also observed in the presence of Ca^{2+} or TFE (about 3,700 and 3,300 degrees, respectively). However, the amount of Ca^{2+}- or TFE-inducible α-helix was the same as that of the native sequence.

Table 2. *Molar ellipticities ($[\Theta]_{222}$) of different peptide analogs*[a]

Peptide	No Ca^{2+}	$+Ca^{2+}$	+TFE	ΔCa^{2+}	ΔTFE
LFIL	6,300	12,700	17,000	6,400	10,700
	(6)	(13)	(17)	(6)	(11)
AAIL	6,100	13,300	20,000	7,200	13,900
	(6)	(13)	(20)	(7)	(14)
LFAA	2,900	9,000	13,700	6,100	10,800
	(3)	(9)	(14)	(6)	(11)
AAAA	2,900	5,400	14,000	2,500	11,100
	(3)	(5)	(14)	(2)	(11)

[a] The experimental conditions used for the CD measurements are shown in Figure 1. +TFE denotes the presence of trifluoroethanol and ΔTFE denotes the trifluoroethanol-induced structure ($[\Theta]_{222}$ in the presence of TFE and the absence of Ca^{2+} minus the $[\Theta]_{222}$ in the absence of Ca^{2+} and TFE). Similarly, ΔCa^{2+} denotes the Ca^{2+}-induced structure. The numbers in parentheses indicate the calculated number of amino acid residues in the α-helical structures.

Simultaneous removal of hydrophobes in the N- and C-terminal helices (AAAA) resulted in similar reduction of original α-helicity as that of removing only the C-terminal hydrophobes (LFAA), but there was only about 40% of the Ca^{2+}-inducible α-helix, as compared to LFIL. The amount of TFE-inducible α-helix remained the same but, like LFAA, the actual molar ellipticity was about 3,000 degrees lower than that of the native sequence.

These results are in agreement with previous findings that the N-terminal region of site III is induced to an α-helical conformation upon binding Ca^{2+} (Nagy et al., 1978; Reid et al., 1981) and can accommodate substitutions in the N-terminal hydrophobes. On the other hand, substitution of the C-terminal hydrophobes seems to permanently disrupt some of its α-helical structure, even in the presence of Ca^{2+} or a strong α-helix-inducing solvent like TFE. Because the C-terminal substitutions were close to the C-terminal end of the peptide, it is reasonable to believe that this permanent uncoiling involves the first few C-terminal residues. It is interesting to note that although alanine is thought to be the strongest helix promoter, LFIL is more helical than LFAA, pointing to the possible roles of intrachain hydrophobic contacts in stabilizing the C-terminal helix (Kinemage 2).

Figure 2 shows the effect that peptide concentration has on molar ellipticity in the presence of Ca^{2+}. In the Ca^{2+}-bound form, the molar ellipticity of AAAA did not seem to change over the wide range of concentrations

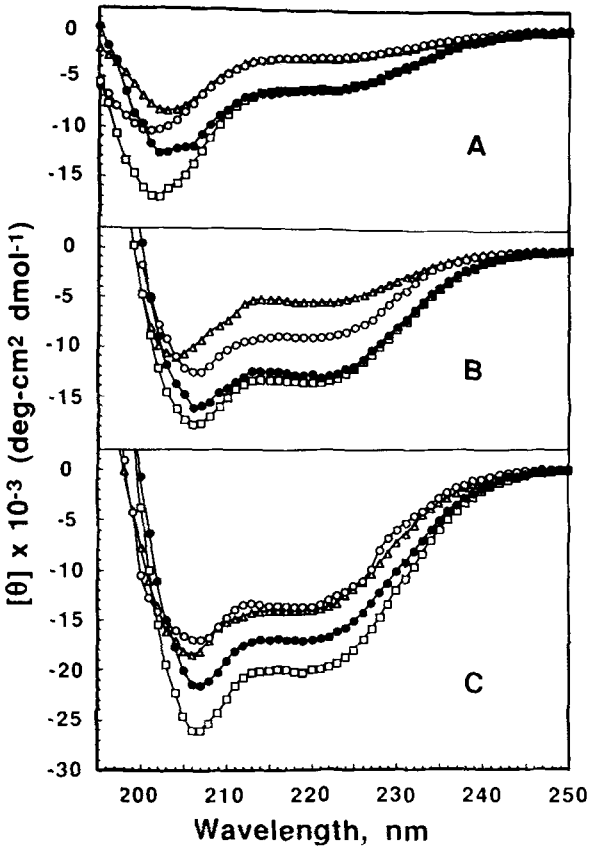

Fig. 1. CD spectra of the different TnC analogs. **A**: Apo-peptides. **B**: In the presence of 20 mM $CaCl_2$. **C**: In the presence of 50% TFE. The peptides were dissolved in 50 mM MOPS containing 100 mM KCl and 20 mM DTT, pH 7.0. Peptide concentrations used for the CD measurements were •, LFIL (3.1×10^{-4} M); □, AAIL (3.2×10^{-4} M); ○, LFAA (2.8×10^{-4} M); △, AAAA (3.1×10^{-4} M). The spectra of the peptide solutions without and with 2 mM EGTA added were very similar, indicating that the samples in the absence of added Ca^{2+} were Ca^{2+} free.

studied. This suggests the presence of a single and noninteracting conformational state in this concentration range. On the other hand, LFAA showed a dramatic decrease in negative molar ellipticity upon decreasing peptide concentration, consistent with a bimolecular, cooperative unfolding process. The molar ellipticity of LFAA at very low peptide concentrations approximates that of AAAA, suggesting that the two peptides are monomeric. As the LFAA concentration is increased this also increases the probability of bimolecular interaction that leads to cooperative folding into a dimeric state with two Ca^{2+}-binding sites. Indeed, analysis of the LFAA data (Fig. 2) using a nonlinear curve-fitting program based on the equation of Ho and DeGrado (1987) showed the degree of association to be 2.0 ± 0.1, clearly suggesting a dimeric structure at high concentration. The transition point from monomeric to dimeric states appears to be around 0.02 mM. AAIL also began to show a decrease in negative molar ellipticity at very low peptide concentrations. Unfortunately, this also approached the minimum limit of detection using CD measurements in very dilute solutions. Finally, the unchanged molar ellipticities of LFIL over a 1,000-fold dilution suggest that a single conformational state exists in this concentration range, a two-site dimer reported previously (Shaw et al., 1990). Due to the insignificant changes in the molar ellipticities of LFIL, AAIL, and AAAA at the concentration ranges studied, no curve-fitting program was used to analyze their data.

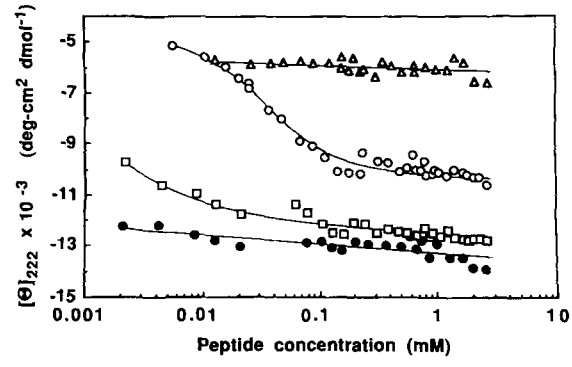

Fig. 2. Concentration-dependence of the molar ellipticities of different TnC peptide analogs at 222 nm. Conditions are described in the Materials and methods. •, LFIL; □, AAIL; ○, LFAA; △, AAAA.

Ca^{2+} affinity

The calcium titration curves of the different TnC analogs (Fig. 3) indicate significant differences in their Ca^{2+} affinities and Table 3 showed a very good correlation between the $[Ca/P]_{1/2}$ and the K_1 values, two of the Ca^{2+}-binding parameters. The analog with the native sequence exhibited the highest affinity, whereas the analog with alanine substitutions in both the N-terminal and C-termi-

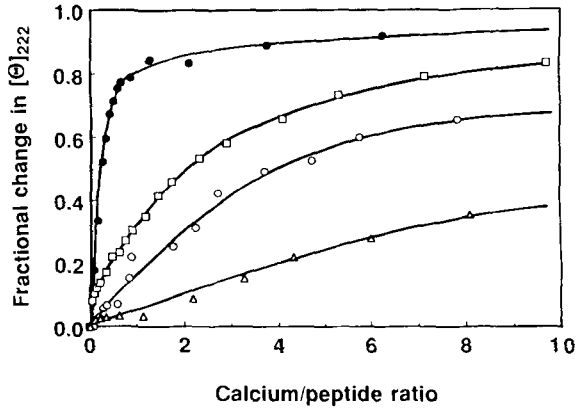

Fig. 3. CD curves of Ca^{2+} titration of different TnC peptide analogs. •, LFIL (1.5×10^{-4} M); □, AAIL (2.0×10^{-4} M); ○, LFAA (1.4×10^{-4} M); △, AAAA (2.1×10^{-4} M).

Table 3. *Ca^{2+}-binding parameters and stability of different TnC peptide analogs*

Peptide	$[Ca/P]_{50}$[a]	K_1[b] (M)	K_{dimer}[b]	$\Delta G_u^{H_2O}$[c] (kcal/mol)
LFIL	0.22	3.3×10^{-6}	2.0×10^{-6}	11
AAIL	2.1	3.7×10^{-4}	6.4×10^{-4}	7
LFAA	3.9	1.0×10^{-3}	2.0×10^{-3}	7
AAAA	13	3.2×10^{-3}[d]	–	3

[a] Estimated from Figure 2 as the calcium/peptide ratio at 50% change in ellipticity after normalizing values to 100% change in the fully Ca^{2+}-saturated (folded) state.

[b] K_1 and K_{dimer} values were determined by a nonlinear curve-fitting routine based on Equations 1–4 (see Materials and methods). K_1 corresponds to the dissociation constant of the first Ca^{2+} bound to the peptide monomer.

[c] $\Delta G_u^{H_2O}$ is the free energy of unfolding in the absence of denaturant.

[d] This value was derived from a curve-fitting routine based on 1:1 Ca:peptide binding described in Equation 2 rather than Equations 2–4 which incorporate peptide dimerization.

nal regions showed the lowest affinity for calcium. The $[Ca/P]_{50}$ value for LFIL was consistent with the previous observation (Shaw et al., 1991a) that the peptide with the native sequence requires only a half molar equivalent of Ca^{2+} to complete the folding process. That is, one molecule of Ca^{2+} can induce complete folding and association of two peptide monomers.

Removal of the N-terminal (AAIL) and C-terminal (LFAA) hydrophobes resulted in a 100- and 300-fold reduction in Ca^{2+} affinity, respectively, compared to that of the native sequence (LFIL). However, simultaneous removal of hydrophobes in both regions (AAAA) resulted in even greater reduction in Ca^{2+} affinity, that is, 1,000 times less than that of the native sequence. Consistent with the α-helicity calculated from their CD spectra, these results suggest that both the N- and C-terminal hydrophobes play an important role in the stabilization of the Ca^{2+}-binding site. These results are also consistent with previous observations by Tsuji and Kaiser (1991) that when two Leu residues in the α-helical region of calbindin-D_{9K} pseudo EF hand were substituted with Gln or Ala, the analogs completely lost their affinity for calcium.

Effect of hydrophobic interactions

There seems to be a general agreement that the pairing of EF hands in native calcium-binding proteins promotes conformational stability of both sites and enhances their affinity for Ca^{2+} (Sekharudu & Sundaralingam, 1988; review by Marsden et al., 1990). For example, the low Ca^{2+} affinities of sites I and II in the N-terminal domain of TnC compared to those of sites III and IV are believed to be at least partly due to fewer interactions (cooperativity) between those former sites (Beckingham, 1991). This might be the reason why the analog with the N-terminal hydrophobes removed (AAIL) exhibited a 100-fold decrease in Ca^{2+} affinity despite its similar helicity over that of the native sequence (LFIL). The decrease in hydrophobic interactions between the α-helices of AAIL decreased the stability of the dimer compared to that of the native sequence. The fact that AAAA has much lower Ca^{2+} affinity and is not dimerized points to the role of intrachain hydrophobic interactions. Substitutions of hydrophobic residues in proteins (Kellis et al., 1988; Matsumura et al., 1988) and in synthetic two-stranded α-helical coiled-coils (Hodges et al., 1990) showed dramatic changes in their overall stability. Molecular modeling using the X-ray structure of TnC (Herzberg & James, 1985; Satyshur et al., 1988), the model of the dimeric two-site domain of synthetic site III of TnC (Shaw et al., 1990), and the NMR structure of site IV (Kay et al., 1991) show an extensive hydrophobic core between the hydrophobes in the N-terminal helix of one monomer and C-terminal helix of the other monomer and vice versa (Kinemage 3), due to the head-to-tail orientation of the Ca^{2+}-binding sites. The molecular models in Figure 4 show the hydrophobic core created by the interaction of hydrophobes from the N-terminal helix of one helix-loop-helix Ca^{2+}-binding site with the C-terminal helix of the other helix-loop-helix Ca^{2+}-binding site along with the hydrophobic residue Ile-115 from the Ca^{2+}-binding loop that enters the hydrophobic core. The extensive Van der Waals interactions of Leu-98, Phe-102, Ile-121, and Leu-122 are indicated in Table 4. For example, Leu-98 and Phe-102 are simultaneously involved in at least 12 different hydrophobic residue contacts (6 are shown in Table 4), whereas Ile-121 and Leu-122 are involved in at least 20 contacts (10 are shown in Table 4). Therefore, substitution of these hydrophobes with Ala results in a substantial decrease in the Van der Waals interactions. In addition, the substitutions also result in a modification of the packing in the hydrophobic core that lead to changes in the polypeptide backbone not only in the α-helices but in the loop region as well (Fig. 5). Any changes in the hydrophobic interactions between the α-helices could also affect Ile-115, which is in the Ca^{2+}-binding loop and enters the hydrophobic core along with Leu-98 and Phe-102 from the N-terminal helix and Leu-118, Ile-121, and Leu-122 from the C-terminal helix of the other chain (Fig. 4; Table 1). Thus, the polypeptide backbone of the Ca^{2+}-binding loop could change and affect Ca^{2+} binding. For example, the data in Table 4 show that Phe-102aN (chain a, N-terminal helix) interacts with Leu-118bC (chain b, C-terminal helix) and Ile-115bL (chain b, Ca^{2+}-binding loop). Therefore substitution of Phe-102 with Ala would affect the interactions (Fig. 4; Kinemage 3) of Phe-102 (green) with Leu-118 (red) and Ile-115 (brown) in the other chain, and these residues in turn have other inter-

Fig. 4. Molecular models showing the hydrophobic side chains involved in the hydrophobic core between two Ca^{2+}-binding sites as a symmetric two-site dimer in a head-to-tail arrangement in the presence of Ca^{2+}. The top panel shows a ribbon presentation of the C-terminal helix (residues 115–126) of one helix–loop–helix Ca^{2+}-binding unit (chain 1) with the N-terminal helix (residues 93–105) of the other helix–loop–helix Ca^{2+}-binding unit (chain 2). The hydrophobic side chains are colored as follows: Ile-115 (orange), Leu-118 (red), Ile-121 (blue), Leu-122 (pink) chain 1; Leu-98 (yellow) and Phe-102 (green) chain 2. The left and right views differ by 180° rotation. The polypeptide backbone is colored white. The bottom panel shows a space-filling model of the presentations shown in the top panel. The models were prepared from the X-ray coordinates of turkey TnC (Herzberg & James, 1985) as follows: Residues 93–126 were used with the side chains at 99 and 100 changed to correspond to the chicken TnC site III sequence (E-99 to Ala-99 and D-100 to N-100, respectively), and C-101 was changed to A-101 and Phe-112 to Tyr-112 as shown in Table 1. Residues 129–162 were used, keeping the polypeptide backbone and C_β coordinates and mutating the side chains to those of site III described above. The site III homodimer was then energy minimized. Only the section described above is displayed here.

Table 4. *Hydrophobic interactions in the native two-site dimer*[a]

Residue[b]	Interchain interactions[c]	Intrachain interactions[c]
Leu-98 aN	Leu-118 bC Leu-122 bC	
Ala-99 aN	Ile-115 bL	
Ala-101 aN	Leu-118 bC Leu-122 bC	Phe-105 aN
Phe-102 aN	Ile-115 bL Leu-118 bC	Phe-105 aN Ile-113 aL
Phe-105 aN	Leu-118 bC Leu-122 bC	Ala-101 aN Phe-102 aN Ile-113 aL Ile-121 aC
Tyr-112 aL	Tyr-112 bL	
Ile-113 aL	Ile-113 bL Leu-118 bC	Phe-102 aN Phe-105 aN Leu-118 aC Ile-121 aC
Ile-115 aL	Ala-99 bN Phe-102 bN	
Leu-118 aC	Leu-98 bN Ala-101 bN Phe-102 bN Phe-105 bN Ile-113 bL Ile-121 bC	Ile-113 aL Ile-121 aC Leu-122 aC
Ile-121 aC	Leu-118 bC	Phe-105 aN Ile-113 aL Leu-118 aC Leu-122 aC
Leu-122 aC	Leu-98 bN Ala-101 bN Phe-105 bN	Leu-118 aC Ile-121 aC

[a] The X-ray coordinates for the backbone and side chains of sites III and IV of troponin C were used except where the side chains were mutated in site IV to give the site III homodimer. After the substitutions were made, the dimer was energy minimized to remove unfavorable side-chain contacts and optimize amino acid geometry and van der Waals interactions (no constraints).

[b] The residues are denoted by their sequence number and whether from chain a or chain b in the two-site homodimer. N, C, and L denote the N-terminal helix, C-terminal helix, and Ca^{2+}-binding loop, respectively (Table 1). Listed in this table are the interactions with hydrophobes from chain a only, and, therefore, only half of the expected interactions in the two-site homodimer are shown. The boxes denote the positions that were substituted by Ala in this study.

[c] Favorable van der Waals distances were observed in the range of 2.3–3.2 Å.

actions (Leu-118 [red] has intrachain interactions with Ile-121 [blue] and Leu-122 [pink], for example).

Guanidine hydrochloride (GdnHCl) denaturation

The GdnHCl denaturation curves of TnC peptide analogs (Fig. 6A) showed that all alanine-substituted analogs have much lower conformational stability than LFIL, the native analog. For example, at the same LFIL and AAIL concentration of 1.35 mg/mL, the concentration of GdnHCl required to denature half of the molecules ($[GdnHCl]_{1/2}$) differs by about 2 molar. The stabilities of LFAA and AAAA are even lower. When the observed free energy of unfolding (ΔG_u) was plotted

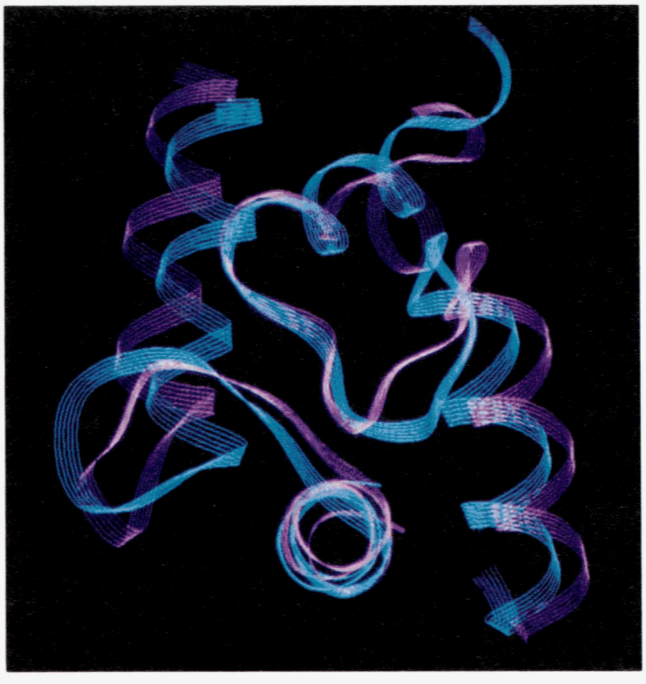

Fig. 5. Backbone superimposition of the analog (AAAA, blue) homodimer on the native homodimer (LFIL, pink) in a ribbon display. The AAAA homodimer model was prepared as described in Figure 4.

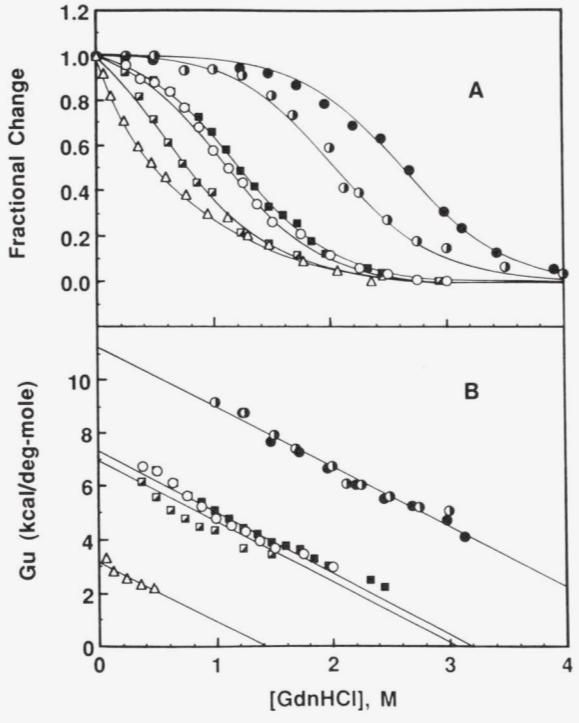

Fig. 6. **A**: Denaturation curves of different concentrations of TnC peptide analogs in GdnHCl in the presence of 20 mM $CaCl_2$. **B**: Corresponding plots of the free energy (ΔG_u) versus [GdnHCl] at different concentrations of TnC peptide analogs. Calculations were made based on a two-state model ($F_2 \rightleftharpoons 2U$) described in the Materials and methods. ●, LFIL, 1.35 mg/mL; ◑, LFIL, 0.08 mg/mL; ■, AAIL, 1.92 mg/mL; ◪, AAIL, 1.35 mg/mL; ○, LFAA, 1.92 mg/mL; △, AAAA, 2.00 mg/mL.

against GdnHCl concentration (Fig. 6B), extrapolations to obtain the free energy of unfolding in the absence of GdnHCl, $\Delta G_u^{H_2O}$ (Greene & Pace, 1974) indicated that the peptide with the native sequence (LFIL) was very stable in its folded structure. The $\Delta G_u^{H_2O}$ value of 11 kcal/mol (Table 3) is comparable to that of a 32-residue α-helical peptide comprising the dimerization domain of transcriptional factor LFB1 (De Francesco et al., 1991) and the dimeric Arc repressor protein (Bowie & Sauer, 1989). Removal of the N-terminal hydrophobes (AAIL) or the C-terminal hydrophobes (LFAA) resulted in structural destabilization by about 4 kcal/mol ($\Delta G_u^{H_2O} = 7$ kcal/mol), although it is still comparable to the free energy of unfolding for small globular proteins (Pace, 1975; Ahmad & Bigelow, 1982). This suggests that there is a large destabilization of the dimer upon substitution of just two hydrophobes each in the flanking α-helices. It should be noted that although only two hydrophobes were actually substituted at a time in AAIL and LFAA, this would drastically affect the multiple hydrophobic residue contacts observed in the native sequence as a result of the two peptide monomers interacting in a head-to-tail arrangement (Table 4). Similarly, Hodges et al. (1990) previously observed that in the association of two 35-residue monomers, which form a two-stranded α-helical coiled-coil, stabilized by nine pairs of Leu–Leu interchain hydrophobic interactions, the substitution of two Leu residues by two Ala residues in each chain of the homodimer completely destabilized the coiled-coil structure. This substitution of four large hydrophobes by four Ala residues is directly analogous to the two Ca^{2+}-binding site homodimer used in this study (AAIL or LFAA). Zhou et al. (1992) have shown that a single Ala substitution in each chain of the above coiled-coil homodimer (a Leu–Leu pair to an Ala–Ala pair) resulted in a 3 kcal/mol destabilization ($\Delta G_u^{H_2O}$ 9.7 kcal/mol for the native molecule to $\Delta G_u^{H_2O}$ of 6.7 kcal/mol for the Ala analog).

Simultaneous removal of both pairs of hydrophobes in the flanking α-helices (AAAA) resulted in a much less stable peptide analog ($\Delta G_u^{H_2O} = 3$ kcal/mol). This energy probably corresponds to a unimolecular unfolding of the helix–loop–helix structure, as intermolecular hydrophobic interactions are expected to be very weak, and the dimeric structure was not observed.

Folding pathway

Based on calcium titration data monitored by two-dimensional (2-D) ^1H NMR spectroscopy, Shaw et al. (1990, 1991a) proposed a possible pathway of the Ca^{2+}-induced folding of the native peptide that eventually leads to the

formation of a dimeric two-site domain (see Equation 1 in the Materials and methods). This model was based on the observation that only one-half equivalent of Ca^{2+} is required to completely fold the site III homodimer (Shaw et al., 1991a) or sites III/IV heterodimer (Shaw et al., 1992b). Two-dimensional ^1H NMR data showed no evidence of the existence of a Ca^{2+}-free folded monomer. Structural changes upon the addition of the second Ca^{2+} appear to be spectroscopically silent because no significant difference in NMR spectra was observed when a solution containing half an equivalent of Ca^{2+} was titrated with another half equivalent of Ca^{2+} (Shaw et al., 1991a, 1992b). It is quite possible that most of the spectroscopic changes (NMR or CD) monitored during Ca^{2+} titration involve only the peptide species U, F-Ca^{2+}, and F_2-Ca^{2+} in Equation 1. Therefore, the next obvious question is whether a Ca^{2+}-bound folded monomer (F-Ca^{2+}) exists as a stable intermediate.

The GdnHCl denaturation curves of the different peptide analogs (Fig. 6A) appear to have single inflection points, suggesting that the intermediate(s) of folding, such as F-Ca^{2+}, does not exist long enough to be detectable. The same figure also showed an increase in structural stability with an increase in peptide concentration. This also rules out the possibility of unimolecular folding and is consistent with dimer formation. Finally, we can test for the absence of stable folding intermediates by assuming a purely two-state mechanism, $F_2 \overset{K_u}{\rightleftharpoons} 2U$, and comparing the ΔG_u versus [GdnHCl] plots at different peptide concentrations. In the absence of stable intermediates the slopes and intercepts of such plots would be constant (Pace, 1986; Bowie & Sauer, 1989; Pastore et al., 1991). Similar plots (Fig. 6B) showed that, within experimental error, a line of the same slope and intercept can each be drawn for the two concentrations of LFIL and of AAIL. These data are consistent with our original contention (Shaw et al., 1991a) of a transient existence of a Ca^{2+}-bound monomer and that the dimerization process involves a concerted folding mechanism.

Conclusion

Through the use of a synthetic peptide approach, we have demonstrated the importance of hydrophobic residues in the N- and C-terminal regions in stabilizing the α-helical structures and the formation and stabilization of a two-site dimeric domain. We believe that the same mechanism is true for the association and the resulting high affinity of Ca^{2+}-binding sites III and IV in skeletal TnC.

Materials and methods

Peptide synthesis and purification

Four 34-residue peptide analogs of calcium-binding site III were synthesized by solid-phase synthesis on copoly (styrene, 1% divinylbenzene) benzhydrylamine hydrochloride resin (Institute Armand Frappier, Laval, Quebec, Canada) using standard *t*-boc chemistry on an Applied Biosystems peptide synthesizer Model 430A (Foster City, California). The peptides were cleaved from the resin by reaction with hydrogen fluoride (20 mL/g resin) containing 10% anisole and 2% 1,2-ethanedithiol for 50 min at −5 °C. The crude peptides were purified by reversed-phase high performance liquid chromatography (HPLC) using a Varian Vista Series 5000 liquid chromatograph (Varian, Walnut Creek, California) equipped with a Synchropak RP-P semipreparative C_{18} column (25 cm × 1 cm internal diameter [ID], 6.5 μm, 300 Å; SynChrom, Linden, Indiana). The purity of the fractions was verified by reinjecting aliquots into a Hewlett-Packard 1090 Series II HPLC equipped with an Aquapore RP-300 analytical column (220 mm × 4.6 mm ID, 7 μm; Applied Biosystems). The amino acid composition and concentration of the peptides were verified on a Beckman Model 6300 amino acid analyzer system equipped with a 25-cm ion-exchange column and a postcolumn ninhydrin calorimetric detection. Data were processed and stored on a Beckman System Gold 5.1 data system. The identity and purity of the peptides were further confirmed using a Bio-Ion 20 plasma desorption mass spectrometer at the Protein Engineering Network of Centers of Excellence (PENCE) laboratory.

CD spectroscopy

Stock solutions (≅1 mM) were prepared by dissolving the peptides in 50 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS) containing 100 mM KCl and 20 mM dithiothreitol (DTT) at pH 7.0. Aliquots were diluted with the same buffer to give 150–250-μM peptide solutions that were used for Ca^{2+} titrations and CD spectroscopy. All CD spectra were obtained by using a JASCO J-500C Spectropolarimeter equipped with a JASCO DP500N data processor.

The CD spectra of the Ca^{2+}-free and the Ca^{2+}-bound peptides were determined in the absence and presence of 10 mM Ca^{2+}, respectively. A peptide solution was considered Ca^{2+} free when its CD spectra did not change significantly upon the addition of 2 mM of (ethylenebis(oxyethylenenitrilo))tetraacetic acid (EGTA). The CD spectra of the Ca^{2+}-free peptides were also determined in the presence of 50% TFE.

The concentration dependence of the molar ellipticities of the different TnC peptide analogs was measured at 222 nm. Stock solutions (10 mg/mL) of peptides were prepared in 50 mM Tris/100 mM KCl/20 mM $CaCl_2$/5 mM DTT, pH 7.0. Different aliquots were diluted in the same buffer, and the ellipticities of the solutions were read using 0.01-, 0.02-, 0.05-, and 0.2-cm cells. The readings were then normalized to molar ellipticities calculated from the 0.01-cm cell.

Calcium titrations

Separate aliquots of the peptide solutions were then titrated with a standard $CaCl_2$ solution, and the change in molar ellipticity was monitored at 222 nm ($[\Theta]_{222}$). The fraction of peptide molecules chelated to calcium (f_{Ca}) was calculated from the equation $f_{Ca} = ([\Theta]_{222} - [\Theta]_U)/([\Theta]_F - [\Theta]_U)$, where $[\Theta]_U$ and $[\Theta]_F$ are the molar ellipticities of the unfolded and folded states, respectively. Based on Equation 1,

$$U + Ca^{2+} \rightleftharpoons F\text{-}Ca^{2+} + U \rightleftharpoons F_2\text{-}Ca^{2+} + Ca^{2+} \rightleftharpoons F_2\text{-}Ca_2^{2+}, \quad (1)$$

it was previously suggested that if the calculated f_{Ca} were plotted against $[Ca_t]/[P_t]$, the values of K_1, K_2, and K_{dimer} could be calculated from nonlinear curve-fitting of the experimental data (Shaw et al., 1991a). Here, U and F are the peptides in the unfolded and folded states, respectively; $[Ca_t]$ and $[P_t]$ are the total calcium and total protein concentrations, respectively; whereas K_1, K_2, and K_{dimer} are dissociation constants from Equation 1 and defined previously (Shaw et al., 1991a) as

$$U + Ca^{2+} \rightleftharpoons F\text{-}Ca^{2+} \qquad K_1; \quad (2)$$

$$F\text{-}Ca^{2+} + U \rightleftharpoons F_2\text{-}Ca^{2+} \qquad K_{dimer}; \quad (3)$$

$$F_2\text{-}Ca^{2+} + Ca^{2+} \rightleftharpoons F_2\text{-}Ca_2^{2+} \qquad K_2. \quad (4)$$

In the case where K_{dimer} is much greater than K_1, these equations simplify to the 1:1 peptide-metal complex shown in Equation 2 (Shaw et al., 1992b). The calcium/peptide ratio at half saturation $[(Ca/P)_{1/2}]$ was determined graphically from plots of the calcium/peptide ratio against fractional change in $[\Theta]_{222}$.

Method of denaturation

There are several available methods to unfold or denature proteins. The method of choice is GdnHCl denaturation because it provides maximum unfolding that is likely to be reversible (Greene & Pace, 1974). From the GdnHCl denaturation curve it is possible not only to estimate and compare conformational stability (Greene & Pace, 1974; Lee & Timasheff, 1974; Schellman, 1978; Pace & Vanderburg, 1979; Shortle, 1989) of closely related proteins or peptides but also to draw deductions about the mechanism of protein folding (Tanford, 1968; Aune & Tanford, 1969; Azuma et al., 1972; Wong & Tanford, 1973; Yutani et al., 1979; Matthews & Crisanti, 1981).

It was previously proposed that the dimerization of a 34-residue synthetic peptide representing site III of skeletal TnC involves at least two intermediates (Shaw et al., 1991a). This makes calculations of the true free energy of folding (or unfolding) complicated. However, important information can be obtained if the folding pathway can be made to fit a two-state mechanism, $F_2 \overset{K_u}{\rightleftharpoons} 2U$, where F is the folded state and U is the unfolded state. This can be accomplished by following the denaturation of the peptide in its Ca^{2+}-bound form (saturating conditions of Ca^{2+}, 20 mM). K_u can be calculated from the equation $K_u = 2P_t[(f_U)^2/(1 - f_U)]$, where P_t is the total peptide concentration and f_U, the fraction of peptide in the unfolded state, is equal to $1 - f_F$. f_F is, in turn, defined as $(\Theta_{222} - \Theta_U)/(\Theta_F - \Theta_U)$. The free energy of unfolding (ΔG_u) can then be calculated from the equation $\Delta G_u = -RT \ln K_u$. The calculated ΔG_u can then be plotted against [GdnHCl] and, in the absence of stable intermediates, the slopes and intercepts of the lines should be constant at any peptide concentration (Bowie & Sauer, 1989; De Francesco et al., 1991). This plot can also be used to calculate the free energy of unfolding in the absence of denaturant ($\Delta G_u^{H_2O}$) by using the equation $\Delta G_u = \Delta G_u^{H_2O} - m[\text{GdnHCl}]$, where m is the slope of the line (Greene & Pace, 1974).

We therefore incubated different concentrations of peptides in different concentrations of GdnHCl in the same MOPS buffer for at least 3 h at 25 °C, and then their CD spectra were determined. From their molar ellipticities at 222 nm, the fraction of peptide molecules that are folded at a particular GdnHCl concentration was then calculated in similar manner as in Ca^{2+} titration.

Calculation of the α-helix content

Nagy et al. (1978) showed that the α-helical content of CB9 (the native cyanogen bromide fragment of TnC) could be estimated from $[\Theta]_{222}$, allowing for the lengths of the α-helical segments (Chen et al., 1974). Reid et al. (1981) later proposed a simplified method to estimate the fraction of α-helix (f_H) of a peptide as $f_H = [\Theta]_{222}/[\Theta]_H^\infty(1 - k/\bar{n})$, where $[\Theta]_H^\infty$ is the maximum mean residue ellipticity of a helix of infinite length and k is the wavelength-dependent constant (Chen et al., 1974), which are 39,500° and 2.57, respectively, and $\bar{n}$ is the average number of residues per helical segment, taken as 9 in this peptide (Nagy et al., 1978; Reid et al., 1981).

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