Specific Formation of a Heterodimeric Two-Site Calcium-Binding Domain from Synthetic Peptides

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The fundamental architectural element of many calcium-binding proteins is the two-site calcium-binding domain, comprised of a pair of helix–loop–helix metal ion binding sites which interact via hydrophobic residues at the interface of the two sites.[1] These properties allow interactions between the sites to be investigated using synthetic peptides,[2] and we have applied this technique to study sites III and IV of the muscle protein troponin-C (TnC). We have recently shown that a 34-residue site III peptide of TnC (SCIII) undergoes calcium-induced folding and association to form a noncovalent two-site homodimer similar in structure to the carboxyl terminal domain of TnC.[3,4] A similar observation has been made for a proteolytic site IV fragment of TnC.[5] In this work we use ^{1}H NMR spectroscopy to probe the preferential formation of a site III/site IV heterodimer (i.e., synthesis of the carboxyl terminal domain of TnC) from two synthetic peptides comprising sites III (SCIII, residues 93–126) and IV (SCIV, residues 129–162) of TnC (Figure 1).

The ^{1}H NMR spectra of calcium-free peptides SCIII and SCIV or an equimolar mixture of peptides SCIII and SCIV show a lack of chemical shift dispersion that is typical of peptides in random coil conformation, in agreement with circular dichroism[6] and calorimetric studies[7] of TnC which show that sites III and IV are unstructured in the absence of calcium. Calcium binding to SCIII or SCIV causes significant changes in its ^{1}H NMR spectrum (Figure 2A,B) resulting from the transition of a "random coil" structure to a helix–loop–helix conformation and the association of two of these motifs to form a two-site homodimer, either Ca_2-$SCIII_2$[3,4] or Ca_2-$SCIV_2$. Calcium addition to a mixture of SCIII and SCIV peptides also results in changes throughout the ^{1}H NMR spectrum (Figure 2C) which are very different from those of Ca_2-$SCIII_2$ (Figure 2A) and Ca_2-$SCIV_2$ (Figure 2B). In particular, several new resonances appear in the aromatic region of the spectrum (6.2–7.5 ppm) and downfield of the residual HDO signal (4.9–5.4 ppm). These changes in the spectrum of SCIII/SCIV and detailed calcium titration experiments[8] suggest that calcium binding and a significant *structural* change in both peptides has occurred with stoichiometric formation of the two-site SCIII/SCIV heterodimer (Ca_2-SCIII/SCIV) in preference over Ca_2-$SCIII_2$ and Ca_2-$SCIV_2$.

To confirm the identity of the Ca_2-SCIII/SCIV heterodimer, we prepared a tryptic fragment (TR_2C, residues 92–162) from rabbit skeletal TnC[9,10] containing sites III and IV. The ^{1}H NMR spectra of Ca_2-SCIII/SCIV (Figure 2C) and calcium-saturated

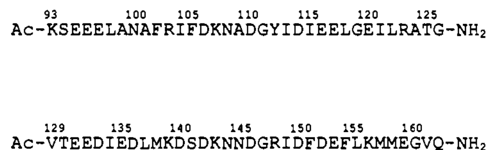

Figure 1. Sequences for SCIII (top) and SCIV (bottom) peptides from chicken skeletal troponin-C with C101A and F112Y replacements. Peptides were synthesized, using an Applied Biosystems 430A peptide synthesizer, and purified using reversed-phase HPLC, and compositions were verified by amino acid analysis and plasma desorption mass spectrometry.

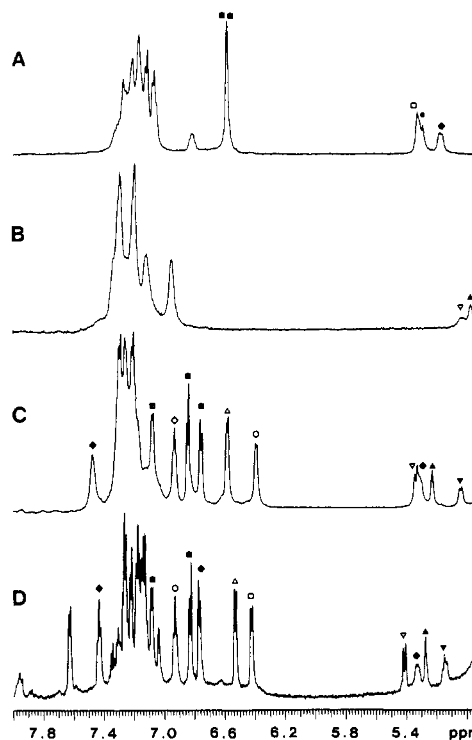

Figure 2. Portions of the ^{1}H NMR spectra of (A) 4 mM Ca_2-$SCIII_2$, 50 mM KCl, 30 mM imidazole-d_4, pH 7.3; (B) 2.5 mM Ca_2-$SCIV_2$, 100 mM KCl, pH 7.2; (C) 2 mM Ca_2-SCIII/SCIV, 100 mM KCl, pH 7.2; and (D) ca. 100 μM Ca_2-TR_2C, 100 mM KCl, pH 7.2. Assigned resonances indicated are the following: F102 δCH (○), F102 εCH (◇), F151 δCH (△), F154 δCH (⊡), F154 εCH (◇), Y112 δCH (■), Y112 εCH (●), Y112 αCH (◆), I113 αCH (▼), D114 αCH (□), I149 αCH (▽), and D150 αCH (▲). The assignments are based on the sequence-specific assignments of Ca_2-$SCIII_2$ and Ca_2-SCIII/SCIV and published assignments for the calcium-saturated site IV dimer[5] and Ca_2-TR_2C.[14,15] Spectra were acquired on either a Varian VXR-500 spectrometer (A, B) or a Varian Unity 600 spectrometer (C, D) in D_2O at 30 °C, and vertical scales were adjusted to give the same apparent peptide concentrations.

TR_2C (Ca_2-TR_2C, Figure 2D) are remarkably similar in the aromatic (6.2–7.5 ppm) and downfield shifted αCH (4.9–5.4 ppm) spectral regions. For Ca_2-SCIII/SCIV the ^{1}H NMR spectrum has been completely assigned[11] and compared to the corresponding resonances previously assigned in TnC[12] and Ca_2-TR_2C.[13] The assigned aromatic resonances from F102 (δCH), F151 (δCH), Y112 (δCH and εCH), and F154 (εCH) of Ca_2-SCIII/SCIV are in agreement with previous assignments in rabbit skeletal TnC[12] and the TR_2C fragment.[13]

The four downfield-shifted αCH resonances present in Ca_2-SCIII/SCIV have been identified as those of Y112 (5.30 ppm), I113 (5.04 ppm), I149 (5.33 ppm), and D150 (5.23 ppm) (Figure 2C) and are similar to those shown for Ca_2-TR_2C (Figure 2D),

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in agreement with the assignments of Y112 (5.29 ppm), I113 (5.05 ppm), and I149 (5.39 ppm) in rabbit skeletal TnC.[12] The downfield shifts of these four resonances and the observation of characteristic crosspeaks in two-dimensional NOE experiments[14] suggest their involvement in a short three-residue antiparallel β-sheet between sites III (positions 112–114) and IV (positions 148–150) which has been observed in the X-ray structures of TnC.[15] An analogous β-sheet is also observed in the site III[3] and site IV[5] homodimers.

The ^{1}H NMR spectrum of Ca_2-SCIII/SCIV (Figure 2C) is nearly identical to that of Ca_2-TR_2C (Figure 2D) and quite distinct from those of the homodimers Ca_2-$SCIII_2$ (Figure 2A) and Ca_2-$SCIV_2$ (Figure 2B), indicating the preferential formation of a two-site SCIII/SCIV heterodimer similar in fold to the carboxyl terminal domain of TnC. These results show that, at least for calcium-binding proteins, synthetic peptides can be folded and assembled specifically into stable protein domains, similar to results obtained for coiled coils,[16] zinc fingers,[17] and fragments of BPTI.[18]

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Experimental Studies on the Hook and Ladder Approach to Molecular Knots: Synthesis of a Topologically Chiral Cyclized Hook and Ladder[1]

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The colored figure-of-eight knot illustrated Figure 1 is the simplest known topological rubber glove[2] and represents the only class in the topological hierarchy of molecular chirality not yet realized in an actual molecular structure.[1] This type of knot cannot result from the Möbius ladder approach to synthsis of knots and links,[3-5] suggesting exploration of a new topological approach, the "hook and ladder",[2c] with the ultimate goal being creation of a

(1) This paper is part 8 in a series on topological stereochemistry. Previous papers in the series: Walba, D. M. A Topological Hierarchy of Molecular Chirality and other Tidbits in Topological Stereochemistry. In *New Developments in Molecular Chirality*; Mezey, P. G., Ed.; Kluwer Academic Publishers: Boston, 1991; pp 119–129 and references therein.
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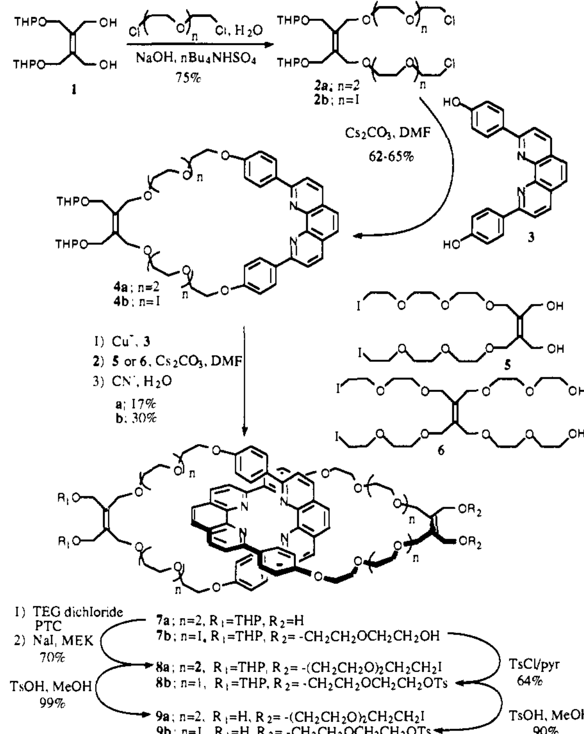
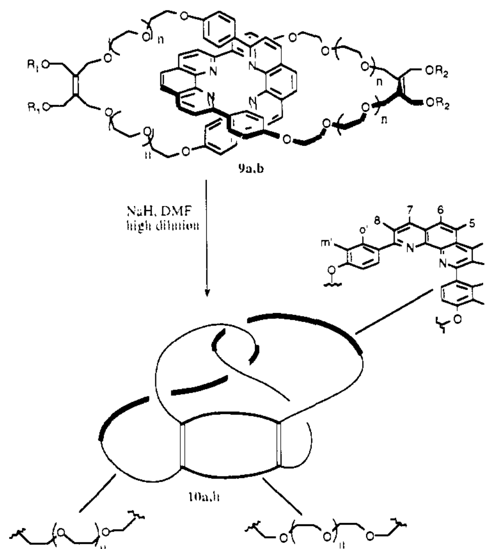
Figure 1. Topological rubber glove.

Scheme I. Synthesis of Hook and Ladders **9a** and **9b**



Scheme II. Synthesis and Proposed Conformation of the Cyclized Hook and Ladder Compounds **10a** and **10b**



molecular topological rubber glove. Herein we report the first synthesis of a molecular hook and ladder and its bismacrocyclization to afford the topologically novel $^1/_4$-twist cyclized hook and ladder.

The key hook and ladder intermediate is actually a catenane (the hook) functionalized with rungs and uprights on each ring (the ladder). Furthermore, the ends of the uprights are func-

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