

Solution Structure of the D/E Helix Linker of Skeletal Troponin-C: As Studied by Circular Dichroism and Two-Dimensional NMR Spectroscopy

Weontae Lee*, N. Rama Krishna[†], G. M. Anatharamaiah[‡], and Herbert C. Cheung[†]

Department of Biochemistry, College of Science, Yonsei University, Seoul 120-740, Korea

[†]*Department of Biochemistry and Molecular Genetics,*

[‡]*University of Alabama at Birmingham, Birmingham, Alabama 35294-2041, U.S.A.*

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We have synthesized a 17-residue peptide with the amino acid sequence RQMKEDAKGKSEEELAD corresponding to residues 84-100 of chicken skeletal troponin C. This stretch of the protein sequence is in the middle one-third of the 32-residue 9-turn α -helix that connects the two globular domains of the dumbbell-shaped molecule and includes the D/E linker helix. We describe here the solution conformation of the helix linker as studied by circular dichroism (CD) and two-dimensional nuclear magnetic resonance (2-D NMR) spectroscopy. The NOE connectivities together with the vicinal $^3J_{\text{NH}}$ coupling constants suggest that the peptide exists in a fast conformational equilibrium among several secondary structures: a nascent helix near the N-terminus, a helix, and a substantial population of extended and random coil forms. In addition, two interresidue α - α NOEs are observed suggesting a bent structure with a bend that includes the single glycine in position 92. These results are consistent with the ideas that in neutral solution the D/E linker region of the central helix in troponin C can adopt a helical conformation and the central helix may have a segmental flexibility around Gly 92.

Introduction

Activation of skeletal and cardiac muscle requires the binding of calcium to troponin. The troponin-tropomyosin complex, which is bound to the actin filament, acts as the regulatory system that modulates actomyosin ATPase and force generation in these two types of muscle. Troponin is comprised of three nonidentical subunits: Troponin I, troponin C, and troponin T. Troponin C (TnC, Mr \approx 18,800) from skeletal muscle has four calcium-binding sites, and the other two subunits have no known affinity for the cation. Two sites (sites I and II) are specific for calcium ions with a low affinity ($K_d = 2 \times 10^5 \text{ M}^{-1}$), and the other two sites (sites III and IV) bind both calcium with a high affinity ($K_d = 2 \times 10^7 \text{ M}^{-1}$) and magnesium competitively. The low-affinity sites are the primary regulatory sites, and calcium binding to these sites triggers the contractile cycle. The binding of activator calcium to TnC enhances the affinity of troponin I for TnC by a factor of 100.¹ This calcium-induced enhancement occurs concomitantly with a weakening of the interaction of troponin I with actin. On the basis of studies with oligopeptides, it has been suggested that a common sequence of troponin I (residues 104-115) may interact with TnC only in the presence of bound activator calcium and with actin in its absence.² Since the linkage between troponin I and TnC within troponin may be the main calcium signal transmitter,^{3,4} it is important to elucidate the structural features that are involved in these two proteins in the transfer of the calcium signal from TnC to distant sites on the actin filament.

The reported crystal structures of TnC from chicken⁵ and turkey⁶ skeletal muscle are very similar and show the protein to have an unusual dumbbell shape. The amino- and carboxy-terminal regions are each folded into a globular domain which contains several EF-hand motifs and two calcium-binding sites. Sites I and II are located in the N-ter-

минаl domain and sites III and IV in the C-terminal domain. The two domains are linked by a 32-residue 9-turn α -helix (residues 75-105), and the overall length of the protein is about 75 Å with an axial ratio of about 3:1. The middle one-third (residues 87-97) of this long helix is exposed fully to solvent and not in contact with neighboring molecules, whereas the helical segments at both ends of the long helix are either partially or completely buried in the globular domains. The helical linker in the dumbbell-shaped structure is a unique structural feature whose function is not immediately apparent. It is unclear how the central helix is stabilized in the crystal. The long helix is slightly bent by 7° near the middle, presumably because of the presence of a glycine in position 92.⁷ However, NMR studies^{9,10} showed that the structure of interconnecting linker is disordered at 40 °C in calcium saturated condition. The NMR data also indicated that residues 84-94 were too highly flexible to get structural information. This feature raises the possibility that the helix may not be fully extended and may possess some segmental flexibility if the protein is in solution. In fact, several lines of evidence suggest possible communication between the two domains in isolated TnC.⁸

We have synthesized several oligopeptides with amino acid sequences that correspond to the middle segment of the central helix of chicken skeletal TnC. These peptides serve as models for the central helix. In the present paper, we report 2-dimensional NMR (nuclear magnetic resonance) studies of one such 17-residue peptide corresponding to amino acid residues 84-100 in the protein sequence. As we expected, the results shows that the peptide has no secondary structure in neutral solution at room temperature. However, at a lower temperature several structural features can be detected, suggesting the existence of an ensemble of several secondary structures that are in a rapid equilibrium.

Experimental Section

Peptide synthesis and purification. A peptide with the amino acid sequence NH₂-Arg-Gln-Met-Lys-Glu-Asp-Ala-Lys-Gly-Lys-Ser-Glu-Glu-Glu-Leu-Ala-Asp-COOH(C₈₄₋₁₀₀) was synthesized by the solid-phase method¹¹ on an Advanced Chem Tech peptide synthesizer 200, using a benzhydrylamine resin (Bachem Fine Chemicals, Torrance, CA) (0.565 mmol of NH₂/g of resin) as the solid support. The α -amino groups of the amino acids were protected with the tert-butyloxycarbonyl (Boc) group. The C-terminal amino acid, Boc-Asp (OBZL), was attached to the solid support through a 4-(oxymethyl)-phenacetyl group.¹² This procedure yields a more stable linkage than the conventional benzyl ester linkage. The following blocking groups were used for side chain protection: γ -benzyl esters for aspartic and glutamic acids, 2-chlorobenzoyloxycarbonyl for lysine, tosyl for arginine, and benzyl ether for serine. The amino acid couplings were carried out using dicyclohexylcarbodiimide and 1-hydroxybenzotriazole. The synthesized peptide was released from the solid support by using a modified HF procedure.¹³ After treatment with HF, the peptide was extracted into 50% acetonitrile/water containing 0.1% trifluoroacetic acid. Crude peptide was purified by reversed-phase HPLC on a C-18 column, and the purity was ascertained by analytical HPLC, amino acid sequencing, and amino acid analysis (Table 1). The sequencing was performed on an Applied Biosystem automated sequencer, and amino acid composition was determined with samples hydrolyzed in 6 N HCl at 110 °C for 24 h, using the phenylisothiocyanate method.

Circular dichroism. Circular dichroism (CD) spectra of C₈₄₋₁₀₀ were obtained from an AVIV 62DS circular dichroism spectrometer equipped with a thermoelectric temperature controller and linked to a 80386 microprocessor-based AT/PC for data acquisition and reduction. Ten scans were obtained at each condition and averaged to increase the signal/noise ratio. Peptide (50 μ g/mL) was dissolved in 20 mM Tris, pH 7.0 and 5.6. Peptide concentrations were determined using quantitative amino acid analysis. The spectra were recorded using a pathlength of 2 mm.

NMR spectroscopy. Nuclear magnetic resonance (NMR) experiments were performed with the peptide C₈₄₋₁₀₀ dissolved in both H₂O and D₂O on a Bruker AM-600 spectrometer operating at a proton frequency of 600.138 MHz. The peptide concentration was in the range of 2-3 mM. Additional data were obtained with a WH-400 spectrometer e-

quipped with an Aspect 3000 computer. The experiments were done at 21 °C and 5 °C, and the temperatures were calibrated using a methanol standard as previously described.¹⁴ COSY (homonuclear two-dimensional correlated spectroscopy) experiments¹⁵⁻¹⁷ were performed in the absolute value mode, whereas NOESY (nuclear overhauser effect spectroscopy) experiments¹⁸ were carried out in the phase-sensitive mode using the time proportional phase incrementation method (TPPI) of Marion & Wuthrich.¹⁹ The strong water resonance was suppressed by gated irradiation during the 1.2 s delay time and also during the mixing period for the NOESY experiments. The COSY and NOESY spectra at 600 MHz were collected as 512t₁ experiments, each with 2K data points and 64 transients. Mixing times ranging from 100 to 800 ms were used for 2D-NOESY at 5 °C.

Small flexible linear peptides such as C₈₄₋₁₀₀ have relatively short correlation times compared to globular proteins of molecular weights greater than 5,000. As a result, longer mixing times are typically needed before NOESY cross peaks begin to manifest in the spectrum. From the NOESY growth curve, we have ascertained that spin-diffusion effects are not significant even for 600-800 ms mixing times. The interesting cross peaks observed in the spectra (e.g. Figure 4) are due to direct effects since no other cross-peaks from protons that could mediate the intensity through spin diffusion were observed in the spectra. Long mixing times in the range of 400-800 ms have typically been used for small peptides by other laboratories also.²⁰⁻²² The relayed COSY experiments^{23,24} were performed at 400 and 600 MHz as 512t₁ experiments, each with 2K data points, using mixing times of 30 and 35 ms. Phase-sensitive COSY experiments¹⁹ were collected at 600 MHz as 2048 complex points for each of 1024t₁ values. The chemical shifts were all referenced to internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS).

All NMR spectra were transferred to a microVAX II computer for processing using the FTNMR program (Hare Research Inc., Woodinville, WA). Both shifted ($\pi/4$) sine and unshifted sine-squared weighting functions were applied to both dimensions prior to Fourier transformation. The first row was half-weighted prior to Fourier transformation in the t₁ dimension.²⁵ The data matrix was zero-filled to give 2-D spectra of 2K \times 2K real points, while phase-sensitive COSY data were processed to 4K \times 4K data matrices.

Results and Discussions

Circular dichroism of peptide. The CD spectra of the peptide C₈₄₋₁₀₀ obtained at pH 7.0 and 25 °C showed characteristics of a random coil with a small positive mean residue ellipticity of +945 at 222 nm, indicating that the peptide possessed little secondary structure in benign solution. We compared the CD spectrum of the peptide in 90% TFE with that obtained in aqueous solution to determine whether secondary structure could be induced. The shape of the spectrum obtained in TFE was different (Figure 1), with a negative mean residue ellipticity of -5732.

Identification of amino acid spin system. In the C₈₄₋₁₀₀ sequence, there is only one Gly and one Leu residue. Glycine was identified by the distinctive finger print pattern

Table 1. Amino acid content of troponin C peptide C₄₋₁₀₀

Amino acid	Experimental	Theoretical
Asx	2.0	2
Glx	5.2	5
Ser	1.0	1
Gly	1.1	1
Arg	1.1	1
Ala	2.3	2
Met	1.0	1
Leu	1.1	1
Lys	3.0	3

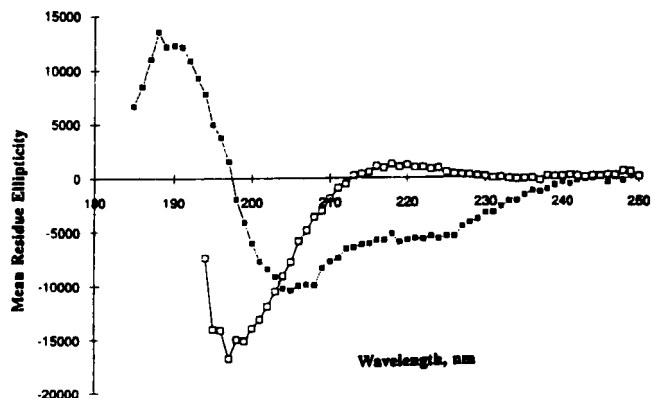


Figure 1. Circular dichroism spectra of peptide C_{84-100} at 25 °C, pH 7.0 (open squares) and in 90% TFE (closed squares).

in the H_2O COSY spectra and by the cross peaks between geminal protons in the D_2O COSY spectra. Identification of the lone Leu residue was done using connectivities from relay COSY and double relay COSY spectra in both D_2O and H_2O solvents. The two alanine residues have been identified by their characteristic methyl group resonances and relayed connectivities in H_2O solution (Figure 2). Three AMX spin systems, one from serine and two from aspartate residues, were identified in COSY and relay COSY spectra in D_2O . These preliminary resonance assignments served as starting points for the sequence-specific assignment procedure.²⁶

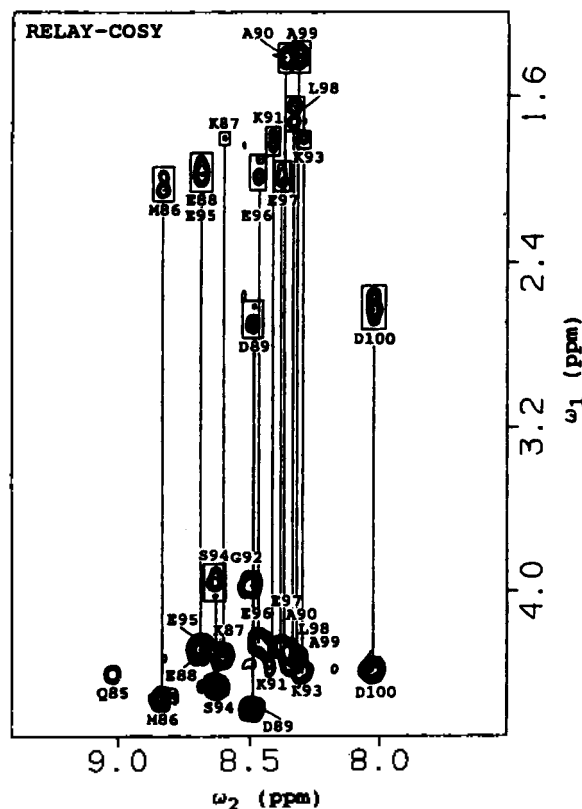


Figure 2. 400 MHz relay COSY proton NMR spectrum of peptide C_{84-100} in 90% $H_2O/10\%$ D_2O at pH 5.6 and 5 °C showing relayed connectivities (mixing time 30 ms). The $NH-C^{\alpha}H$ relay cross peaks are enclosed by squares.

Sequence-specific assignments. Sequential resonance assignments were made using 2-D COSY and NOESY spectra in 90% $H_2O/10\%$ D_2O solution. These spectra are shown in Figure 3A and 3B. The $d_{\alpha N}(i, i+1)$ connectivities were observed for all of the residues at both 21 °C and 5 °C. Sequential assignments for the backbone protons were completed by following $d_{\alpha N}$ connectivities from $NH-C^{\alpha}H$ COSY cross peaks of known amino acids. The complete side chain proton assignments for some residues could not be completed due to resonance overlap. The proton chemical shifts for the peptide determined at 5 °C are summarized in Table 2.

Secondary structural elements. At room temperature the proton chemical shifts for the individual residues in C_{84-100} are in excellent agreement with the corresponding random coil values.²⁶ As expected for a highly flexible peptide with 17 residues, $NH-C^{\alpha}H$ coupling constants for all the residues are of the order of 6.5-7 Hz. Even though $d_{\alpha N}$ contacts could be observed at a mixing time of 800 ms, no other intersidue contacts were observed at room temperature. However, when the sample temperature was lowered to 5 °C, a number of additional interresidue NOE contacts were observed, indicating the formation of some secondary structures. With the exception of Gln 95 (residue

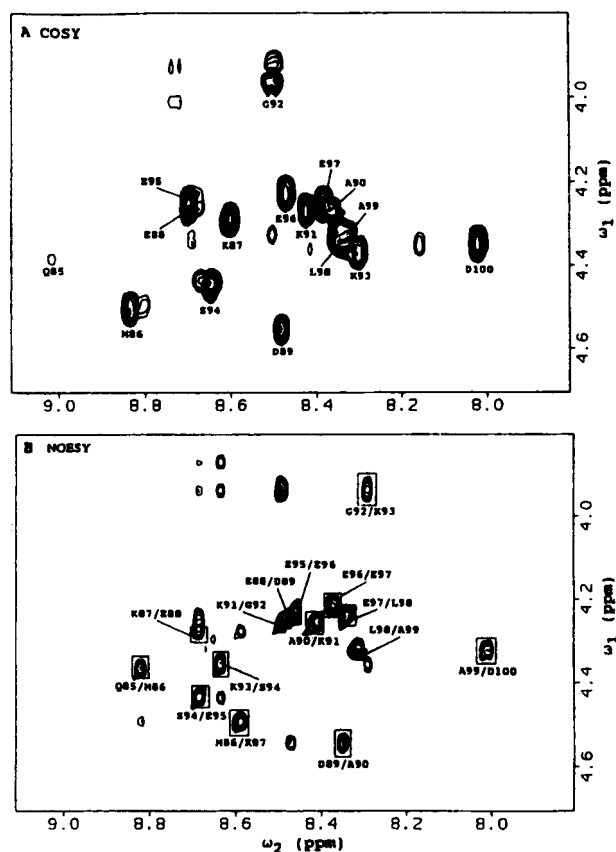


Figure 3. (A) 600 MHz 2-D COSY fingerprint region (unsymmetrized) of peptide C_{84-100} showing the $NH-C^{\alpha}H$ cross peaks in 90% $H_2O/10\%$ D_2O at 5 °C, pH 5.6. (B) 2-D NOESY unsymmetrized spectrum (mixing time 400 ms) of the same sample at 5 °C. The $d_{\alpha N}(i, i+1)$ NOESY cross peaks are enclosed by squares. The $d_{\alpha N}(i, i+3)$ contact between residues 92 and 95 is weak and not shown here.

Table 2. Proton NMR chemical shift assignments for troponin C peptide C₄₋₁₀₀. Chemical shifts are expressed in ppm from internal DSS. The sample was maintained at 5 °C, pH 5.6

Residue	NH	C ^α H	C ^β H	Other	³ J _{HNG} (±0.8 Hz)
Arg84		4.05	1.91, 1.91	C ^γ H ₂ 1.60, 1.65 C ^δ H ₂ 3.22 N ^ε H ₂ 7.34	
Gln85	9.01	4.38	1.97, 2.02	C ^γ H ₂ 2.37, 2.58 N ^ε H ₂ 7.02, 7.73	9.4
Met 86	8.83	4.51	1.99, 2.06	C ^γ H ₂ 2.56, 2.62	7.4
Lys87	8.60	4.28	1.76, 1.82	C ^γ H ₂ 1.44, 1.45 C ^δ H ₂ 1.68 C ^ε H ₂ 3.00 N ^ε H ₂ 7.63	6.1
Glu88	8.70	4.26	1.96, 2.03	C ^γ H ₂ 2.28, 2.32	6.9
Asp89	8.48	4.55	2.66, 2.70		7.3
Ala90	8.26	4.27	1.39		6.1
Lys91	8.44	4.27	1.79, 1.85	C ^γ H ₂ 1.48, 1.40	7.0
Gly92	8.50	3.92			11.7
Lys93	8.30	4.37	1.76, 1.83	C ^γ H ₂ 1.40, 1.43	7.4
Ser94	8.64	4.44	3.89, 3.95		7.1
Glu95	8.70	4.25	2.08, 2.03	C ^γ H ₂ 2.26, 2.30	6.9
Glu96	8.47	4.23	1.94, 2.03	C ^γ H ₂ 2.27, 2.29	6.6
Glu97	8.28	4.26	1.94, 2.02	C ^γ H ₂ 2.27, 2.32	7.2
Leu98	8.24	4.33	1.68, 1.57	C ^γ H ₂ 1.65 C ^δ H ₂ 0.87, 0.92	7.6
Ala99	8.32	4.34	1.39		6.8
Asp100	8.02	4.35	2.56, 2.66		6.4

2 in the peptide), all other vicinal NH-C^αH coupling constants remained essentially the same, suggesting retention of significant conformational flexibility. The amide proton resonance region of the 2-D NOESY spectrum in 90% H₂O/10% D₂O at 5 °C and pH 5.6 is shown in Figure 5. The observation of sequential d_{NN}(i, i+1) and d_{αN}(i, i+1) NOEs indicates folded structures. The amide proton resonances of Glu 97, Leu 98, and Ala 99 residues are too close to resolve NOEs among them. Three d_{αN}(i, i+2) connectivities, one d_{αN}(i, i+3) and two d_{αβ}(i, i+3) NOEs indicative of helical structures are observed. Figure 4 shows two α-α NOE connectivities. These data indicate a bent structure involving residues 89-94. All these NOE connectivities are summarized in Figure 6. Most of the NOEs shown in this figure were measured at a mixing time of 400 ms, except the d_{αβ}(i, i+3) contact between residue 95 and 98 which was observed at 500 ms mixing time.

At room temperature the chemical shifts of the individual residues suggest the peptide to be a random coil in aqueous solution. This finding is consistent with CD data which show a small positive mean residue ellipticity at 222 nm. The small negative ellipticity observed with the peptide dissolved in a helix-promoting solvent suggests that the helicity increases in this solvent.

The d_{NN} NOE contacts suggest a nascent helix near the amino terminus. A helical conformation is suggested by the medium range NOE contacts [d_{αN}(i, i+2), d_{NN}(i, i+3), and d_{αβ}(i, i+3)] and the d_{NN} contacts in the segment of residues 89-98. This helix is in a fast equilibrium with a substantial po-

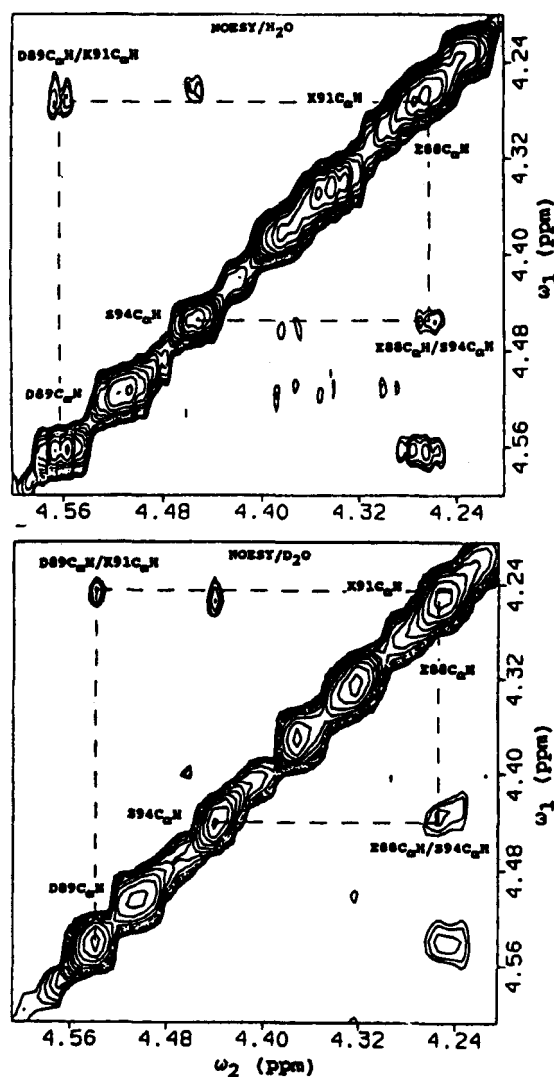


Figure 4. 600 MHz 2-D NOESY proton NMR spectra (mixing time 800 ms) in (A) 90% H₂O/10% D₂O and in (B) D₂O solutions at 5 °C and pH 5.6. The spectra show two interresidue α-α NOE contacts, suggesting bent structures. The NOESY spectrum of the sample in (A) with a mixing time of 400 ms also shows these contacts (data not shown).

pulation of extended and random coil forms and some bent forms. These bent forms are suggested by our observation of two α-α contacts (Figure 4). Two pairs of residues (88 and 94, 89 and 91) are involved in the observed interresidue α-α NOEs. If the peptide bends in the region of Gly 92, the motion can easily bring about the first α-α NOE connectivity. Since the peptide in solution does not have a stable secondary structure and is possibly interconverting between different conformers some of which could have salt bridges that are different from the ones observed in the crystal structure,²⁷ an α-α NOESY contact between residues 89 and 91 is not unexpected.

Discussions

Short peptides (≤20 residues) have been predicted by the Zimm-Bragg equation²⁸ and the host-guest data of random copolymers of amino acids²⁹ not to have detectable helical

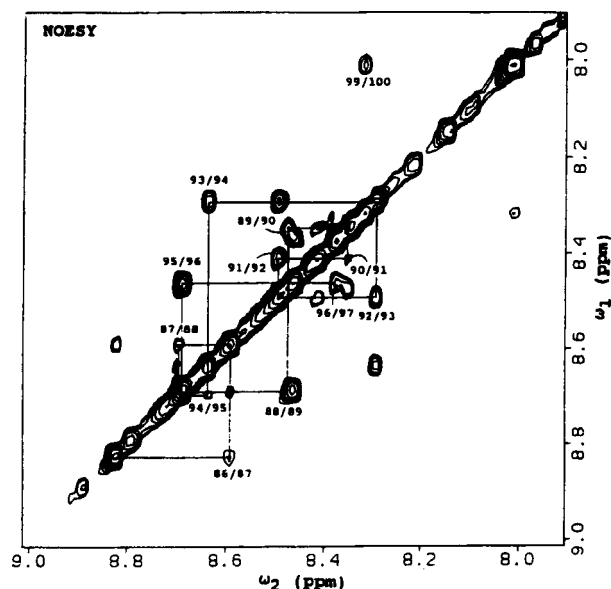


Figure 5. 2-D NOESY spectrum showing $d_{NN}(i, i+1)$ contacts between residues 86 to 97 and 99, 100 of peptide C_{84-100} at 5 °C and pH 5.6. The mixing time was 800 ms. These peaks were also observed at 400 ms mixing time (data not shown), but were much weaker in intensity.

content in water. However, a number of studies from different laboratories have detected folded conformations for short flexible peptides. Folded conformations have been detected in penta- and octapeptides on the basis of retarded amide hydrogen exchange rates.^{30,31} Recent experiments have established that the α -helix formed by the ribonuclease C-peptide (13 residues) is largely stabilized by favorable interaction between charged side chains and the helix dipole although other interactions, such as salt bridge formation, may also play a contributory role.³² A synthetic immunogenic 19-residue peptide corresponding to the C-helix of myohemerythrin exists as a nascent helix composed of a conformational ensemble of turn-like structures.²¹ Other recent NMR studies have also indicated at least partial helix formation by peptides in aqueous solution.³³⁻³⁵ These studies

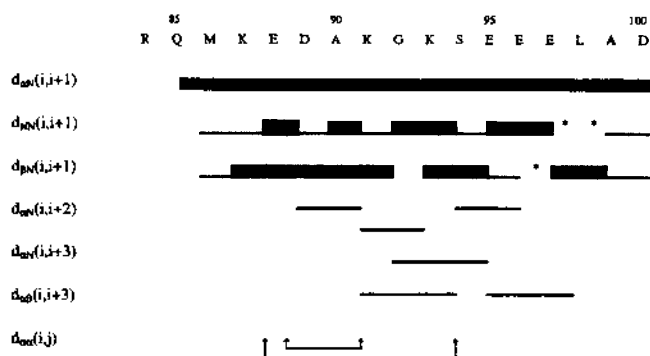


Figure 6. Summary of the NOE connectivities identified in peptide C_{84-100} at 5 °C, pH 5.6. The mixing time was 400 ms (see text). Unresolved region due to resonance overlap is indicated by *. Intensities of observed NOEs are classified by the thickness of the lines. Arrows represent the two interresidue α - α NOE contacts identified in both H_2O and D_2O solutions.

have clearly demonstrated that short peptides can form helices or partial helices in water, and this formation is favored at a low temperature near 0 °C. Our findings of helical structures with C_{84-100} in water are consistent with these previous reports.

If the dominant contributory factor to the stabilization of a short peptide is favorable interaction of charged side chains with the helix dipole,³⁶ the isolated sequence of C_{84-100} may not have an optimal helix stability. The N-terminal residue of the peptide has a side chain that is positively charged, and the C-terminal residue has a negatively charged side chain. The positive charges at the amino end are near the positive pole, and the negative charges at the carboxyl end are near the negative pole of the macrodipole generated by the peptide bond dipole moments. The repulsive interactions at the two ends of the peptide with the helix dipole can result in a destabilization of the helix. However, Lys 98 is two residues from the C-terminus and should provide some stabilizing effect on the macrodipole at this end of the peptide. While the side chains at the N- and C-termini in the free peptide may have less than optimal interaction with helix dipole, the longer segment of the central helix (residues 75-105) in TnC may be more favorably stabilized due to the presence of other different amino acid residues at the two ends of the segment. There are several potential salt bridges between charged side chains spaced three or four residues apart that can stabilize the helix. The residues of the helical in the peptide suggested by NOE contacts are within a segment where potential electrostatic and/or salt bridge interactions can occur (Asp89-Lys93, Lys91-Glu95, and Lys93-Glu96). In fact, these same and additional salt bridges have been invoked as the major interactions that can stabilize the middle segment of the TnC central helix and are responsible for the observed extended D/E helix in the crystal structure of the protein.^{9,27} Most of the amino acid residues of C_{84-100} are of relatively high helix forming propensity, except Gly92 and Ser94, which are in the middle of the helix. These two residues prefer turn-like structures and can induce conformational changes that may affect the overall conformation of the peptide. If the bent structure is in a fast equilibrium with other conformations, the segmental flexibility induced by these residues can further reduce the α -helicity. When the various helix-disrupting and helix-forming factors are taken into consideration, it appears that a segment of C_{84-100} can exist in a helical conformation in aqueous solution. The observed relatively low helicity in water and TFE is a manifestation of the balance of the various stabilizing and destabilizing interactions.

Low-angle X-ray scattering³⁷ and fluorescence resonance energy transfer³⁸ studies have shown that TnC has a solution conformation that is compatible with a segmental flexibility in the central linker. Heteronuclear multi-dimensional NMR studies for calcium saturated skeletal troponin-C have also supported that the interconnecting linker has small number of NOE constraints due to conformational disorder at 40 °C.¹⁰ The present NMR results suggest that the segmental flexibility of the central helix could arise from a short stretch involving Gly92. In spite of the flexibility, the middle one-third of the central helix can exist in a helical conformation in an aqueous environment. This flexibility can provide a mechanism for communication between the two

domains of the protein and this communication, as has been suggested, may be involved in the interaction with troponin I to initiate the activation process.

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