serine proteinase inhibitors, suggest that hirudin is a representative of a new family of inhibitors (*21*).

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8. A 2.3 Å resolution set of intensity data was collected with a FAST area detector at 5.2-kW x-ray power. The data were corrected for absorption using equivalent reflections to determine an absorption ellipsoid and were evaluated on-line with the program MADNES [A. Messerschmidt and J. W. Pflugrath, *J. Appl. Crystallogr.* **20**, 306 (1987); 164,250 measurements, 23,521 independent reflections (91%), $R_{merge} = \Sigma(I - \langle I \rangle)/\Sigma I = 0.042$ between averaged Friedel pairs, 60% measured between 2.3 to 2.4 Å resolution]. Patterson methods were used to determine the orientation of the thrombin molecule in crystals of the complex. The rotation search was performed with the thrombin structure of PPACK–α-thrombin (*4*) with PROTEIN [W. Steigemann, thesis, Technische Universitat München (1974)]. The rotation function was calculated in Patterson space (7565 peaks, 8.0 to 3.0 Å shell) and the highest peak corresponded to the correct solution (7.7σ above the mean). An angular refinement around the highest peak in steps of 0.5° increased it to 8.1σ. The positioning of the thrombin molecule in the unit cell was achieved by a translation search with programs written by Lattman [E. Lattman, *Methods Enzymol.* **115B**, 55ff (1985), modified by one of us (R.H.)] with 8.0 to 3.0 Å resolution intensity data. The highest peaks (~13σ) in all of the Harker sections occurred at consistent locations to position the molecule at $x = 67.42$, $y = 3.00$, and $z = 52.91$ Å in the correct enantiomorphic space group. The R value (defined as $\Sigma||F_o| - |F_c||/\Sigma|F_o|$) of this solution was 0.39. The rotational and the translational parameters were refined with the rigid-body refinement program TRAREF [R. Huber and M. J. S. Schneider, *Appl. Crystallogr.* **18**, 165 (1985)] reducing R to 0.34. A 3.0 Å ($2F_o - F_c$) map calculated with Sim-weighted model phases [G. A. Sim, *ibid.* **12**, 813 (1959)] showed the thrombin molecule clearly and electron density for most of hirudin. The model of hirudin determined by NMR (7) proved helpful in tracing the backbone of the NH_2-terminal domain; however, there was also density for the COOH-terminal of hirudin. The model of the complex was refined with the energy restraint–crystallographic refinement program EREF [A. Jack and M. Levitt, *Acta Crystallogr.* **A34**, 931 (1978)] with interactive graphics interventions. In the final stages individual temperature factors were included along with 207 solvent molecules at stereochemically reasonable positions based on electron and difference density maps. The R value of this structure is 0.193 for 21,960 reflections between 7.0 and 2.3 Å resolution. The rms deviations from ideal values for bond lengths and angles are 0.013 Å and 2.4°, respectively.
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10. The turn is hinged to Gly residues on either side, which might contribute to the flexibility observed by NMR in solution (7). An optimal superposition of 38 Cα atoms of the hirudin structure, h-Thr^4-Leu^{30}, h-Glu^{38}-Pro^{48}, determined by NMR and in the complex, gave an rms deviation of 0.86 Å. The close correspondence suggests that the structure of the NH_2-terminal domain is basically unaltered when complexed with α-thrombin.
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Calcium-Induced Peptide Association to Form an Intact Protein Domain: ^{1}H NMR Structural Evidence

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The 70-residue carboxyl-terminal domain of the muscle contractile protein troponin-C contains two helix-loop-helix calcium (Ca)-binding sites that are related to each other by approximate twofold rotational symmetry. Hydrophobic residues from the helices and a short three residue β sheet at the interface of the two sites act to stabilize the protein domain in the presence of Ca. A synthetic 34-residue peptide representing one of these sites (site III) has been synthesized and studied by H-1 nuclear magnetic resonance (NMR) spectroscopy. In solution this peptide undergoes a Ca-induced conformational change to form the helix-loop-helix Ca-binding motif. Two-dimensional nuclear Overhauser effect spectra have provided evidence for the formation of a β sheet and interactions between several hydrophobic residues from opposing helices as found in troponin-C. It is proposed that a symmetric two-site dimer similar in tertiary structure to the carboxyl-terminal domain of troponin-C forms from the assembly of two site III peptides in the Ca-bound form.

A FUNDAMENTAL ASPECT OF SUCCESSFUL protein design is an understanding of the processes that stabilize the self-assembly of proteins. Although the correct folding of a protein is ultimately determined by its amino acid sequence, the most important energy contributions that determine protein assembly are derived from hydrophobic interactions, hydrogen bonding, and electrostatic forces (*1*). In many cases, specific information about these interactions can be obtained from the three-dimensional (3-D) structure of a protein as determined by 2-D NMR spectroscopy or x-ray crystallography.

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One approach to study the effects that hydrophobic and electrostatic interactions have on protein structure is to use synthetic peptides to represent specific portions of a protein (*2*). Peptides are much smaller than proteins and generally exist in random coil conformations. However, under suitable conditions peptides may adopt well-defined conformations more representative of their structures found in the native protein (*3*). We used this approach to study the effects that metal-ion binding has on the conformational changes that occur in the Ca-binding protein troponin-C (TnC). This muscle protein contains four highly homologous Ca-binding sites each arranged in a helix-loop-helix motif about 30 amino acids in length (*4*). These sites are organized pairwise to

form two two-site domains that have been shown through x-ray crystallographic techniques to each have an approximate twofold rotational symmetry (*5, 6*). We have been concerned primarily with the high-affinity sites of TnC, sites III and IV, which are located in the carboxyl-terminal domain of the protein. The homology of the primary sequences of these sites and their symmetric relation has suggested that these sites are probably a result of gene duplication. It has been suggested that the short β strand present between these two sites signifies that folding of these two sites is interdependent (*7*).

As a first step in determining the conformational change that occurs upon metal-ion binding in TnC, we have synthesized a 34-residue peptide representing the high-affinity Ca-binding site III of chicken skeletal TnC (SCIII, Fig. 1) and have studied it by high-resolution ^{1}H NMR spectroscopy. This peptide represents the E helix, Ca-binding site III, and the F helix (residues 93 through 126), or about one half of the carboxyl-terminal domain. We have completely assigned the NMR spectrum of the Ca-saturated form in order to determine the 3-D solution structure of this peptide and compare it to the structure of site III in the native protein.

In the absence of Ca, the ^{1}H NMR spectrum of SCIII is characteristic of an unstructured random coil conformation (Fig. 1). There are no resonances downfield of the residual HDO signal and all of the γCH_3 and δCH_3 groups of Ile and Leu form a series of large unresolved resonances centered at ~0.85 ppm. Upon addition of Ca to SCIII, changes occur throughout the spectrum, such as the shifting of the αCH resonances of Y112, I113, and D114 (*8*) to a much lower field (5.2 to 5.3 ppm) and the shifting of the δCH_3 groups of I113, I121, and γCH_3 groups of I104 and I121 (0.2 to 0.5 ppm). Further, the linewidths of many of the resonances become visibly broader in the Ca form than in the apo form. These observations are indicative of a significant structural change of the peptide as well as tight Ca binding (*9*). The complete sequential resonance assignment of Ca-saturated SCIII was accomplished with 2-D ^{1}H NMR techniques, and the secondary structure of the peptide was determined from an analysis

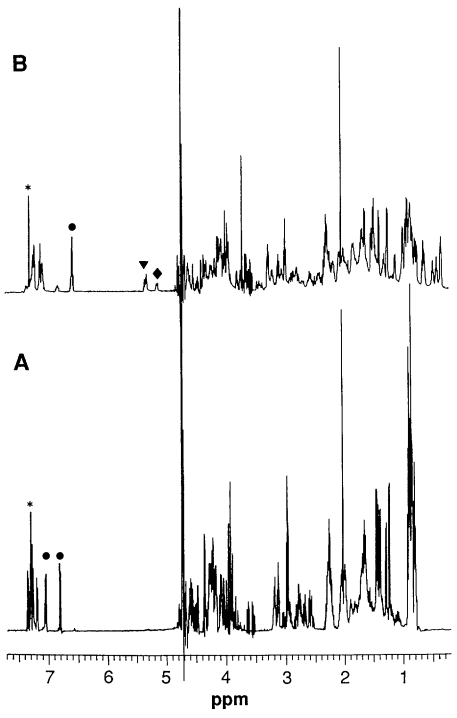


Fig. 1. 500-MHz ^{1}H NMR spectra of 430 μM SCIII in D_2O, 50 mM KCl, and 30 mM imidazole at pH 7.35. The spectra show (**A**) apo-SCIII and (**B**) SCIII and 428 μM $CaCl_2$. ^{1}H NMR spectra were obtained at 30°C on a Varian VXR 500 spectrometer with a spectral width of 5200 Hz, acquisition time of 2 s, and relaxation delay of 2 s. Each spectrum is plotted on the same vertical scale and with the same data processing. Resonances marked are Y112 aromatic protons (●), Y112 αCH (♦), and I113 αCH and D114 αCH (▼). The peak marked (*) results from the 4,5 protons in imidazole.

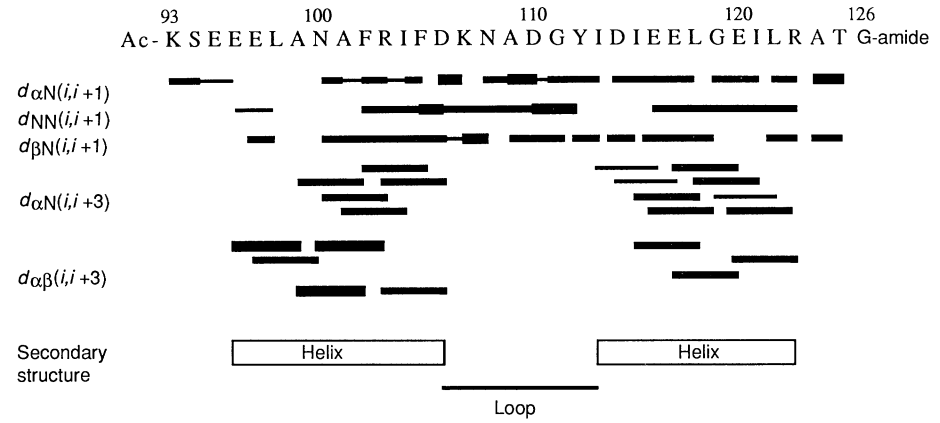


Fig. 2. Summary of the sequential connectivities involving NH, αCH, and βCH protons for SCIII. Abbreviations such as $d_{\alpha N}(i,j)$ correspond to an observed nOe between the αCH proton at position *i* and the NH proton at position *j*. The thickness of the lines corresponds to the intensity of the observed nOe (*10*). The secondary structural elements defined by the nOe connectivities are also indicated (bottom).

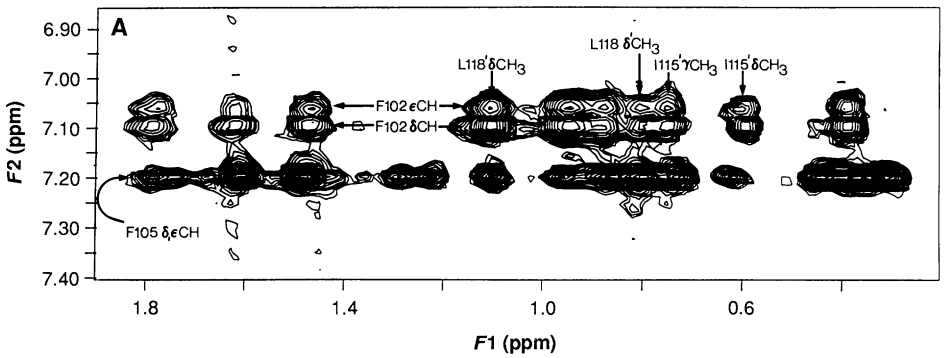
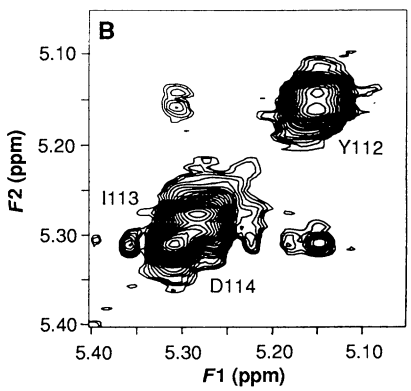


Fig. 3. (**A**) A portion of the 300-ms NOESY spectrum of 4.0 mM SCIII in 80% H_2O–20% D_2O at pH 7.3, 15°C, acquired at 500 MHz (*19*). The spectrum shows nOe cross peaks arising between the aromatic side chains of F102 and F105 (along *F2*) of SCIII and the methyl side chains of I115′ and L118′ (along *F1*) from SCIII′. (**B**) Portion of a NOESY spectrum under the same conditions as (A) but in D_2O and with a 200-ms mixing time. The spectrum shows the Y112 αCH, I113 αCH, and D114 αCH diagonal peaks and the Y112 αCH, D114′ αCH cross peaks characteristic of the involvement of these residues in a β sheet. The spectra were unsymmetrized and processed with 4 K by 4 K data points. In spectrum (B) splitting of the Y112 αCH diagonal peak and cross peak occurs in the *F2* dimension due to greater resolution in that dimension.

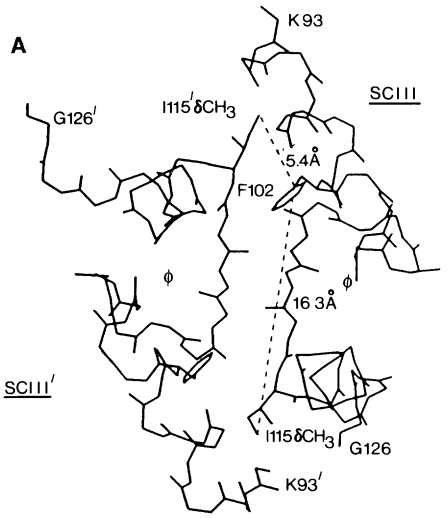


of the short-range nuclear Overhauser effects (nOes) (*10*). A summary of these connectivities is found in Fig. 2. Several strong $d_{\alpha N}(i,i + 3)$ or $d_{\alpha\beta}(i,i + 3)$ cross peaks, indicative of α helix, are present from residues 96 to 106 and from residues 113 to 123. This result clearly indicates that two separate well-defined helices are present. An intense stretch of $d_{NN}(i,i + 1)$ cross peaks is found spanning residues 101 to 112. The lack of $d_{\alpha N}(i,i + 3)$ or $d_{\alpha\beta}(i,i + 3)$ cross peaks for residues 106 to 112 shows that this portion of the peptide is not α helical. The $d_{NN}(i,i + 1)$ cross peaks suggest that these residues are arranged so that the amide protons of each residue are all relatively close in space (*11*). This result would be expected if a Ca-binding loop were formed similar to that found in x-ray analyses of TnC (*5, 6*). It is clear from this interpretation that the peptide has formed the essential helix-loop-helix motif required for Ca binding as is found in the crystal structures of several Ca-binding proteins (*12*).

The most dramatic observation in the NOESY spectra of the Ca-bound form of SCIII is the inordinate number of nOe cross peaks made by the F102 and F105 aromatic ring residues of the E helix to other residues (Fig. 3A). Upon analysis of these cross peaks it became apparent that many of these interactions were to side chains of several hydrophobic residues from the F helix. Specifically, interactions and therefore close internuclear distances were noted between L98 and G119, A99 and I115, A101 and L118, and F102 with both I115 and L118. These long-range nOes appeared at first sight to be inconsistent with the conclusion that the SCIII peptide had a similar tertiary structure to that found in the native protein. Several strong long-range nOes representing distances (r) of 3.5 to 4.5 Å were present between these residues. In the x-ray structure of TnC these distances range from 13 to 17 Å and would be unobservable by nOe experiments since the nOe cross peak intensity is related to distance by a factor of $1/r^6$. However, these observed nOes could be explained if the peptide had formed a dimer in solution identical in structure to the head-to-tail arrangement of sites III and IV in TnC (Fig. 4A). In this case, one SCIII (residues 93 to 126) peptide would have dimerized with a second identical molecule (SCIII′) representing site IV of TnC and comprising residues 129 to 162 (Fig. 4B). As in TnC, this assembly would require the hydrophobic interaction of residues from two pairs of helices and the formation of a short three-residue β sheet between the two Ca-binding sites. We have used these two criteria to show that SCIII is forming a symmetrical two-site dimer similar in structure to the carboxyl-terminal domain of TnC.

The x-ray structures of TnC and several other Ca-binding proteins (*12*) have revealed that the residues at the interface of two adjacent helix-loop-helix subunits interact favorably to form a highly conserved hydrophobic core (*13*). In TnC, hydrophobic residues at the interface of helix E of site III form a largely aromatic cluster with residues from helix H of site IV. Similarly, an aliphatic core is formed between helix F and helix G of the two sites. Strynadka and James (*12*) have shown that the predominant hydrophobic interactions occur between the side chains of F102 with F151 and F154 and of L98 with F154 at the E-H helix interface. At the F-G helix interface, L118 and I115 interact primarily with I134 and M138. For SCIII, the formation of a symmetric dimer would necessitate that residues I134, M138, F151, and F154 of site IV would be coincident with residues L98′, F102′, I115′, and L118′ of SCIII′ (Fig. 4B). In consonance with the x-ray studies, intense nOe cross peaks were found between the aromatic side chain of F102 and the αCH, γCH3, and δCH3 groups for I115′ (Figs. 3A and 4B) and the βCH_2, δCH_3, and $\delta CH_{3'}$ groups of L118′. Furthermore, nOe cross peaks were also observed between the δCH_3 groups of L98 of SCIII and the βCH_2 and NH protons of L118′ and the NH protons of G119′ of SCIII′. In addition, interactions were also noted for: A99 αCH with I115′ δCH3; A99 βCH_3 with L122′ and L118′ δCH_3 groups; A101 NH and βCH_3 with L118′ δCH_3 groups; and A101 βCH_3 with L122′ δCH_3 groups (Fig. 4). All of these interactions are hydrophobic in nature and are consistent with x-ray findings for TnC, suggesting that the assembly of two SCIII peptides has occurred to form a symmetric two-site dimer.

The second criterion used to verify the assembly of a SCIII dimer was the formation of a β sheet between two SCIII peptides. In the x-ray structures of TnC an antiparallel β sheet has been identified between residues 112 to 114 of site III and 148 to 150 of site IV (*5, 6*). In a SCIII dimer, the analogous residues would be Y112, I113, and D114 of SCIII and Y112′, I113′, and D114′ of SCIII′ (Fig. 4B). Evidence that a β sheet was forming between these residues was derived from three characteristic NMR phenomena (*14*): (i) the low-field NH resonances of residues in a β sheet arising from hydrogen-bonding patterns; (ii) the low-field αCH resonances resulting from the preferred conformation of residues in a β sheet; and (iii) the presence of a single nOe between two αCH residues on opposite strands that are uniquely close in space. The presence of all three of these criteria strongly suggest that



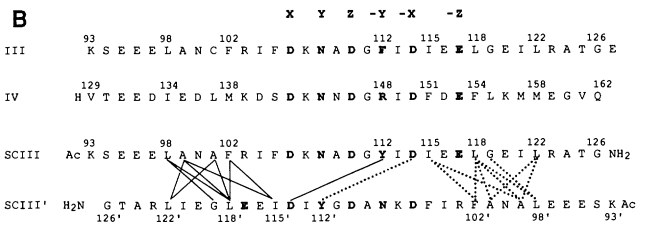


Fig. 4. (**A**) Proposed arrangement of SCIII and SCIII′ peptides forming a head-to-tail dimer (α-carbon backbone shown) as determined from NMR data. The dimer model was generated with x-ray coordinates from turkey TnC for residues 93 to 126 and 129 to 162 (*5*). The program MUTATE (written by R. Reid) was used to correct the amino acid sequence in each case. The x-ray interchain distance for F102 γC to I115 δCH_3 (16.3 Å) is compared to the much shorter interchain distance for F102 γC to I115 δCH_3 (5.4 Å) in agreement with the nOe data; Ca ions are shown as spheres. (**B**) The primary amino acid sequences (*8*) of sites III and IV of chicken skeletal TnC (*20*) and the sequence for the synthetic site III peptide SCIII are shown on the top three lines (SCIII′ is identical to SCIII). Peptide SCIII is amino terminal–acetylated and carboxyl terminal–amidated. The positions ±**X**, ±**Y**, and ±**Z** correspond to the metal-ion coordinating ligands. The four helices for the two sites in TnC encompass the following residues; helix E, 95 to 105; helix F, 115 to 125; helix G, 131 to 141; and helix H, 151 to 162 (*12*). The bottom two lines show the head-to-tail arrangement of SCIII and SCIII′ in the dimer. The sequence of SCIII′ is reversed (that is, from the carboxyl-terminal end to the amino-terminal end) to more clearly show the interactions and to more closely represent the alignment of residues as found for sites III and IV in the TnC x-ray structures (*5, 6*). The interchain nOes showing some of the interactions between SCIII and SCIII′ are indicated by the solid and dotted lines.

the residues are involved in a β-sheet structure. In SCIII there are four amide resonances found at very low field: G111 NH (10.4 ppm); Y112 NH (8.0 ppm); I113 NH (9.4 ppm); and D114 NH (8.8 ppm). These latter three residues, Y112 to D114, also have significantly shifted αCH resonances located at 5.2 (Y112) and 5.3 ppm (I113 and D114). All of these ^{1}H NMR assignments for SCIII are in excellent agreement with those of Ca-bound rabbit TnC (*15*). In x-ray studies of TnC, the αCH protons of D114 and R148 and F112 and D150 of the β sheet are proximate, and in solution an nOe is present between these two protons (*15*). For SCIII, the analogous contact would be between D114 and Y112′ (and Y112 and D114′). An nOe between these protons was observed (Fig. 3B), indicating that the β sheet is present between SCIII peptides in solution (*16*).

In this report we present conclusive ^{1}H NMR evidence that two 34-residue site III peptides from TnC have assembled only in the presence of Ca to form a symmetric dimer in solution (*17*). The nOe results also suggest that the structure of the dimer is similar, if not identical, to that for the carboxyl-terminal domain of TnC. These results substantiate the use of synthetic peptides to study the folding and assembly of proteins and allow fundamental studies for protein design to be explored with this synthetic protein. Of particular interest for this study is the energetics of the hydrophobic interactions between helices of the SCIII peptides. In effect, we have simplified the 70-residue carboxyl-terminal domain of TnC to 34 residues without sacrificing tertiary structure. This should allow the interaction of drugs and other components of the troponin complex (*18*) to be efficiently studied with this peptide alone as the carboxyl-terminal half of TnC.

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15. S. Tsuda, Y. Hasegawa, M. Yoshida, K. Yagi, K. Hikichi, *Biochemistry* **27**, 4120 (1988).
16. The x-ray structure of turkey TnC (*5*) reveals that the β sheet formed between sites III and IV forms only two hydrogen bonds between residues I113 and I149. Furthermore at either end of the β sheet a water molecule separates the two strands. As a result the intermolecular distance between the two strands is larger than for a classical antiparallel β sheet. The average distance between α-carbons of residues 112 and 150 and of 114 and 148 is 4.72, implying 2.76 Å for the α-protons. From Fig. 3B, the calculated distance measured from volume integration is 2.97 Å, in good agreement with the x-ray findings.
17. Further support for the assembly of two peptides is derived from Ca titration experiments. During these experiments, the upfield-shifted aromatic resonances and several methyl resonances (Fig. 1) are monitored by 1-D ^{1}H NMR as a function of added Ca. We observe only two species in slow exchange; the unfolded apo-peptide and the structured dimer. Titration curves have shown that only 1 mol of Ca is required to fold 2 mol of SCIII peptide. Upon addition of a second mole of Ca we observe no further spectral changes in these resonances. These observations are consistent with a conformational change in SCIII upon binding of Ca, dimerization of a Ca-bound SCIII with an apo SCIII and subsequent conformational change in the apo SCIII, and finally binding of a second Ca molecule to the SCIII dimer. The resonances used to monitor these interactions reflect changes in the conformation of the peptides and especially the formation of the hydrophobic core of the dimer, more so than simply the binding of Ca. It is possible that other regions of the ^{1}H spectrum are more sensitive to the presence of Ca (that is, the β-methylene protons of coordinating amino acids) and would show slight differences in the dimer with one molecule of Ca. This report reflects nOe spectra for two-peptide, two-Ca dimer.
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19. The nOe build-up curves from NOESY spectra taken at 75, 150, and 300 ms showed that the nOe intensity was approximately linear with the mixing time for the cross peaks shown.
20. Residue 100 in chicken TnC, originally thought to be Asp, has been subsequently shown to be Asn (L. B. Smillie, personal communication).

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Characterization of Naturally Occurring Minor Histocompatibility Peptides Including H-4 and H-Y

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Minor histocompatibility (H) antigens can be peptides derived from cellular proteins that are presented on the cell surface by major histocompatibility complex (MHC) class I molecules. This is similar to viral antigens, because in both cases cytotoxic T lymphocytes (CTLs) recognize artificially produced peptides loaded on target cells. Naturally processed minor H peptides were found to be similar to those artificial CTL-epitopes, as far as size and hydrophobicity is concerned. The peptides studied were isolated from a transfectant that expressed a model CTL-defined antigen, β-galactosidase, from male cells that express H-Y, which has been known operationally since 1955, and from cells that express H-4, known since 1961.

MHC CLASS I–RESTRICTED CTLs are essential for immunity to intracellular pathogens (*1*). In addition, CTLs are also involved in rejection of transplanted foreign tissues. If tissue donor and recipient are matched for MHC genes, but not for other genes, recipient CTLs may recognize donor minor H antigens (*2*). CTLs are thought to recognize complexes of MHC class I molecules and peptides derived from cellular proteins, since MHC-restricted CTLs can recognize artificially produced peptides on suitable target cells and since

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