Interactions between Paired Calcium-Binding Sites in Proteins: NMR Determination of the Stoichiometry of Calcium Binding to a Synthetic Troponin-C Peptide†

Gary S. Shaw, Linda F. Golden, Robert S. Hodges, and Brian D. Sykes*

Contribution from the Department of Biochemistry and MRC Group in Protein Structure and Function, University of Alberta, Edmonton, Alberta T6G 2H7, Canada.
Received January 8, 1991

Abstract: Calcium binding to a synthetic 34-residue peptide (SCIII) representing a single calcium-binding site has been determined with use of ^{1}H NMR spectroscopy. In solution this peptide undergoes a calcium-induced conformational change consistent with the transition from a "random coil" to helix–loop–helix motif. In addition, previous two-dimensional ^{1}H NMR studies have shown that the calcium-bound form of SCIII is a two-site dimer. In this work, calcium titration data revealed that the stoichiometry for calcium binding to SCIII was 1:2 Ca:peptide ratio, indicating that the binding of a single calcium enables the folding of two polypeptide chains. This mechanism is consistent with other protein–protein association pathways that display a cooperative transition. In addition, the calcium dissociation constants for the binding of two calciums were determined and found to be 3 μM and >1 mM. The latter constant was verified with use of a novel NMR indicator, diglycolic acid, to probe calcium binding.

Calcium-binding proteins form an important class of regulatory proteins that control such diverse processes as muscle contraction, enzyme activation, and cell growth. For many of these metabolic events the key steps are the binding of calcium to the protein, the induction of a significant conformational change, and the triggering of a biological response. The common structural feature of many calcium-binding proteins is the metal ion binding site. This contiguous sequence of about 30 amino acids forms a highly conserved helix–loop–helix structural motif upon calcium coordination.[1-3] Frequently, this helix–loop–helix binding site is paired with a second metal ion binding site forming a two-site domain. X-ray crystallographic studies have shown that several calcium-binding proteins such as parvalbumin,[3,4] troponin-C,[5,6] calmodulin,[7] and calbindin D_{9K}[8] possess these paired domains, which have further been found to have a pseudo-2-fold rotational symmetric relationship between calcium-binding sites. The calcium affinities and stoichiometries of these calcium-binding proteins have been measured by equilibrium dialysis[9] and recently by spectroscopic techniques such as NMR spectroscopy,[10-13] fluorescence, and circular dichroism.[14,15]

One approach for investigating the calcium-binding properties of these proteins is to employ synthetic peptides representing a single contiguous helix–loop–helix sequence.[16-18] This technique has been used successfully for studying the calcium-binding properties of some calcium-binding proteins.[19] In particular, Hodges and co-workers have studied peptides representing site III from the skeletal muscle protein, troponin-C (TnC).[16,18] In the C-terminal domain of TnC, site III is paired with site IV[5,6] (a similar arrangement occurs in calmodulin[7]). This approach has allowed the calcium-binding properties of a single site to be compared to those for a two-site domain in order to ascertain possible interactions between sites. For example, it was found that the calcium dissociation constant for the isolated site III peptide (K_d = 3.8 × 10^{-6} M)[16] was approximately 200-fold higher than the dissociation constant for a tryptic fragment containing both sites III and IV (K_d = 1.9 × 10^{-8} M),[15] suggesting that a positive interaction exists between sites III and IV in troponin-C which enhances their calcium-binding properties and stabilizes the intact domain.

Interactions that occur in a folded protein domain can be hydrophobic, electrostatic, or hydrogen bonding in nature and are not restricted to calcium-binding proteins, but in general contribute significantly to the folding pathway of the protein.[20] However, most studies dealing with these forces that stabilize proteins have concentrated on non-calcium binding proteins, although there are a few exceptions.[21] As with the earlier work by Hodges on calcium-binding proteins, a great deal of interest has focussed on the use of model peptides to assess protein-folding events and their implications for protein design.[22-27] Specifically, the interactions

† This study was supported by a grant from the Medical Research Council of Canada and an Alberta Heritage Foundation for Medical Research Fellowship (G.S.S.).

(1) Marsden, B. J.; Shaw, G. S.; Sykes, B. D. *Biochem. Cell Biol.* **1990**, *68*, 587.
(2) Reid, R. E.; Hodges, R. S. *J. Theor. Biol.* **1980**, *84*, 401.
(3) Kretsinger, R. H.; Nockolds, C. E. *J. Biol. Chem.* **1973**, *248*, 3313.
(4) (a) Swain, A. L.; Kretsinger, R. H.; Amma, E. L. *J. Biol. Chem.* **1989**, *264*, 16620. (b) Declercq, J. P.; Tinant, B.; Parello, J.; Etienne, G.; Huber, R. *J. Mol. Biol.* **1988**, *202*, 349.
(5) Herzberg, O.; James, M. N. G. *J. Mol. Biol.* **1988**, *203*, 761.
(6) Satyshur, K. A.; Rao, S. T.; Pyzalska, D.; Drendel, W.; Greaser, M.; Sundaralingam, M. *J. Biol. Chem.* **1988**, *263*, 1628.
(7) Babu, Y. S.; Bugg, C. E.; Cook, W. J. *J. Mol. Biol.* **1988**, *203*, 191.
(8) Szebenyi, D. M. E.; Moffat, K. J. *J. Biol. Chem.* **1986**, *261*, 8761.
(9) Potter, J. D.; Gergely, J. *J. Biol. Chem.* **1975**, *250*, 4628.
(10) (a) Shelling, J. G.; Hofmann, T.; Sykes, B. D. *Biochemistry* **1985**, *24*, 2332. (b) Shelling, J. G.; Sykes, B. D. *J. Biol. Chem.* **1985**, *260*, 8342.
(11) Golden, L. F.; Corson, D. C.; Sykes, B. D.; Banville, D.; MacManus, J. P. *J. Biol. Chem.* **1989**, *264*, 20314.
(12) Williams, T. C.; Corson, D. C.; Oikawa, K.; McCubbin, W. D.; Kay, C. M.; Sykes, B. D. *Biochemistry* **1986**, *25*, 1835.
(13) Linse, S.; Brodin, P.; Drakenberg, T.; Thulin, E.; Sellers, P.; Elmdén, K.; Grundström, T.; Forsen, S. *Biochemistry* **1987**, *26*, 6723.
(14) Drabikowski, W.; Brzeska, H.; Venyaminov, S. Y. *J. Biol. Chem.* **1982**, *257*, 11584.
(15) Leavis, P. C.; Rosenfeld, S. S.; Gergely, J.; Grabarek, Z.; Drabikowski, W. *J. Biol. Chem.* **1978**, *253*, 5452.
(16) Reid, R. E.; Gariépy, J.; Saund, A. K.; Hodges, R. S. *J. Biol. Chem.* **1981**, *256*, 2742.
(17) Reid, R. E. *Int. J. Pept. Protein Res.* **1987**, *30*, 613.
(18) Gariépy, J.; Sykes, B. D.; Reid, R. E.; Hodges, R. S. *Biochemistry* **1982**, *21*, 1506.
(19) (a) Borin, G.; Pezzoli, A.; Marchiori, F.; Peggion, E. *Int. J. Pept. Protein Res.* **1985**, *26*, 528. (b) Borin, G.; Ruzza, P.; Rossi, M.; Calderan, A.; Marchiori, F.; Peggion, E. *Biopolymers* **1989**, *28*, 353. (c) Buchta, R.; Bordi, E.; Fridkin, M. *Int. J. Pep. Protein Res.* **1986**, *28*, 289. (d) Malik, N. A.; Anantharamaiah, G. M.; Gawish, A.; Cheung, H. C. *Biochem. Biophys. Acta* **1987**, *911*, 221. (e) Kanellis, P.; Yang, J.; Cheung, H. C.; Lenkinski, R. E. *Arch. Biochem. Biophys.* **1983**, *220*, 530. (f) Marchiori, F.; Borin, G.; Chessa, G.; Cavaggion, G.; Michelin, L.; Peggion, E. *Hoppe-Seyler's Z. Physiol. Chem.* **1983**, *364*, 1019.
(20) (a) Ghelis, C.; Yon, J. In *Protein Folding*; Horecker, B., Kaplan, N. O., Marmor, J., Scheraga, H. A., Eds.; Academic Press: New York, **1982**; pp 136-176. (b) Jaenicke, R. *Prog. Biophys. Mol. Biol.* **1987**, *49*, 117.
(21) (a) Akke, M.; Forsen, S. *Proteins* **1990**, *8*, 23. (b) Linse, S.; Brodin, P.; Johansson, C.; Thulin, E.; Grundstrom, T.; Forsen, S. *Nature* **1988**, *335*, 651. (c) Wendt, B.; Hofmann, T.; Martin, S. R.; Bayley, P.; Brodin, P.; Grundstrom, T.; Thulin, E.; Linse, S.; Forsen, S. *Eur. J. Biochem.* **1988**, *175*, 439.

0002-7863/91/1513-5557$02.50/0

within a protein domain have been monitored by studying the association of synthetic peptide fragments. It has been found that peptide association can be driven by the hydrophobic interactions which stabilize the folded protein such that a cooperative transition occurs between an unfolded polypeptide chain and an assembly of folded subunits. This may also be the case for some calcium-binding proteins such as troponin-C and calmodulin. In the absence of calcium the C-terminal domains of these proteins are largely unfolded.[14,15] Calcium binding induces a significant increase in secondary structure, accompanied by a large structural reorganization of the protein. In the C-terminal domains of troponin-C and calmodulin, X-ray studies have shown that a short β-sheet and numerous hydrophobic contacts are formed at the interface of the two calcium-binding sites (III and IV) when occupied by calcium.[5-7] This framework has been found to be a common element of calcium-binding proteins which have pairs of calcium-binding sites.[28] On the basis of these observations, it has been suggested that folding a pair of calcium-binding sites should be interdependent[8,29] similar to the cooperative transition found for the synthetic peptide association studies.[22-26] This observation has been corroborated by calorimetric studies which show that the calcium-saturated forms of troponin-C and calmodulin unfolded as a "cooperative block".[30]

In order to study the folding pathway of the C-terminal domain of troponin-C we have taken a "protein design" approach which utilizes synthetic peptides representing a single calcium-binding site in the protein. Recently, we have shown that calcium binding to a synthetic site III troponin-C peptide results in a large conformational change accompanied by a self-association process.[31] Two-dimensional ^{1}H NMR studies have provided evidence that the calcium-bound form of the peptide is a stable two-site dimer similar in structure to the C-terminal domain of troponin-C. However, we found that the stoichiometry of calcium binding to this peptide is quite unusual.[31] Subsequently, a report by Kay et al.[32] has shown that calcium binding to site IV of troponin-C also forms a symmetric dimer but only in the presence of a large excess of calcium. In both of these cases two hydrophobic clusters of amino acids were identified in the folded calcium-bound state, similar to those found from X-ray studies of troponin-C, suggesting that their formation is the driving force for peptide self-association. The mechanism for this calcium-induced peptide folding may yield an understanding of the folding in a calcium-binding protein such as troponin-C or for protein association pathways in general. In this study, we have used ^{1}H NMR spectroscopy to follow calcium binding and peptide association of a site III peptide from troponin-C. The stoichiometry for calcium binding and the dissociation constants obtained indicate that binding of a single calcium ion causes a cooperative structural transition from unfolded monomer to folded dimer.

Experimental Section

Materials and Methods. Deuterated imidazole-d_4 was purchased from MSD Isotopes. Calcium chloride and potassium chloride were "Purotronic" grade (99.9975%) and were obtained from Alfa Chemicals. Diglycolic acid was obtained from Aldrich and was recrystallized twice from water and then lyophilized prior to use. The synthesis of the 34-residue peptide (SCIII) comprising residues 93-126 of chicken skeletal TnC was carried out with use of stepwise solid-phase techniques.[33] Two modifications to the sequence were made; cysteine 101 was replaced by alanine to prevent possible intermolecular disulfide bond formation, and phenylalanine 112 was replaced by tyrosine. The amino acid sequence for SCIII is

93 100
Acetyl-Lys-Ser-Glu-Glu-Glu-Leu-Ala-Asn-Ala-Phe-Arg-Ile-Phe-Asp-
110 120
Lys-Asn-Ala-Asp-Gly-Tyr-Ile-Asp-Ile-Glu-Glu-Leu-Gly-Glu-Ile-Leu-
126
Arg-Ala-Thr-Gly-amide

^{1}H NMR Experiments. The binding of calcium to SCIII was monitored by high-field ^{1}H NMR spectroscopy with use of a Varian VXR-500 spectrometer. Samples were typically about 200-500 μM peptide in a volume of 500 or 600 μL of buffer. Typical acquisition parameters were the following: spectral width = 5200 Hz, pulse width (90°) = 15 μs, acquisition time = 2.0 s, temperature = 30 °C. Suppression of the H_2O (or HDO) resonance was accomplished with use of a 2.0-s presaturation pulse. All spectra were referenced to the trimethylsilyl resonance of DSS at 0.00 ppm.

Calcium Titration of SCIII. A solution of SCIII was prepared by dissolving 1.5 mg of lyophilized SCIII in 800 μL of 99.9% D_2O containing 50 mM KCl and 30 mM imidazole-d_4. A chelex solution (1 g/3 mL) was prepared in D_2O, and a 400-μL aliquot of this was filtered to prepare D_2O-washed chelex beads which were added to the SCIII solution. The pH was adjusted to 8.65 (uncorrected) and the solution heated at 65 °C for 45 min. The SCIII solution was rapidly filtered through a 0.45-μm Millipore filter into an Eppendorf tube and the pH adjusted to 7.35 (uncorrected). The concentration of SCIII was determined in triplicate by amino acid analysis by comparing alanine and leucine peak areas to those of a standard 8-nmol sample. The final concentration of apo-SCIII was found to be 431 ± 6 μM.

A calcium stock solution (102.9 mM) was prepared in D_2O containing 50 mM KCl, and its calcium concentration was determined from EDTA titration with murexide indicator or by inductively coupled-plasma (ICP) spectroscopy. Diluted calcium solutions were prepared from this stock solution in D_2O containing 50 mM KCl. Calcium additions (1-5 μL) were made from stock 10.3 or 102.9 mM calcium solutions with use of a calibrated 10-μL Hamilton syringe. Additions were made directly to the NMR tube and the sample was thoroughly mixed. The solution was equilibrated for at least 15 min and a spectrum was acquired. The total volume change during the experiment was 31 μL from an initial volume of 600 μL of SCIII to a final volume of 631 μL. The concentration of SCIII in the NMR tube after the titration was completed was also determined in triplicate by amino acid analysis as described above and found to be 410 ± 19 μM in excellent agreement with that expected (410 μM) on the basis of dilution. The final calcium concentration of this sample was determined by ICP spectroscopy and found to be consistent with that expected on the basis of the volumes of each calcium stock solution added.

Calcium Titration of SCIII in the Presence of Diglycolic Acid. A stock solution of 100.0 mM diglycolic acid (DGA) was prepared by dissolving 134.1 mg of DGA in 10 mL of D_2O. A 400 μM DGA sample for calcium titration was prepared by diluting 4.0 μL of this solution to 1.0 mL with D_2O which was 50 mM KCl and 30 mM imidazole. A 500-μL aliquot was removed and a calcium titration carried out and analyzed by ^{1}H NMR spectroscopy at 25 °C and pH 7.37 (uncorrected). An 11.0 mM stock calcium solution was used for calcium additions. From these data, a standard curve of DGA chemical shift vs $[Ca_t]/[DGA]$ was obtained where $[Ca_t]$ represents the total Ca^{2+} concentration.

A solution comprised of both DGA and SCIII was also prepared. A 500-μL sample containing 289 μM SCIII was prepared in a similar manner as previously described, and 1.5 μL of the stock 100.0 mM DGA solution was added to the SCIII solution. The final DGA concentration was 300 μM. The pH of the sample was adjusted to 7.23 (uncorrected).

Concentration-Dependence Studies. The dependence of the association equilibrium involved in forming the SCIII dimer was assessed by using a 500-μL sample of SCIII (177 μM) in the buffer previously described. The sample was titrated with a 10.3 mM calcium solution to a Ca:peptide ratio of 0.56. Aliquots of the sample were then removed in succession and replaced with an equal volume of buffer. In this manner, a dilution series comprised of six samples of SCIII was prepared, ranging in concentration from 177 to 14 μM but having identical Ca:peptide ratios. For each sample a ^{1}H NMR spectrum was acquired and the composition of apo- and calcium-bound peptide analyzed.

Calcium Dissociation Constants. The mechanism for calcium binding and peptide association will be shown to be comprised of three interde-

(22) Bowie, J. U.; Sauer, R. T. *Biochemistry* **1989**, *28*, 7139.
(23) Ho, S. P.; DeGrado, W. F. *J. Am. Chem. Soc.* **1987**, *109*, 6751.
(24) Oas, T. G.; Kim, P. S. *Nature* **1988**, *336*, 42.
(25) Regan, L.; DeGrado, W. F. *Science* **1988**, *241*, 976.
(26) DeGrado, W. F.; Wasserman, Z. R.; Lear, J. D. *Science* **1989**, *243*, 622.
(27) Kaumaya, P. T. P.; Berndt, K. D.; Heidorn, D. B.; Trewhella, J.; Kezdy, F. J.; Goldberg, E. *Biochemistry* **1990**, *29*, 13.
(28) Strynadka, N. C. J.; James, M. N. G. *Annu. Rev. Biochem.* **1989**, *58*, 951.
(29) Kretzinger, R. H. *Cold Spring Harbour Symp. Quant. Biol.* **1987**, *52*, 499.
(30) (a) Tsalkova, T. N.; Privalov, P. L. *Biochim. Biophys. Acta* **1980**, *624*, 196. (b) Tsalkova, T. N.; Privalov, P. L. *J. Mol. Biol.* **1985**, *181*, 533.
(31) Shaw, G. S.; Hodges, R. S.; Sykes, B. D. *Science* **1990**, *249*, 280.
(32) Kay, L. E.; Forman-Kay, J. D.; McCubbin, W.; Kay, C. E. *Biochemistry* **1991**, *30*, 4323.
(33) Hodges, R. S.; Semchuk, P. D.; Taneja, A. K.; Kay, C. M.; Parker, J. M. R.; Mant, C. T. *Pept. Res.* **1988**, *1*, 19.

pendent equilibria, eq 1–3, where P, P*Ca, P^*_2Ca, and $P^*_2Ca_2$ represent apo-SCIII, folded calcium-bound SCIII monomer, folded SCIII dimer with a single calcium, and the calcium-saturated dimer, respectively.

$$K_1 = \frac{[P][Ca]}{[P^*Ca]} \quad (1)$$

$$K_d = \frac{[P^*Ca][P]}{[P^*_2Ca]} \quad (2)$$

$$K_2 = \frac{[P^*_2Ca][Ca]}{[P^*_2Ca_2]} \quad (3)$$

The total concentration of peptide, $[P_t]$, can be represented in terms of [P], [P*Ca], $[P^*_2Ca]$, and $[P_2{}^*Ca_2]$ and is given by

$$[P_t] = [P] + [P^*Ca] + 2[P^*_2Ca] + 2[P^*_2Ca_2]$$

which upon rearrangement becomes

$$[P] = [P_t] - [P^*Ca] - 2[P^*_2Ca] - 2[P^*_2Ca_2] \quad (4)$$

Similarly, the total calcium concentration, $[Ca_t]$, is

$$[Ca_t] = [Ca] + [P^*Ca] + [P^*_2Ca] + 2[P^*_2Ca_2]$$

Substituting in eq 4 for the unknowns $[P^*_2Ca]$ and $[P^*_2Ca_2]$ from eqs 2 and 3 we arrive at

$$[P] = [P_t] - [P^*Ca] - \frac{2[P^*Ca][P]}{K_d} - \frac{2[P^*Ca][P][Ca]}{K_dK_2} \quad (5)$$

By substituting for [P][Ca] from eq 1 and rearranging, eq 5 is converted to its quadratic form, eq 6.

$$[P^*Ca]^2\frac{2K_1(K_2 + [Ca])}{K_dK_2[Ca]} + \frac{[P^*Ca](K_1 + [Ca])}{[Ca]} - [P_t] = 0 \quad (6)$$

A solution to [P*Ca] can be obtained from eq 6 if $[P_t]$, K_1, K_2, K_d, and [Ca] are known. Values for [P], $[P^*_2Ca]$, and $[P^*_2Ca_2]$ can then be calculated from eqs 1–3, respectively. The integral of any resonance from calcium-bound SCIII at each pair of the calcium titration corresponds to a fractional intensity, f, based on the total concentration of SCIII, $[P_t]$, such that

$$f = \frac{[P^*Ca] + 2[P^*_2Ca] + 2[P^*_2Ca_2]}{[P_t]}$$

The calculated curve of f versus $[Ca_t]/[P_t]$ was plotted and values of K_1, K_2, and K_d were chosen to fit the observed data. This was done by fitting all combinations of equilibrium constants as follows: 0.1 $\mu M \le K_1 \le 100$ μM, 1 $\mu M \le K_2 \le 1$ mM, and 0.1 $\mu M \le K_d \le 100$ μM, using a step size of log $K = 0.5$. The free calcium concentrations [Ca] used were selected on the basis of the value of K_1 selected. Typically 500 free calcium concentrations were used per calculation. The best fit curve for calcium binding to SCIII was determined from the K_1, K_2, and K_d values which yielded the lowest standard deviation between the calculated and observed data.

Diglycolic Acid Dissociation Constant. Calcium binding to diglycolic acid was based on the equilibrium shown in eq 7 where L and M represent the free acid and calcium, respectively, and LM is the complex

$$L + M \rightleftharpoons LM \quad (7)$$

The dissociation constant K_{DGA} was derived from eq 8 with use of previously described methods.[34,35]

$$K_{DGA} = \frac{[L][M]}{[LM]} \quad (8)$$

Results and Discussion

Stoichiometry of Calcium Binding to SCIII. In the apo-state, SCIII possesses relatively narrow spectral lines and a near degeneracy of many proton resonances (Figure 1). For example, in the aliphatic region all of the γCH_2, γCH_3, and δCH_3 resonances from the apo species appear as unresolved multiplets between 0.75 and 0.95 ppm. Other groups of resonances fall within well-defined regions in chemical shift as has been reported by Bundi and Wüthrich for "random coil" peptides.[36] These observations suggest that apo-SCIII exists as a "random coil" in solution, consistent with conclusions reached for other calcium-binding proteins[18,35,37] and zinc-binding peptides[38] in the absence of a bound metal ion.

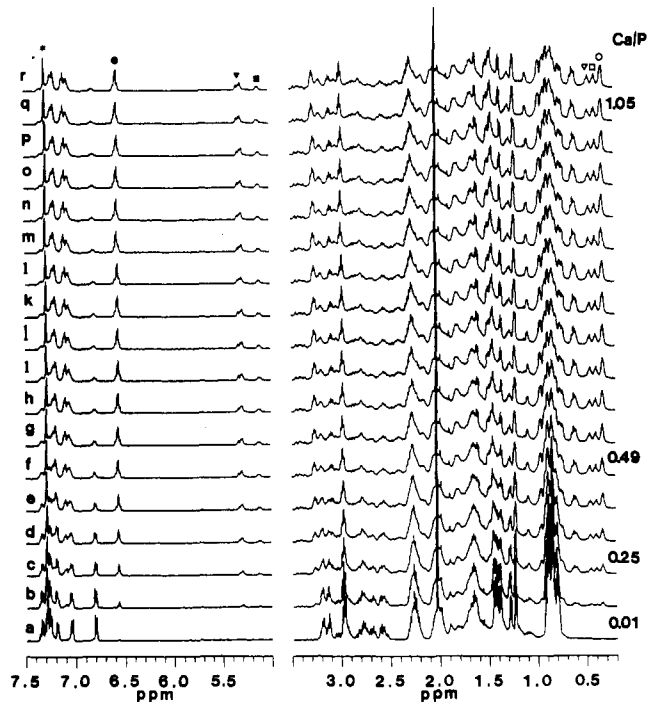

Figure 1. 500-MHz ^{1}H NMR spectra of 430 μM SCIII, shown at the following Ca:peptide ratios: (a) 0.01, (b) 0.13, (c) 0.25, (d) 0.33, (e) 0.41, (f) 0.49, (g) 0.53, (h) 0.57, (i) 0.61, (j) 0.65, (k) 0.69, (l) 0.73, (m) 0.77, (n) 0.81, (o) 0.85, (p) 0.93, (q) 1.05, (r) 1.17. Some specific ratios (Ca/P) are highlighted on the right side of the figure. Resonances for calcium-bound SCIII are marked as follows: I121 δCH_3 (▽), I113 δCH_3 (□), I121 γCH_3 (○), Y112 αCH (■), I113 αCH and D114 αCH (▼), Y112 δ,ϵ protons (●). The resonance marked with an asterisk results from the 4,5 protons of the imidazole ring in the buffer. Conditions are described in the Experimental Section.

The addition of calcium to SCIII resulted in dramatic changes in the ^{1}H NMR spectrum as shown in Figure 1. As the Ca:peptide ratio is increased, new resonances appear and increase in intensity throughout the entire spectrum, consistent with slow exchange kinetics as has been observed for other calcium-binding proteins.[12,13,37,39,40] These resonances also become more disperse, especially those of I104 γCH_3, I113 γCH_3, and I121 γCH_3 and that of I121 γCH′ which is found at −0.38 ppm in calcium-bound SCIII. Significant changes in chemical shift also occur for Y112 αCH, I113 αCH, and D114 αCH which in the calcium-bound form appear as multiplets between 5.2 and 5.3 ppm.[31] In the aromatic region of the spectrum, particularly diagnostic resonances to follow as a function of calcium concentration are those of Y112. In the apopeptide, and δ and ϵ protons are found as a pair of doublets at 7.1 and 6.8 ppm, respectively. Upon addition of calcium, these resonances shift to higher field and appear nearly degenerate at about 6.6 ppm. It should be noted that a resonance at 6.8 ppm which is present in calcium-bound SCIII (F102 ζCH) is also present in the apo-species, but is partially obscured by the Y112 ϵCH doublet.

Calcium binding to SCIII was quantitated by plotting the integral of several resonances from the calcium-bound species as a function of the Ca:peptide ratio. These data were normalized to arrive at the average curve shown in Figure 2. The stoi-

(34) Williams, T. C.; Shelling, J. G.; Sykes, B. D. In *NMR in the Life Sciences*; Bradbury, E. M.; Nicolini, C., Eds.; Plenum Press: New York, **1985**; pp 93–103.
(35) Marsden, B. J.; Hodges, R. S.; Sykes, B. D. *Biochemistry* **1988**, *27*, 4198.
(36) Bundi, A.; Wüthrich, K. *Biopolymers* **1979**, *18*, 285.
(37) Drakenberg, T.; Forsen, S.; Thulin, E.; Vogel, H. J. *J. Biol. Chem.* **1987**, *262*, 672.
(38) Párraga, G.; Horvath, S. J.; Eisen, A.; Taylor, W. E.; Hood, L.; Young, E. T.; Klevit, R. E. *Science* **1988**, *241*, 1489.
(39) Corson, D. C.; Williams, T. C.; Kay, L. E.; Sykes, B. D. *Biochemistry* **1986**, *25*, 1817.
(40) Tsuda, S.; Hasegawa, Y.; Yoshida, M.; Yagi, K.; Hikichi, K. *Biochemistry* **1988**, *27*, 4120.

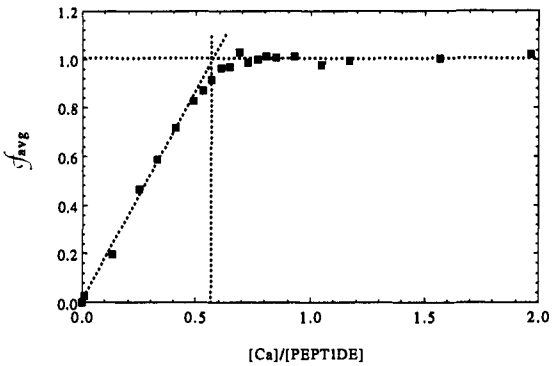

Figure 2. Calcium titration plot for 430 μM SCIII derived from Figure 1. Resonances for Y112 aromatic protons, Y112 αCH, I113 αCH and D114 αCH, I121 δCH_3, I113 δCH_3, and I121 γCH_3, were integrated and averaged to determine f_{av}. Two dashed lines indicate the slopes for the data at low and high Ca:peptide ratios. The vertical dashed line represents the intersection point of these two lines. Conditions are described in the Experimental Section.

chiometry of calcium binding was determined from an intersection of the initial slope at small Ca:peptide ratios and the slope at maximum intensity. As shown in Figure 2, these two lines intersect near the 0.5 Ca:peptide ratio.

The stoichiometry of 0.5 Ca:peptide for SCIII suggests that a simple 1:1 calcium–peptide complex cannot be the species observed during the titration. In other studies where calcium binding to a peptide or protein fragment has been monitored spectroscopically, the stoichiometry usually coincides to the number of calcium ions bound per number of binding sites in the protein. This has been shown for troponin-C, which has four calcium binding sites, and where the stoichiometry is 4:4 Ca:sites. In the tryptic fragments of troponin-C (TR_1C, TR_2C) which each contain two sites, the stoichiometries are 2:2 Ca:sites.[15] However, this is not the case for a thrombin fragment (TH1) which contains three sites and shows a stoichiometry of 1:3 Ca:sites when structure formation is monitored by circular dichroism spectroscopy but has been shown with use of a calcium-specific electrode to be capable of binding three molecules of calcium. This latter results shows that the binding of one calcium is sufficient to fold the entire three-site fragment.[15]

Peptides and protein fragments containing one calcium-binding site have also had their calcium-binding properties assessed. For example, the peptides CB8 (TnC, site II), CB9 (TnC, site III), TH2 (TnC, site IV), and TR_3E (CaM, site IV) have all been shown to bind calcium and dissociation constants have been calculated for these peptides assuming a 1:1 stoichiometry.[14,15,41] In these cases, spectroscopic data have been plotted vs the free calcium concentration and fitted in order to calculate the dissociation constants. While this method is very accurate for determining the K_{Ca} it does not reflect the stoichiometry of calcium binding.

Mechanism for Calcium Binding. The SCIII peptide has only one calcium-binding site. During the calcium titration experiment, calcium binding to SCIII results in a conformational change in SCIII. The new resonances appearing in the ^{1}H NMR spectra of SCIII (Figure 1) are a reflection of the conformational change per unit of calcium added. A stoichiometry of 0.5 Ca:peptide for SCIII suggests that 1 equiv of calcium is sufficient to induce a conformational change in 2 equiv of SCIII and is similar to observations described above for the TH1 fragment of troponin-C where 1:3 stoichiometry was observed.[15]

Three distinct mechanisms for calcium binding to SCIII are possible, but all are not consistent with experimental data. A first mechanism would involve apo-SCIII existing as a dimer in solution. Calcium binding to one subunit could occur and induce a conformational change in both subunits, giving rise to the 1:2 stoichiometry as observed from Figure 2. However, the narrow lines

(41) Nagy, B.; Potter, J. D.; Gergely, J. *J. Biol. Chem.* **1978**, *253*, 5971.

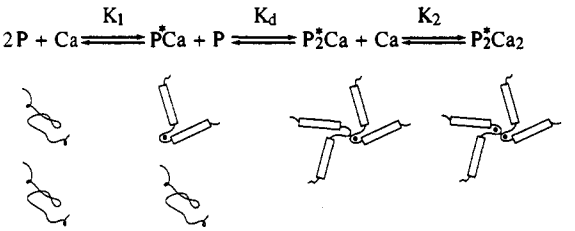

Figure 3. The proposed mechanism for calcium binding and association of SCIII. Helices are represented by open rectangles and bound calcium by solid dots. Apo-SCIII (P) binds to calcium to form a helix–loop–helix (P^*Ca) which associates with a second apo-SCIII peptide (P) to form the helix–loop–helix dimer (P^*_2Ca) and finally binds a second calcium ion to form $P^*_2Ca_2$. In P^*_2Ca and $P^*_2Ca_2$ a 2-fold rotational axis perpendicular to the plane of the paper runs through the center of the dimer. In the three-dimensional structure[31] the symmetry related pairs of helices pack against each other. Equilibrium constants K_1, K_d, and K_2 are described in the Experimental Section.

in the ^{1}H NMR spectrum of apo-SCIII (Figure 1) suggest that the peptide does not exist as a dimer at this point nor as a monomer–dimer equilibrium. Sedimentation equilibrium studies have also shown that apo-SCIII (i.e. chicken troponin-C, residues 93–126) is a monomer.[42] A similar result has been found for the site IV rabbit troponin-C peptide (TH2, residues 121–159) studied by Kay et al.[32] Furthermore, since apo-SCIII appears to be a "random coil" in solution a specific dimerization of two SCIII peptides would not be expected without a significant conformational change as has been found for other peptide association pathways.[22-27,43] A second mechanism would have SCIII as an unstructured monomer in solution. Upon calcium binding a dramatic conformational change occurs to form the helix–loop–helix motif. At the concentrations used for these NMR studies, two of these calcium-saturated monomers could specifically associate to form a dimer. While this mechanism has several merits, it suffers from the drawback that the stoichiometry of calcium binding would be a 1:1 Ca:peptide ratio.

A mechanism that can fit the experimental data is outlined in Figure 3. As indicated, calcium binds to the unstructured peptide and induces a conformational change to form the helix–loop–helix subunit P^*Ca. This peptide associates with an apo-SCIII molecule, P, causing it to undergo an identical conformational change and forming the dimer P^*_2Ca, which is the calcium-bound species observed by NMR spectroscopy (Figure 1). The dimer, now with only one of two possible sites occupied, can bind a second calcium as it is added, to form $P^*_2Ca_2$.[44] This mechanism warrants a strong stabilizing interaction between the two helix–loop–helix subunits, and since the formation of one helix–loop–helix is able

(42) Shaw, G. S. Unpublished results.
(43) (a) Labhardt, A. M.; Baldwin, R. L. *J. Mol. Biol.* **1979**, *135*, 231. (b) Labbhardt, A. M.; Baldwin, R. L. *J. Mol. Biol.* **1979**, *135*, 245.
(44) The mechanism we have used to fit the data is

$$P + Ca \rightleftharpoons P^*Ca \quad K_1$$

$$P^*Ca + P \rightleftharpoons P^*_2Ca \quad K_d$$

$$P^*_2Ca + Ca \rightleftharpoons P^*_2Ca_2 \quad K_2$$

and is described in the Experimental Section. One can include the equilibrium (described in the text)

$$P^*Ca + P^*Ca \rightleftharpoons P^*_2Ca_2 \quad K_d'$$

in this mechanism with no change in the equations used to fit the data. Since no new species are introduced, the equilibrium constant for this reaction can be related by

$$K_d' = (K_2/K_1)K_d$$

Another reaction pathway that may be possible is

$$P + P \rightleftharpoons P^*_2 \quad K_d''$$

$$P^*_2 + Ca \rightleftharpoons P^*_2Ca \quad K_1'$$

where the equilibrium constants are related to those in our mechanism by

$$K_d''K_1' = K_1K_d$$

However, we see no evidence by NMR or sedimentation equilibrium for the existance of the species P^*_2 at significant concentrations.

to induce structure in a second peptide it indicates that a cooperative transition exists between them. This conclusion is consistent with NMR and X-ray crystallographic studies[28,45,46] of many calcium-binding proteins, which show that two hydrophobic cores between pairs of the helix–loop–helix calcium-binding sites act to stabilize a two-site domain.

The mechanism outlined in Figure 3 is similar to other folding pathways, which do not rely on a metal ion to induce structure. For example, DeGrado and co-workers[23,25,26] have shown that peptide association is the key step in the formation of a four-helix bundle. During this process the conformational transition from random coil to α-helix occurs only upon association and an isolated α-helix cannot be detected in solution. Similarly, Oas and Kim[24] have found that peptides representing an α-helix (residues 43–58) and a β-sheet (residues 20–33) region of BPTI have induced structure only when a disulfide bridge between them is intact. Both of these cases show that peptide association is required for the conformational change to occur. This is similar to the proposed mechanism for SCIII where calcium binding appears to form the one calcium dimer, P_2*Ca, directly. The isolated helix–loop–helix P*Ca must be an intermediate in this process but is not observed during the NMR experiments (Figure 1).

The proposed mechanism in Figure 3 shows that binding of the first equivalent of calcium to SCIII results in large chemical shift changes arising from the large conformational changes which occur in the peptides upon both metal ion binding and peptide association. However, between 0.5 and 1.0 equiv of calcium, there were no significant spectral changes (Figure 1). This indicates that a similar flux in conformation does not occur during this stage. Indeed several calcium-binding proteins and protein fragments have been shown to undergo little structural change upon calcium binding, and thus calcium binding can appear "invisible" by some spectroscopic methods. For example, in rat parvalbumin Williams et al.[12] showed that the ^{1}H NMR spectrum of the apo-form is remarkably similar to that of the metal ion bound form, regardless of whether the bound metal was Ca, Mg, or Lu. Similarly, circular dichroism spectroscopy of the N-terminal domain of troponin-C (TR_1C) shows no ellipticity change upon calcium binding, suggesting little change in secondary structure.[15] In this latter case calcium binding to TR_1C must be monitored with use of a calcium-specific electrode. In SCIII, binding of the second molecule of calcium was assumed since it could not be directly monitored by observing the peptide ^{1}H NMR spectrum. By using the approach described in the Experimental Section and the mechanism in Figure 3, the data shown in Figure 2 were fitted for values of K_1, K_d, and K_2. This procedure proved to be unsatisfactory because several combinations of these equilibrium constants were found to satisfy the data.

A unique solution for K_1, K_d, and K_2 was determined with use of a series of dilution experiments where the Ca:peptide ratio was held constant (0.56) but the peptide and calcium concentrations were altered. The ^{1}H NMR spectra in Figure 4 show that at the highest SCIII concentration (420 μM, spectrum a) the Y112 δ and ϵ aromatic protons for calcium-bound SCIII are present near 6.6 ppm and F102 ζCH is near 6.8 ppm. The Y112 ϵ protons from apo-SCIII, also found at 6.8 ppm as shown in Figure 1, are absent in this spectrum. As the SCIII and calcium concentrations are decreased, the Y112 ϵCH doublet at 6.8 ppm appears (overlapping with the F102 ζCH resonance), indicating the presence of apo-SCIII. The Y112 resonance continues to increase in intensity as the SCIII concentration is further lowered. At the lowest SCIII concentration shown (14.0 μM, spectrum g), the resonance at 6.6 ppm, representing calcium-bound SCIII (P^*_2Ca), corresponds to only about 60% of the total peptide on the basis of peak integration. As was the case in the calcium titration experiment, Figure 1, there are only two species present during the dilution experiment, namely P_2*Ca and P. There is no evidence for an isolated helix–loop–helix P*Ca. These data were incorporated with the data from Figure 2 in order to obtain a series of binding curves, Figure 5, which yielded the dissociation constants $K_1 = 0.003 \pm 0.001$ mM, $K_d = 0.01 \pm 0.004$ mM, and $K_2 \approx 1$ mM.

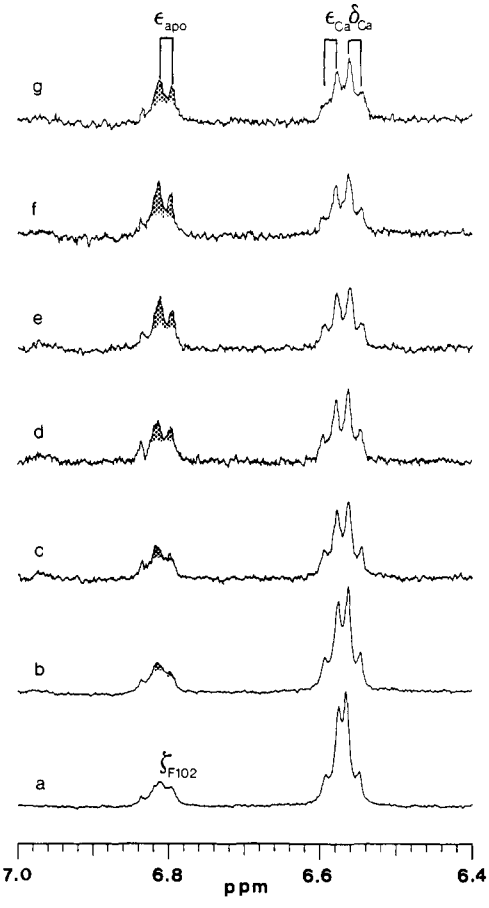

Figure 4. ^{1}H NMR spectra showing the Y112 aromatic region of SCIII at various SCIII concentrations: (a) 420, (b) 177, (c) 85, (d) 44, (e) 26, (f) 17, (g) 14 μM. The Ca:peptide ratio is 0.56 for all cases and the vertical scale was adjusted for concentration. The Y112 δ, ϵ CH resonances for calcium-bound SCIII are present as two doublets centered at 6.57 ppm and are indicated as δ_{Ca} and ϵ_{Ca}. The Y112 ϵCH resonance of apo-SCIII, ϵ_{apo}, is also shown at 6.80 ppm and is shaded at each dilution stage. The Y112 δCH resonance of apo-SCIII is found at lower field and is not shown. Spectrum a also shows the ζ-proton of F102 at 6.805 ppm which is present at the same chemical shift in the absence and presence of calcium in all seven spectra.

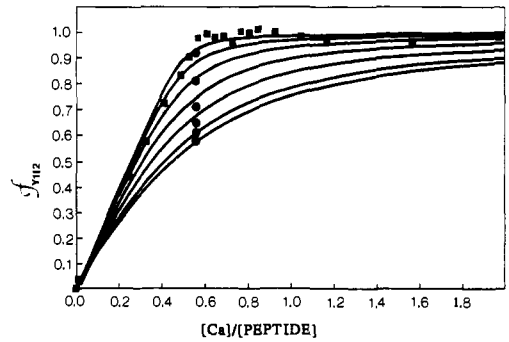

Figure 5. Calcium titration plot for SCIII derived from Figure 1 (■) and Figure 4 (●). Resonances of Y112 aromatic protons were used to assess the relative concentrations of apo and calcium bound SCIII, where f_{Y112} denotes the fraction of calcium-bound SCIII. The six points marked with a solid circle represent SCIII concentrations of 177, 85, 44, 14, 26, and 17 μM, respectively, moving down through the series of curves. The curves shown are those fitted for $K_1 = 0.003$ mM, $K_d = 0.01$ mM, and $K_2 = 1$ mM which yielded the lowest standard deviation from the data.

(45) Levine, B. A.; Mercola, D.; Coffman, D.; Thornton, J. M. *J. Mol. Biol.* **1977**, *115*, 743.
(46) Drabikowski, W.; Dalgarno, D. C.; Levine, B. A.; Gergely, J.; Grabarek, Z.; Leavis, P. C. *Eur. J. Biochem.* **1985**, *151*, 17.

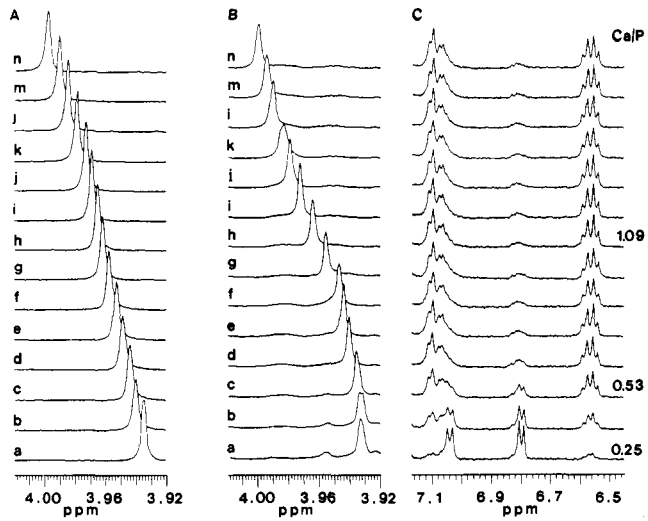

Figure 6. 500-MHz ^{1}H NMR spectra showing the calcium titration of (A) 400 μM DGA and (B, C) 300 μM DGA + 289 μM SCIII. The shifts of the DGA resonance in the absence of SCIII and the presence of SCIII during calcium addition are shown in panels A and B, respectively. The Ca:DGA ratios for panel A are (a) 0.00, (b) 0.06, (c) 0.11, (d) 0.17, (e) 0.22, (f) 0.28, (g) 0.33, (h) 0.38, (i) 0.43, (j) 0.48, (k) 0.61, (l) 0.72, (m) 0.83, (n) 0.93. The Ca:SCIII ratios for panels B and C are (a) 0.25, (b) 0.40, (c) 0.53, (d) 0.67, (e) 0.74, (f) 0.81, (g) 0.95, (h) 1.09, (i) 1.24, (j) 1.38, (k) 1.53, (l) 1.67, (m) 1.78, (n) 1.93. Panel C shows the aromatic region of SCIII for the same data as panel B with some selected Ca/P ratios highlighted.

In order to verify the relative magnitudes of K_1 and K_2 a more direct measure of each was required. However, the lack of a spectral change upon binding of the second calcium to the SCIII dimer presented a significant problem because it represented a spectroscopically invisible step in the process. Other groups have employed fluorescent calcium indicators or calcium-selective electrodes to measure calcium binding in such a process.[13,15,21b,47] We developed a technique using a novel calcium indicator, diglycolic acid (DGA), where calcium binding to DGA could be monitored by ^{1}H NMR spectroscopy. A similar method utilizing EDTA has been used previously to determine the calcium-binding constants for porcine calbindin D_{9k}.[10a]

The selection of DGA as the indicator molecule was based on the calculated values of K_1 and K_2 for SCIII. Inclusion of DGA in the SCIII solution allows for a competition between SCIII and the DGA for calcium, depending on the relative dissociation constants for the two species. Since K_1 = 0.003 mM and $K_2 \approx$ 1 mM for SCIII (from curve fitting, Figure 5) and binding of the second equivalent of calcium (i.e. K_2 step) was the unobservable step it was important to select an indicator with a K_D much larger than K_1 for SCIII so as not to compete with that calcium-binding event. In addition it was preferable to have K_{DGA} similar to K_2 for SCIII so that the calcium competition was dependent on these two constants.

Diglycolic acid is a symmetric molecule, having four methylene protons which are magnetically equivalent resulting in a single resonance in the ^{1}H NMR spectrum at 3.93 ppm (Figure 6A). Binding of calcium to DGA occurs in the fast exchange regime with a stoichiometry of 1:1 Ca:DGA and shifts the DGA resonance to higher frequency. At about 6 equiv of calcium, the resonance appears about 60-Hz downfield of that in the calcium free spectrum. A plot of the change in chemical shift vs Ca:DGA (Figure 7) was analyzed, yielding a calculated dissociation constant, K_{DGA}, of 211 ($\pm$6) μM. This dissociation constant is approximately 70-fold larger than K_1 for SCIII. However, the dissociation constant for DGA is only 5 times smaller than the estimated K_2 for SCIII, making it an ideal indicator to assess the second calcium binding stage.

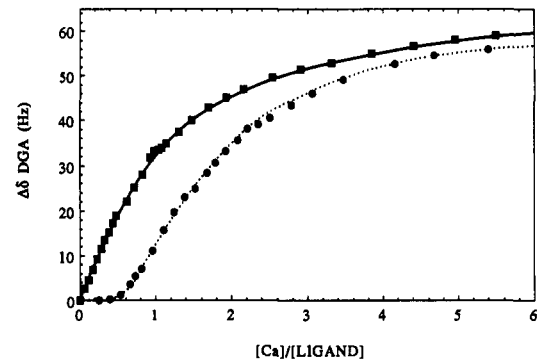

Figure 7. Calcium titration plot for DGA in the absence (■) and presence (●) of SCIII. The data are plotted as the change in chemical shift ($\Delta\delta$) of the DGA resonance from its initial position to that observed at a given calcium concentration vs [Ca]/[ligand] where ligand is DGA (■) and SCIII (●).

The calcium titration of 300 μM DGA and 289 μM apo-SCIII is shown in Figure 6, B and C. As with the spectrum in the absence of SCIII and with no calcium added, the DGA resonance in Figure 6B is at 3.93 ppm. During the early stages of this experiment, added calcium has little effect on the DGA resonance. However, examination of the aromatic region of SCIII in Figure 6C shows the appearance of resonances corresponding to the calcium-bound form of SCIII at 7.06, 7.11, and 6.57 ppm. These latter resonances continue to increase in size until a Ca:peptide ratio of about 0.5 is reached, similar to that observed for the calcium titration of SCIII in the absence of DGA (Figure 1). As the calcium concentration is increased above the 0.5 Ca:peptide ratio these resonances from SCIII remain at constant intensity. It is only during this portion of the titration that the DGA resonance shifts to lower field, indicative of calcium binding.

The chemical shift changes for DGA in the absence and presence of SCIII are plotted in Figure 7. In the presence of SCIII the curve initially shows little change in chemical shift. Above a Ca:peptide ratio of 0.5 the DGA resonance titrates as it did in the absence of SCIII, suggesting calcium binding to DGA is occurring. Analysis of the DGA data for this portion of the curve (Figure 7) yielded a dissociation constant in the presence of SCIII of 205 ($\pm$7) μM for DGA, which is nearly identical with that of DGA alone (211 $\pm$ 6 μM) and shows that binding of calcium to DGA is favored compared to the binding of the second calcium ion to SCIII. Qualitatively, this observation also shows that K_2 for the SCIII dimer must be >10-fold larger than K_{DGA}. A conservative value would be $K_2 \approx$ 2 mM, which is in excellent agreement with that obtained ($\approx$1 mM) from curve fitting of the calcium titration for SCIII (Figure 5).

The dissociation constant K_2 for the binding of calcium to P^*_2Ca can now be used to interpret the calcium titration data in Figures 1 and 3. The highest Ca:peptide ratio shown in Figure 1 is 1.17 for 480 μM total calcium and 411 μM total SCIII. At this stage of the titration it can be calculated (based on K_1, K_d, and K_2) that P^*_2Ca represents >80% of the total SCIII and $P^*_2Ca_2$ is present at <20%. Thus in Figure 1 spectral changes are not obvious between 0.5 and 1.0 equiv of calcium in part because of the small proportion of $P^*_2Ca_2$ formed to this point.

Site–Site Interactions in SCIII. In probing the proposed folding mechanism of SCIII (Figure 3) it is useful to look at site–site interactions and the calcium dissociation constants separately. In the SCIII dimer, two-dimensional ^{1}H NMR studies have revealed that 14 hydrophobic contacts exist between residues at the interfaces of the α-helices in the two polypeptide chains.[31] In the absence of calcium, SCIII is unfolded so that the burying of these hydrophobes upon calcium binding and self-association will contribute greatly to the stability of the dimer. Taking into account the relative individual energies from the burying of these residues,[48] one obtains $\Delta G \approx -55$ kJ/mol for SCIII. While this

(47) Forsen, S.; Linse, S.; Thulin, E.; Lindegarde, B.; Martin, S.; Bayley, P.; Brodin, P.; Grundstrom, T. *Eur. J. Biochem.* **1988**, *177*, 47.

(48) Guy, H. R. *Biophys. J.* **1985**, *47*, 61.

value is not a quantitative measure, its magnitude is similar to those obtained for other pathways[22,23,27] where the interaction of nonpolar residues is the driving force for self-association and suggests this may also be responsible for SCIII dimerization.

The dissociation constant for the binding of calcium to P^*_2Ca (K_2) is approximately 300 times smaller than that for calcium binding to the unfolded peptide, P (K_1). One possibility for this observation is that the presence of a single calcium in the dimer, P^*_2Ca, inhibits further calcium binding via electrostatic repulsion between calcium ions. Complexation studies utilizing model crown ether systems have shown that such a mechanism is possible[49] when the distance between the binding sites is relatively small. However, in the C-terminal domain of troponin-C the calcium ions in calcium-binding sites III and IV are 11.7 Å apart. Since a similar distance is expected in $P^*_2Ca_2$ for SCIII it is unlikely that electrostatic repulsion could account for the observed weak binding of the second calcium ion.

The weaker binding of the second calcium ion could also be a result of decreased mobility of the P^*_2Ca complex compared to the unfolded peptide, P. Such changes in dynamics have been recently assessed calbindin D_{9k}[50] where the association and dissociation rate constants for calcium binding, k_{on} and k_{off}, respectively, have been measured.[51] A similar analysis for SCIII would be valuable for interpretation of the dissociation constants in SCIII. For example, if calcium binding to $P_2{}^*Ca$ is inhibited because the site is performed one might expect the association rate constant for this step (k^2_{on}) to be slower than that for initial calcium binding to P (k^1_{on}). For the binding of the first calcium ion to SCIII no observable line broadening occurs with increasing calcium concentration giving an upper limit of $k^1_{off} < 10\ s^{-1}$. However, this estimate actually corresponds to the product of the calcium-binding step (K_1) and the peptide association (k_d), and an estimate of k^1_{on} is not easily extracted.

The mechanism and stoichiometry for calcium binding to SCIII appears to be different from previous results with synthetic peptides and fragments from troponin-C. One of the most obvious reasons for our observation of a peptide association pathway is the concentration regime used here as compared to other studies. Previous work with either troponin-C and calmodulin peptide fragments were done with use of concentrations ranging from 5 to 20 μM.[14,15,41] In the present study, the peptide association constant was found to be near this range (K_d = 10 μM). This would imply that if peptide association was occurring in earlier works it may have been incomplete due to the concentrations used. As a result, the calculated dissociation constants for calcium would likely be higher than that found in this study. Indeed in one case a dissociation constant of 30 μM has been found for fragment CB9 (site III of troponin-C),[15] which is about 10 fold higher than K_1 = 3 μM for SCIII.

There have also been a few studies[16,17] conducted with troponin-C peptides at concentrations greater than 0.1 mM, where a high degree of peptide association would be expected to occur. As discussed earlier, these studies assumed 1:1 Ca peptide stoichiometry. However, even if peptide association was suspected, binding of a second calcium ion likely would not have been observed since free calcium ion concentrations were not extended high enough to measure the weak binding of the second ion (K_2 = 1 mM). As a result, the reported dissociation constant should be similar to K_1 (3 μM) in this study. This has been found for at least one case where a dissociation constant of 3.8 mM was found.[16]

Acknowledgment. This work arose in part from studies conducted by Jean Gariepy in 1982 at the University of Alberta and whose NMR titrations of a site III rabbit skeletal troponin-C peptide (residues 90–123) showed 0.5 Ca–peptide stoichiometry. We would like to thank Robert Boyko for his assistance with the curve-fitting programs used, Paul Semchuk for his help with peptide synthesis and purification, and Mike Nattriss for amino acid analysis. We are also grateful to Sue Smith for typing this manuscript and to Wendy Findlay and Carolyn Slupsky for helpful discussions.

(49) Rebek, J.; Costello, T.; Marshall, L.; Wattley, R.; Gadwood, R. C.; Onan, K. J. *J. Am. Chem. Soc.* **1985**, *107*, 7481.
(50) Linse, S.; Teleman, O.; Drakenberg, T. *Biochemistry* **1990**, *29*, 5925.
(51) Martin, S.; Linse, S.; Johansson, C.; Bayley, P. M.; Forsen, S. *Biochemistry* **1990**, *29*, 4188.

Crossed-Beam Study of the S_N2 Reaction

Y. F. Yen, R. J. Cross,* and M. Saunders*

Contribution from the Department of Chemistry, Yale University, New Haven, Connecticut 06511-8118. *Received November 19, 1990*

Abstract: We have used crossed molecular beams to study the dynamics of chemiionization S_N2 reactions of the type $RX + R'_3N \rightarrow RR'_3N^+ + X^-$, where RX is an alkyl halide and R'_3N is a nucleophile. We have seen reaction between methyl iodide, ethyl iodide, and *n*-propyl iodide with quinuclidine and between methyl iodide and pyridine and tri-*n*-propylamine. The reactive cross sections have a threshold between 4.4 and 6.0 eV depending on the system. They rise rapidly above the threshold and then level off at higher energies showing no dependence on the vibrational energy of the nucleophile.

Introduction

Nucleophilic substitution reactions are sufficiently important that they are generally introduced early in a course in elementary organic chemistry. The S_N2 reaction, with its picturesque umbrella inversion, is the most important of these. It was characterized by Ingold et al.[1] in the 1930's and has been studied in solution ever since. There have been several studies of the general reaction

$$X^- + CH_3Y \rightarrow Y^- + CH_3X \qquad (1)$$

in the gas phase with use of flowing afterglow,[2] ion cyclotron resonance,[3] and tandem mass spectroscopy.[4] Several groups have calculated the potential-energy surface for this type of reaction.[5,6]

(1) Gleave, J. L.; Hughes, E. D.; Ingold, C. K. *J. Chem. Soc.* **1935**, 236.

(2) Bohme, D. K.; Young, L. B. *J. Am. Chem. Soc.* **1970**, *92*, 7354. Tanaka, K.; MacKay, J. D.; Payzant, J. D.; Bohme, D. K. *Can. J. Chem.* **1976**, *54*, 1643. Bohm, D. K.; Mackay, G. I. *J. Am. Chem. Soc.* **1981**, *103*, 987. Bohme, D. K.; Raksit, A. B. *J. Am. Chem. Soc.* **1984**, *106*, 3447. Bohme, D. K.; Raksit, A. B. *Can. J. Chem.* **1985**, *63*, 3007. Lane, K. R.; Squires, R. R. *J. Am. Chem. Soc.* **1986**, *108*, 7187. DePuy, C. H.; Gronert, S.; Mullin, A.; Birnbaum, V. M. *J. Am. Chem. Soc.* **1990**, *112*, 8650.
(3) Olmstead, W. N.; Brauman, J. I. *J. Am. Chem. Soc.* **1977**, *99*, 4219. Pellerite, M. J.; Brauman, J. I. *J. Am. Chem. Soc.* **1983**, *105*, 2672. Dodd, J. A.; Brauman, J. I. *J. Phys. Chem.* **1986**, *90*, 3559. Baer, S.; Stoutland, P. O.; Braumann, J. I. *J. Am. Chem. Soc.* **1989**, *111*, 4097. Han, C.; Brauman, J. I. *J. Am. Chem. Soc.* **1989**, *111*, 6491. Larson, J. W.; McMahon, T. B. *J. Am. Chem. Soc.* **1984**, *106*, 517. Larson, J. W.; McMahon, T. B. *J. Phys. Chem.* **1984**, *88*, 1083.

0002-7863/91/1513-5563$02.50/0