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Alpha Helix Capping in Synthetic Model Peptides by Reciprocal Side Chain–Main Chain Interactions: Evidence for an N Terminal "Capping Box"

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ABSTRACT **A significant fraction of the amino acids in proteins are alpha helical in conformation. Alpha helices in globular proteins are short, with an average length of about twelve residues, so that residues at the ends of helices make up an important fraction of all helical residues. In the middle of a helix, H-bonds connect the NH and CO groups of each residue to partners four residues along the chain. At the ends of a helix, the H-bond potential of the main chain remains unfulfilled, and helix capping interactions involving bonds from polar side chains to the NH or CO of the backbone have been proposed and detected. In a study of synthetic helical peptides, we have found that the sequence Ser-Glu-Asp-Glu stabilizes the alpha helix in a series of helical peptides with consensus sequences. Following the report by Harper and Rose, which identifies SerXaaXaaGlu as a member of a class of common motifs at the N termini of alpha helices in proteins that they refer to as "capping boxes," we have reexamined the side chain–main chain interactions in a varient sequence using ^{1}H NMR, and find that the postulated reciprocal side chain-backbone bonding between the first Ser and last Glu side chains and their peptide NH partners can be resolved. Deletion of two residues N terminal to the Ser-Glu-Asp-Glu sequence in these peptides has no effect on the initiation of helical structure, as defined by two-dimensional (2D) NMR experiments on this variant. Thus the capping box sequence Ser-Glu-Asp-Glu inhibits N terminal fraying of the N terminus of alpha helix in these peptides, and shows the side chain–main chain interactions proposed by Harper and Rose. It thus acts as a helix initiating signal. Since normal α helix cannot propagate beyond the N terminus of this structure, the box acts as a termination signal in this direction as well.**

Key words: α-helix capping, α-helix initiation, α-helix termination, synthetic peptides, protein folding, circular dichroism, ^{1}H nmr

INTRODUCTION

The classical $(3.6)_{13}$ alpha helix is stabilized by a system of H-bonds linking each peptide residue NH to a CO four positions away.[1] The four positions at either end of a helix cannot maintain this H-bonding pattern, since they lack the donor NH at the C terminus and the acceptor CO groups at the N terminus.[2] In short helices, which occur commonly in globular proteins, interactions at the ends are thought to play an important role in helix stabilization.[2–6] Structural and statistical analyses suggest that polar side chains bond to peptide groups at the ends to stabilize helical structure.[2,7] Experiments with substitutions in proteins[8–10] and in helical peptides[11–13] have confirmed that specific side chains at the ends influence the stability of helices. While Ala is the strongest helix-forming side chain in the middle of a helix, for example, it is destabilizing relative to Ser, Asn or Gly at the N terminus.[11] The three-dimensional structure of a peptide designated S3,

Abbreviations: PAL, Peptide Amide Linker; Fmoc, 9-fluorenylmethoxycarbonyl; BOP, benzotriazolyloxotris (dimethylamino)-phosphonium hexafluorophosphate; HOBt, 1-hydroxybenzotriazole; CD, circular dichroism; 2D NMR, two-dimensional nuclear magnetic resonance; NOESY, two dimensional nuclear Overhauser and exchange spectroscopy; FID, free induction decay; ROESY, rotating frame nuclear Overhauser effect spectroscopy; A, Ala; D, Asp; E, Glu; F, Phe, G, Gly; H, His; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; Y, Tyr.

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TABLE I. Peptide Sequence and Helical Content*

Peptide	Sequence	$-[\theta]222$ f	%
N-cap(position 3)			
Parent S3	YM S EDELKAAEAAFKRHGPT	16,000	50
N3(Asn)	YM N EDELKAAEAAFKRHGPT	14,100	44
G3(Gly)	YM G EDELKAAEAAFKRHGPT	10,580	33
A3(Ala)	YM A EDELKAAEAAFKRHGPT	7,700	24
N3(position 6)			
Parent E6	YMSED E LKAAEAAFKRHGPT	16,000	50
D6(Asp)	YMSED D LKAAEAAFKRHGPT	13,200	41
Q6(Gln)	YMSED Q LKAAEAAFKRHGPT	12,100	38
A6(Ala)	YMSED A LKAAEAAFKRHGPT	12,000	38
G6(Gly)	YMSED G LKAAEAAFKRHGPT	7,000	22
Middle(position 10)			
Parent A10	YMSEDELKA A EAAFKRHGPT	16,000	50
S10(Ser)	YMSEDELKA S EAAFKRHGPT	9,300	29
G10(Gly)	YMSEDELKA G EAAFKRHGPT	5,560	17
Ccap(position 18)			
Parent G18	YMSEDELKAAEAAFKRH G PT	16,000	50
N18(Asn)	YMSEDELKAAEAAFKRH N PT	20,000	63
Truncated peptide S1			
Parent S3	YMSEDELKAAEAAFKRHGPT	16,000	50
S1	SEDELKAAEAAFKRHGPY	14,000	45

*All the peptides in this study were acetylated at the N terminus and amidated at the C terminus, as described previously.[11,12] $[\theta]_{222}$ is the mean residue ellipticity (degree·cm^{2}dmol^{-1}) at 222 nm, 4°C, pH 6. F $=[-[\theta]_{obsd}/40{,}000(n-4)/n] \times 100\%$ = percent helix content.[24] Positions in these peptides are designated N'' N' N_{cap} N1 N2 N3 N4 N5 (Helix Mid)$_4$ C5 C4 C3 C2 C1 C_{cap} C' C''.[7,11]

(Table I) has been determined,[12] in an effort to define the interactions responsible for N terminal capping. This peptide[11] is a member of a series, the sequence of which includes one of the most frequent amino acids at each position identified in the survey of helices in proteins of known structure by the Richardsons.[7] The structure of this peptide, determined by two-dimensional (2D) ^{1}H NMR, reveals features at the N terminus indicative of capping: the Oγ of Ser3 in the chain interacts with the main chain NH of Glu 6.[12]

Harper and Rose[5] have recently reported that sequence patterns such as SXXE occur frequently at the N termini of helices in globular proteins. Inspection of the structures of a number of these sequences reveals reciprocal bonding between the Ser side chain γO and the Glu backbone NH and the Glu side chain CO and the Ser backbone NH.[5] Since we have detected the former interaction in a peptide model,[12] we were motivated to reexamine our NMR model, to see if the latter interaction can be detected. Analysis of the NOESY spectra in another peptide in the series, N18, synthesized originally in order to study capping effects at the C terminus (Table I), reveals the Ser3 NH-Oϵ Glu6 pairing more clearly, because the NMR spectrum of this peptide shows a wider dispersion in chemical shifts than that of S3. The overall helix content of the peptide with N18 is significantly higher than that of S3 based on CD spectroscopy as seen in Table I.

To establish whether or not a capping box is sufficient to initiate a helix, we have synthesized and determined the structure of a new peptide, S1 (Table I), in which the two residues at the N terminus in S3 are deleted, locating the serine at the N terminal residue of the molecule. The C terminal Thr in S3 was changed to Tyr in S1 to allow accurate concentration measurement.[14] Minor changes in the CD spectrum may accompany this shift, due to an "aromatic effect,"[15] but these have no influence on the conclusions of the present study; the presence of capping structures may counter such CD contributions in any case, because both ends of the model peptides include bends.

Structural analysis of the peptide S1 by 2D ^{1}H NMR reveals that the helix initiates at the N terminal Ser1, with clearly defined reciprocal side chain-main chain interactions between Ser1 and Glu 4. In peptides S3 and N18, helix initiation occurs at Ser3, and not at Tyr1 or Met2, although Met is a strong helix-forming side chain in the middle of helices.[16,17] This observation suggests that the sequence Ser-Glu-Asp-Glu controls initiation of alpha helical structure, presumably by means of the specific bonding observed. At the same time, the structure inhibits distal helix formation, since the bend in the chain produced (Fig. 4) is incompatible with helical structure N terminal to the signal itself. Hence the proposed "capping box" motif both initiates alpha helix, avoiding normal fraying at the N

terminus, and acts to terminate helix N terminal to the Ser.

MATERIALS AND METHODS

Peptide Synthesis

The sequences of the peptide models studied are presented in Table I. Peptides were synthesized on a MilliGen/Biosearch 9600 automated synthesizer using PAL[1] resin (from Milligen/Biosearch, Massachusetts) and Fmoc chemistry with BOP and HOBt as coupling reagents. Peptides were purified by reverse phase HPLC as described previously,[16] and the purity of each peptide was checked on a Waters analytical column (Delta Pak C18-100Å). The correct molecular weight of each product peptide was verified by electrospray ionization mass spectroscopy, using a Vestec Model 200 mass spectrometer.

CD Spectroscopy

CD spectra were recorded on an Aviv DS60 spectropolarimeter equipped with an HP 89100A temperature controller. The wavelength of the instrument was calibrated with (+)-10-camphorsulfonic acid.[18] The peptide concentration was determined by the tyrosine UV absorption with ϵ = 1,450 at 275.5 nm, 25°C in 6 M Gn·HCl.[14] CD Measurements were performed in 10 mM KF solutions in a 1 mm pathlength cell at a peptide concentration about 30 μM, pH 6, 4°C, averaging three scans with a step size of 0.5 nm for the spectra shown.

^{1}H NMR Spectroscopy

All ^{1}H NMR spectra were recorded on a Bruker AMX-600 spectrometer at 10°C. Time proportional phase incrementation (TPPI)[19] was used to obtain phase-sensitive NOESY[20] spectra. The water signal was suppressed by using a "jump and return" sequence (90°-t-90°).[21] Each 2D data set contained 512 FIDs with 2K data points each, obtained by recording 48 scans/FID, with a spectral width of 6,204 Hz, and a 2 s recycle delay. Spectra were Fourier transformed in both t_1 and t_2 dimensions after apodization with a skewed sine bell function, typically with a 60° phase shift and skew of 0.5. Zero filling was done in the t_1 dimension to obtain a final matrix of 1,024 × 1,024 real points. The first data point in t_1 was multiplied by 0.5 to suppress t_1 ridges.[22] All data processing was carried out on a Personal Iris 4D/25GT workstation using the FELIX program (Biosym, Inc., San Diego, CA). Peptide samples for NMR experiments were about 4 mM in H_2O/D_2O (85%/15%) at pH 5.5.

RESULTS

Helix Content in Model Peptides

The relative helix content of peptides is most conveniently evaluated by means of CD spectral measurements. In the asymmetric environment of a helix, the strong peptide absorption bands near 200 nm in the UV become optically active; the alpha helix spectrum is characterized by minima at 222 and 208 nm, and a maximum at 195 nm.[23] Figure 1 shows CD spectra of the peptides of this study. The form of the spectra shown can be deconvoluted into a mixture of alpha helix and random coil conformations.[24] Together with the NOESY connectivity patterns seen in S1 and N18, this ensures that we are not dealing with turns or sheets. In Figure 1A, which summarizes a set of substitutions at the presumed Ncap position,[11] no definite isoelliptic point occurs near 206 nm as is seen in many helical peptide models.[16] Lack of an isoelliptic point can be taken to indicate that the peptide bonds in the chain are not in two "states"; this is consistent with formation or loss of a capping structure at the ends. On the other hand, we assume that the signal at 222 nm provides a measure of overall helix content in these chains, as has been observed in previous NMR studies.[11,12,16] The CD spectra suggest that Ala is relatively destabilizing at Ncap relative to Ser, Asn, and Gly. Figure 1B confirms that Ala is more strongly helix stabilizing than Ser or Gly at midhelix sites, as has been observed in other peptide models.[16,17] Alanine is among the most frequent side chains in helices of known proteins[25] and predominates at midhelical positions.[7] Figure 1C shows the results of substitution of the Glu residue three positions C terminal to the Ncap position, corresponding to the putative capping side chain. At position 6, Gly is destabilizing relative to Ala, as in Figure 1B, but the polar side chains Glu and Asp are both more stabilizing than Gln or Ala. The relative stability of the polar side chains corresponds closely to the frequency of their occurrence in capping boxes containing Ser at the Ncap position.[5] Figure 1D shows that the CD signals of S3 and the truncated peptide S1 are similar, consistent with retention of the alpha helical structure present in the latter chain. Our calculations[24] indicate that S1 and S3 have similar helix content (see Table I); the shorter chain length of S1 would be expected to reduce the helix content slightly due to the cooperativity in helix formation.[26]

Reciprocal Capping Interactions in Peptide N18

Table II shows the complete resonance assignment of N18 peptide. Figure 2A shows a section of the 2D NOESY spectrum of N18, a peptide with sequence identical to that of S3 except for the substitution of Asn18 for Gly18 at the presumed Ccap site. As seen in Table I, N18 has a distinctly higher helix content than S3 due to effects at the C terminus.[3] One consequence of this increased helicity is an improvement in dispersion of the fingerprint region of the spectrum (Fig. 2A). As seen in this figure, residues 2

to 7 in the peptide N18 show NOE connectivity (short through space distances)[27] via proximity of the α, β and γ protons in their side chains. This is similar to the spectral behavior of S3,[12] except that several nonstandard cross peaks involving side chain protons are better resolved in N18. Glu 4 shows only one cross peak for the β protons or γ protons connecting its side chain protons and backbone amide, suggesting relatively free rotation. The corresponding protons in the side chain of Glu6 are resolved, indicating formation of structure. Each side chain proton of Glu6 shows cross peaks with the backbone amide of Ser3. The C_β cross peaks are stronger than the C_γ, indicating the former are closer than the latter. This is consistent with a structure in which O_ϵ of Glu6 forms a hydrogen bond with the NH of Ser3.

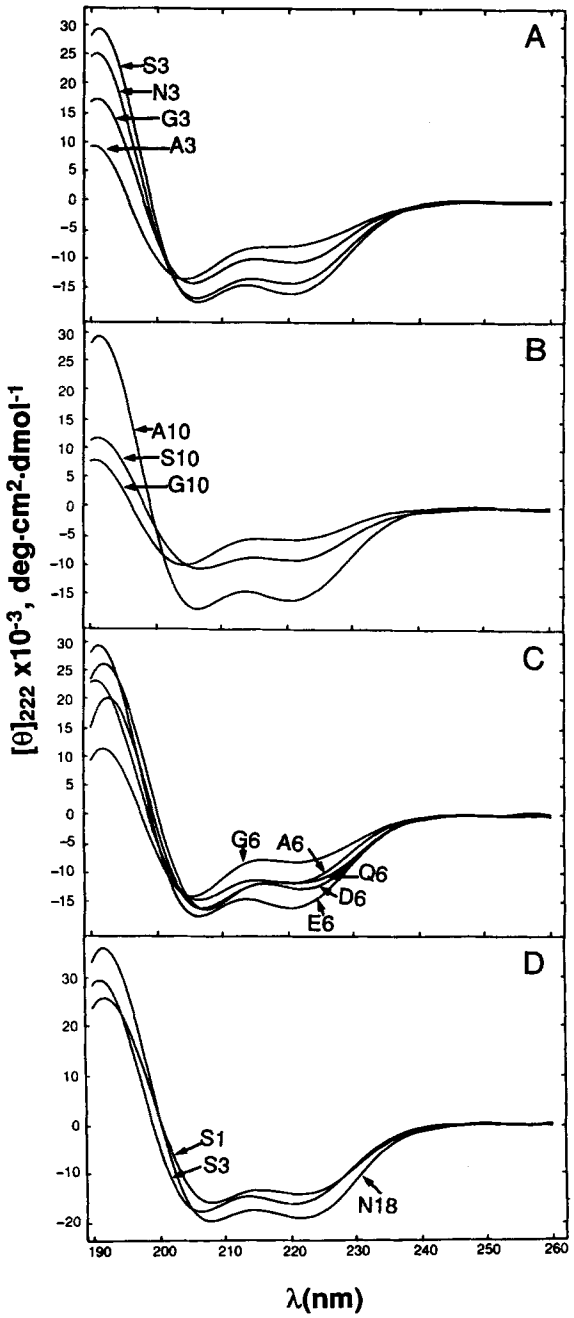


Fig. 1. CD spectra of eleven model peptides in 10 mM KF, pH 6 at 4°C. The parent model peptide sequence in this series is S3 (Table I). Each peptide is identified by a letter followed by a number; the letter is the single letter code abbreviation for the amino acid residue substituted and the number corresponds to the site within the model peptide. The sequence of S1 has Tyr and Met deleted from N terminus of the parent model peptide S3 (Table I). The peptide denoted as S3 (in A), A10 (in B), and E6 (in C) is the parent peptide. **A:** Spectra of S3, N3, G3, and A3. **B:** Spectra of A10, S10, and G10, for comparison of the relative stabilizing order of these three residues in the helix middle. **C:** Spectra of E6, D6, Q6, A6, and G6. No isoelliptic point is observed here. **D:** Spectra of S1, S3, and N18.

In Figure 2A, two well resolved cross peaks connect the two β protons of Ser3 (designated β_1 and β_2 in the figure) to the backbone amide in the same residue. The difference in intensity suggests that β_2 is closer to its amide than β_1. Both β protons show cross peaks with the NH of Glu4; the β_1 cross peak is more intense than that of β_2. In addition, there is a cross peak between NH of Asp5 and β_1 of Ser3, but not β_2. These observations are consistent with a model in which Oγ of Ser3 shares a bifurcated H-bond with the NH groups of Glu6 and Asp5. Once the side chain of Ser3 is fixed by formation of this H-bond, one of its β protons that lies near the NH of Glu4 (β_1) is further from the backbone NH of Ser3 than the other, β_2. This arrangement reconciles all the cross peak intensities observed in this region of the NOESY spectrum. As in the analysis of S3[12], NOE cross peaks ($d_{NN}^{i,i+1}$)[27] connect pairs of adjacent residues in the sequence of N18 from Ser3 to Asn18, confirming that the N terminus of the alpha helix initiates at Ser3.

Are these NOEs specific, or due to spin diffusion? The data shown in Figures 2 and 3 correspond to a mixing time of 250 ms. We have collected spectra at 55 ms and 125 ms (not shown), which show that the NOEs build up at a rate comparable to that in apamin, a helical peptide of 18 residues with two disulfide bonds. In the case of apamin, NOESY build up rates have been measured up to 450 ms.[28] Spin diffusion affected the closest protons (1.75Å apart) only at 300 ms or longer. Even in a rigid molecule the size of S1 or N18, NOE buildup rates are slow, because $\omega\tau_c \sim 1$.[28] We carried out a ROESY experiment, which showed no negative cross peaks (the diagonal is shown positive), confirming the absence of spin diffusion.[29] The helical structure in isolated peptides is much less rigid than in globular proteins; this is seen, for example, in the dramatic effects of a single Gly in the middle of helical peptides,[11] and in the NMR spectra of a Leu → Gly replacement in the middle of another helical model.[30] Each site in these models is partially helix and partially coil, based on analysis of chemical shifts[31] as well as dynamics. Spin diffusion pathways are favored in rigid structures, and attenuated in fluctuating ones.[27] These considerations preclude a major contribution from

TABLE II. ^{1}H Chemical Shifts* of Assigned Resonances of N18 Peptide

Residue	NH	$C_\alpha H$	$C_\beta H$	$C_\gamma H$	$C_\delta H$	Other ^{1}H
Acetyl		1.96				
Y1	8.33	4.50	2.96			6.81/7.12
M2	8.18	4.48	1.98/1.87	2.49		ϵCH_3 2.03
S3	8.62	4.44	4.30/4.02			
E4	9.02	4.10	2.10	2.40		
D5	8.52	4.44	2.66/2.61			
E6	8.01	4.10	2.24/2.07	2.41/2.34		
L7	8.42	4.09	1.78	1.63	0.87	
K8	8.24	4.10	1.90	1.65/1.50	1.70	ϵCH_2 2.99
A9	7.99	4.24	1.50			
A10	8.15	4.23	1.52			
E11	8.25	4.16	2.17/2.12	2.50/2.32		
A12	8.03	4.18	1.50			
A13	7.98	4.13	1.47			
F14	8.06	4.38	3.25			7.29/7.37/7.32
K15	8.04	4.12	1.89	1.49/1.65	1.73	ϵCH_2 3.02
R16	7.83	4.16	1.81	1.70/1.58	3.21	ϵNH 7.27/7.54
H17	8.10	4.74	3.26/3.13			7.30/8.61
N18	8.30	4.96	2.67/2.49			γNH 7.41/6.94
P19†		4.50	2.33	2.04	3.72	
T20‡	8.23	4.33	4.02	1.24		
T20‡	8.52	4.25	—	1.28		
Terminal	7.56/7.28					

*Chemical shifts (in ppm) are measured relative to internal TSP [sodium (trimethylsilyl) propionate].
†Data for trans-Pro19.
‡T20 and T20′ correspond to *trans* and *cis* conformation of Pro19.

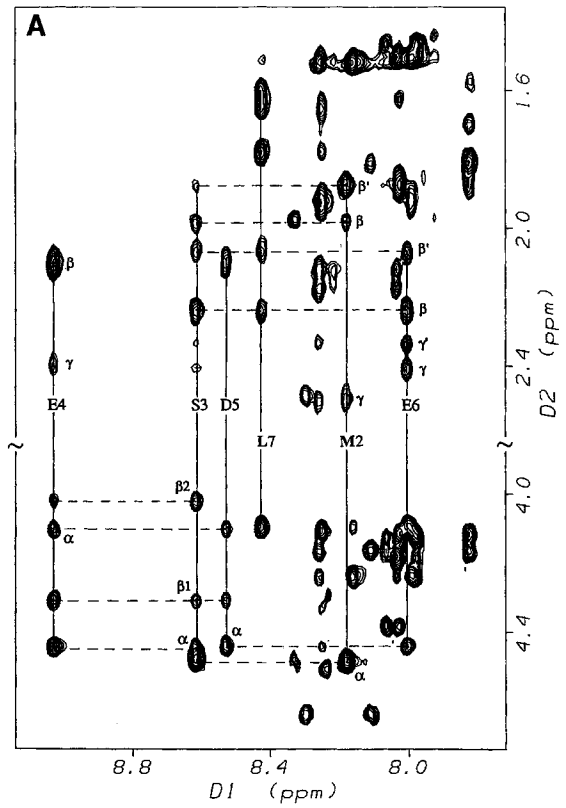
spin diffusion to the NOEs measured in Figures 2 and 3.

Reciprocal Capping Interactions and the Helix Distribution in Peptide S1

To assess the role of the putative capping box in helix initiation, we carried out a 2D ^{1}H NMR analysis of the truncated peptide, S1 (Table I). On the basis of the standard picture of cooperative nucleation in alpha helices, the ends of a helix are expected to "fray"[26] since the individual H-bonds are weak. Fraying of the ends has been demonstrated in NMR studies of helical model peptides.[31–33] The fact that S1 has a CD spectrum close to that of S3 suggests that extensive fraying may not be taking place at the N terminus in these peptides. The amide region of the NOESY spectrum of S1 is shown in Figure 3. The connectivity between the NH of residue i in the peptide and the NH of residue i + 1 is seen to extend from Ser1 to His15, so that the apparent extent of the helix in S1 is similar to that of S3[12] despite the loss of two N terminal residues. Figure 2B shows that the fingerprint region of S1 is very similar to that in N18 (Fig. 2A), so that the structure of the N terminus is preserved. Figure 4 illustrates a molecular model built from the constraints imposed by the NOESY spectrum of S1 (Figs. 2B and 3). The overall structure is similar to that we previously derived for this region in S3,[12] but includes the additional constraints discussed above which locate the side chain of Glu4 near the backbone amide of Ser1. The N terminus of N18 would include Tyr1 and Met2 oriented as shown in our model of S3.[12]

DISCUSSION

The hypothesis that sequences such as SerXaa-XaaGlu or variants constitute a "capping box"[5] is tested here in two ways. First, the presence of the sequence Ser-Glu-Asp-Glu in the peptide N18 gives rise to a pattern of short distances in the NOESY spectra (Fig. 2A) consistent with the reciprocal Ser3-Glu6 side chain–main chain interactions postulated by Harper and Rose.[5] As seen in the model for the N terminal region of S1 (Fig. 4), O_ϵ of Glu4 is H-bonded to the amide of Ser1, while the O_γ of Ser1 forms a bifurcated H-bond with the NH of Asp3 and Glu4. Second, analysis of the truncated peptide S1 shows that these interactions are maintained when the N terminal Tyr-Met side chains are removed from S3, preserving the N terminal structure while the alpha helix initiates again at the Ser residue. This is not the behavior anticipated for helical peptides;[31] Rohl et al.[33] demonstrated by hydrogen exchange that fraying should smoothly increase the opening probability by factors of (1/s) or about 0.66 for alanine, as the end of a helix is approached. Fraying is clearly seen in a study of the hydrogen exchange rates of peptide groups in melittin;[32] the rates increase progressively for 4–6 residues at either end of the chain. The effectively complete inhibition of fraying from the N terminus in peptide S1 argues that the interactions we detect in the Ser-Glu-Asp-Glu tetrad in S1 or N18



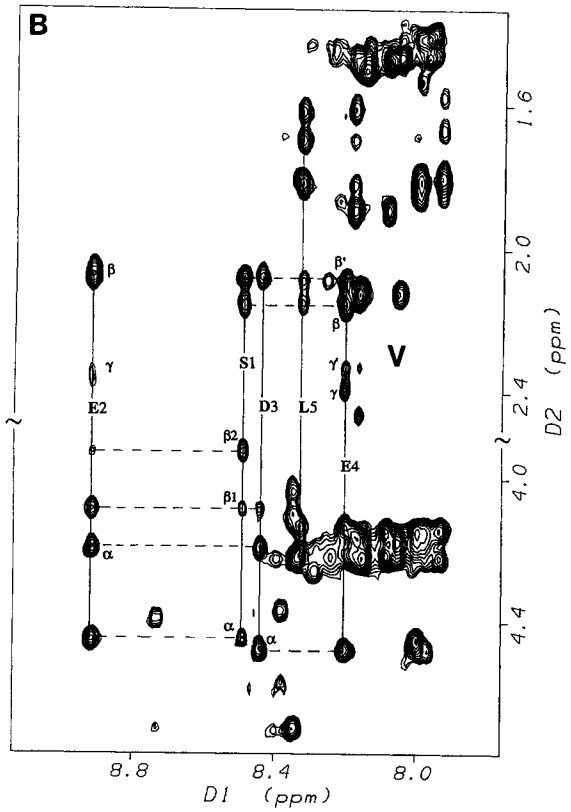


Fig. 2. **A:** Fingerprint region of NOESY spectra of N18. Spectra was recorded with a mixing time of 250 ms at 10°C, pH 5.5. The solid lines connect the cross peaks between the amide proton designated and its own α, β, and γ protons. The dashed lines connect the cross peaks between side chain and amide protons of neighboring residues in the capping box. **B:** Fingerprint region of NOESY spectra of peptide S1 with Ser as the first residue of the chain. The spectrum was recorded with a mixing time of 250 ms at 10°C, pH 5.5. The solid lines and dashed lines have the same significance as in A.

are responsible for helix initiation, as hypothesized in the model.[5] We see no indication that either the Glu or the Asp side chains within the tetrad are structurally organized, further supporting the hypothesis that the operative interaction is between the first Ser and last Glu residues of SerXaaXaaGlu, hence representing a capping box. It is worth emphasizing that normal α helix cannot propagate in the N terminal direction from this structure; the axis is bent sharply, so that the structure can be considered as a termination signal for helix distal to the box. The presence of a single N terminal capping box thus exerts both positive and negative control on the helix.

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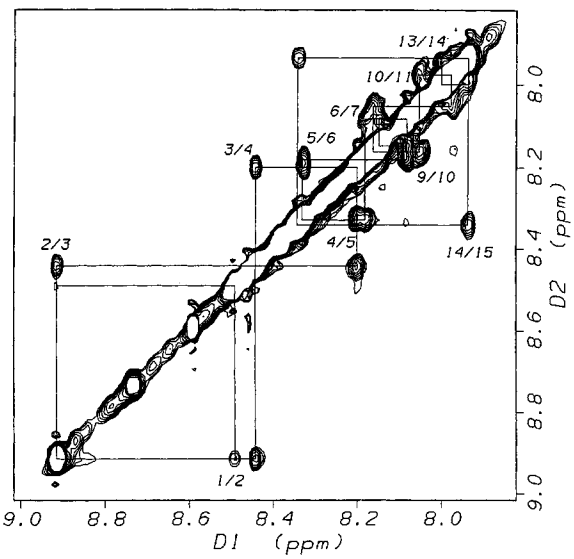

Fig. 3. Amide-amide region of NOESY spectrum of S1 peptide with a mixing time of 250 ms at 10°C, pH 5.5. The $d_{NN}^{i,i+1}$ cross peaks between two adjacent amide protons are indicated by the sequence number of these two adjacent residues.

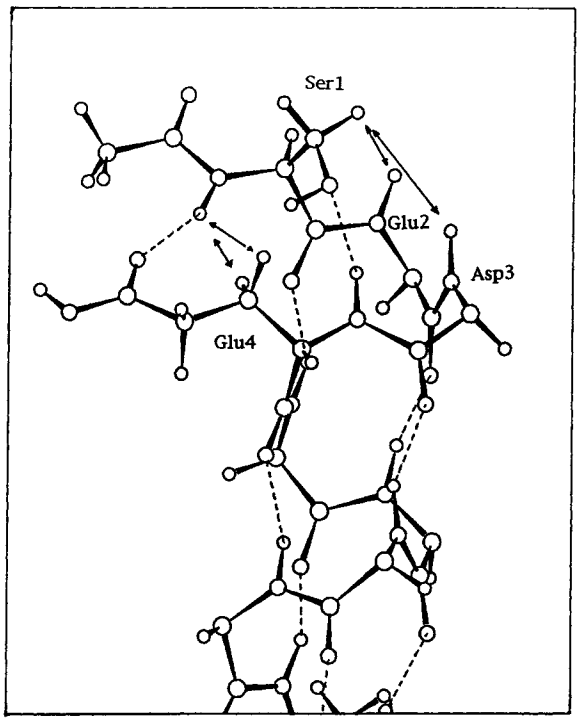

Fig. 4. Molecular model of capping box of peptide S1 assembled from the distance constraints imposed by the NOEs shown in Figures 2 and 3. Only side chains at Ncap(Ser) and N3(Glu)[7] positions are shown in this view. Arrows indicate those NOEs observed for side chain and backbone amide protons of S1. The model was made on a personal Iris 4D/25GT workstation using Insight II (Biosym Inc.).

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