Structure of the Propeptide of Prothrombin Containing the γ-Carboxylation Recognition Site Determined by Two-Dimensional NMR Spectroscopy†

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ABSTRACT: The propeptides of the vitamin K dependent blood clotting and regulatory proteins contain a γ-carboxylation recognition site that directs precursor forms of these proteins for posttranslational γ-carboxylation. Peptides corresponding to the propeptide of prothrombin were synthesized and examined by circular dichroism (CD) and nuclear magnetic resonance spectroscopy (NMR). CD spectra indicate that these peptides have little or no secondary structure in aqueous solutions but that the addition of trifluoroethanol induces or stabilizes a structure containing α-helical character. The maximum helical content occurs at 35–40% trifluoroethanol. This trifluoroethanol-stabilized structure was solved by two-dimensional NMR spectroscopy. The NMR results demonstrate that residues −13 to −3 form an amphipathic α-helix. NMR spectra indicate that a similar structure is present at 5 °C, in the absence of trifluoroethanol. Of the residues previously implicated in defining the γ-carboxylation recognition site, four residues (−18, −17, −16, and −15) are adjacent to the helical region and one residue (−10) is located within the helix. The potential role of the amphipathic α-helix in the γ-carboxylation recognition site is discussed.

The vitamin K dependent proteins prothrombin, factor VII, factor IX, factor X, protein C, and protein S are essential for the regulation of blood coagulation (Furie & Furie, 1988). The precursor forms of these proteins contain 10–12 glutamic acid residues that must be posttranslationally modified to γ-carboxyglutamic acid (Gla)[1] residues in order to generate fully functional, mature protein. Carboxylation of these Glu residues takes place in the rough endoplasmic reticulum and is carried out by the vitamin K dependent carboxylase (Carlisle & Suttie, 1980). The precursor forms of the vitamin K dependent proteins have amino-terminal propeptide extensions, residues −18 to −1, that share significant sequence homology (Diuguid et al., 1986; Pan & Price, 1985) and contain a recognition element, termed the "γ-carboxylation recognition site" (Jorgensen et al., 1987; Foster et al., 1987).

Evidence demonstrating the role of the propeptide in designating the vitamin K dependent proteins for carboxylation includes the following. Naturally occuring variants of factor IX with mutations in the propeptide region are only partially carboxylated (Diuguid et al., 1986; Ware et al., 1989). Factor IX and prothrombin are not carboxylated if the propeptide is deleted or if conserved residues within it are mutated (Jorgensen et al., 1987; Huber et al., 1990). Synthetic peptides containing the propeptide sequence of prothrombin or factor IX and portions of the Gla domain are efficiently carboxylated in vitro (Ulrich et al., 1988; Hubbard et al., 1989a). Synthetic peptide analogues of the propeptides bind directly to the vitamin K dependent carboxylase (Hubbard et al., 1989b). Point mutations at the two most highly conserved residues in the propeptide, −16 (substitution of Ala for Phe) and −10 (substitution of Glu for Ala), significantly reduce carboxylation of recombinant factor IX in vivo (Jorgensen et al., 1987), and mutations at residues −18, −17, −15, or −10 significantly reduce carboxylation of recombinant prothrombin (Huber et al., 1990). This last finding suggests that these residues are directly involved in forming the recognition site.

Secondary structural analysis of the propeptide sequence of the vitamin K dependent proteins by the method of Chou and Fasman (1978) suggests the presence of α-helical structure in this region. It is apparent that such an α-helix would have amphipathic character as a result of the spacing of hydrophobic and hydrophilic residues in the propeptide sequence. An amphipathic helix could play an important structural role in the interaction of the propeptide with either the carboxylase or the membrane of the endoplasmic reticulum. We have examined the secondary structure of synthetic propeptide analogues by CD and NMR to better understand the interaction of the propeptide with the vitamin K dependent carboxylase.

Materials and Methods

Synthesis of Peptides. Peptides were synthesized on an Applied Biosystems Model 430A peptide synthesizer (Merrifield, 1965) as previously described (Ulrich et al., 1988; Hubbard et al., 1989a). The cleaved, deprotected peptides were purified by high-performance liquid chromatography on a Waters HPLC system. The sequence of each peptide was verified by automated Edman degradation on an Applied Biosystems Model 470 protein sequencer.

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[1] Abbreviations: Gla, γ-carboxyglutamic acid; CD, circular dichroism; NMR, nuclear magnetic resonance; DQF, double quantum filtered; COSY, correlated spectroscopy; TOCSY, totally correlated spectroscopy; NOESY, nuclear Overhauser effect spectroscopy; NOE, nuclear Overhauser effect; TFE, 2,2,2-trifluoroethanol; TSP, 3-(trimethylsilyl)propionic acid.

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-18 -15 -10 -5 -1 +1

His-Val-Phe-Leu-Ala-Pro-Gln-Gln-Ala-Arg-Ser-Leu-Leu-Gln-Arg-Val-Arg-Arg Peptide I

His-Val-Phe-Leu-Ala-Pro-Gln-Gln-Ala-Arg-Ser-Leu-Leu-Gln-Arg-Val-Arg-Arg-Tyr Peptide II

His-Val-**Ala**-Leu-Ala-Pro-Gln-Gln-Ala-Arg-Ser-Leu-Leu-Gln-Arg-Val-Arg-Arg Peptide III

His-Val-Phe-Leu-Ala-Pro-Gln-Gln-**Glu**-Arg-Ser-Leu-Leu-Gln-Arg-Val-Arg-Arg Peptide IV

FIGURE 1: Synthetic peptide analogues of the propeptides of prothrombin. These peptides are competitive inhibitors of the carboxylation of substrates that contain the carboxylation recognition site. The numbering of the residues is negative, starting from the amino terminus of mature prothrombin. The bold residues in peptides III and IV indicate mutations that impair expression of the carboxylation recognition site.

Circular Dichroism Measurements. CD measurements were recorded from 250 to 200 nm on a Jasco 500 CD spectrometer interfaced to a Packard-Bell computer for signal averaging and processing. The peptide spectra obtained in a 1-cm cell were averaged for 10 scans. θ represents mean residue ellipticity values (deg cm^2 dmol^{-1}) calculated from a mean residue molecular weight of 134–142, depending upon the primary structure of each peptide.

CD samples were 40 μM in peptide and 0.1 M in NaCl. The solution was adjusted to a pH meter reading between 7.4 and 7.7, uncorrected for the presence of organic solvent.

NMR Spectroscopy. NMR spectra were recorded on a Bruker AM 400 spectrometer equipped with an Aspect 3000 computer and digital phase shifters. Phase-sensitive 2D data were recorded by using time-proportional phase incrementation (Marion & Wüthrich, 1983; Rance et al., 1983). Typical 2D data sets contained 512 FIDs with 2K data points each, between 64 and 128 scans/FID, a sweep width of 4000 Hz, and a recycle time of at least 1.3 s. Data sets were zero filled to 1K data points in t_1. Data were processed on a Sun 3/60 workstation with the program FTNMR (Hare Research, Inc.). The data were multiplied by a shifted sine bell window function in both dimensions. The first data point in t_1 was multiplied by 0.5 to suppress t_1 noise (Otting et al., 1986). An automatic baseline correction was applied to NOESY data in the t_2 dimension prior to transformation in t_1. All chemical shifts are relative to 3-(trimethylsilyl)propionic acid (TSP).

Double-quantum-filtered (DQF) COSY spectra were recorded with the pulse sequence t_0–90°–t_1–90°–δ–90°–t_2, where t_1 is the evolution time, t_2 is the acquisition time, and δ is a fixed delay of 3 μs (Rance et al., 1983). DQF single-relayed COSY spectra were recorded with the pulse sequence t_0–90°–t_1–90°–δ–90°–τ–180°–τ–90°–δ–90°–t_2, where δ is a fixed delay of 3 μs and τ was 20 ms. TOCSY spectra were recorded with the pulse sequence t_0–90°–t_1–SL_x–(MLEV17)–SL_x–t_2, where SL_x was a 2.5-ms trim pulse along the x axis (Bax & Davis, 1985). The MLEV17 pulse sequence was repeated to give mixing times of 65–195 ms. NOESY data were collected with the pulse sequence t_0–90°–t_1–90°–τ_m–90°–t_2 (Jeener et al., 1979; Bodenhausen et al., 1984). Solvent suppression was achieved by presaturation of the H_2O resonance for all of the experiments except for NOESY. In the NOESY experiments the observation pulse was replaced with a 90° jump–return pulse (Plateau & Guêron, 1982). Spectra were recorded at several mixing times between 75 and 400 ms; the secondary structure was determined with a 200-ms (or shorter) mixing time, for which the effects of spin diffusion were negligible. The NOESY spectra at 5 °C in H_2O were recorded on a Bruker AMX 600 spectrometer with presaturation to suppress the water resonance. NMR samples, which were 20 mM in peptide, were prepared by dissolving the peptide in H_2O, adjusting the pH to 3.0 with HCl, and adding TFE to 35% (v/v).

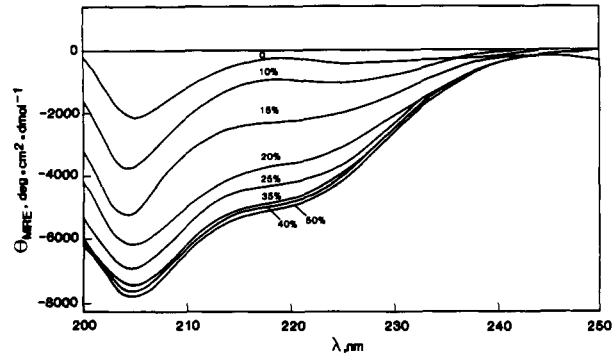

FIGURE 2: Effect of TFE on the circular dichroism spectra of peptide I. Mean residue ellipticity of the peptide was measured with increasing concentration of TFE, as indicated.

Reagents. CD samples were prepared with 2,2,2-trifluoroethanol (TFE; gold label) from Aldrich. For NMR samples, 2,2,2-trifluoroethyl alcohol-d_3 (99.1 atom % D) was purchased from MSD Isotopes. ^2H_2O (99.9%) and TSP were from Aldrich.

RESULTS

Peptide Preparation and Analysis. Four peptides were prepared for analysis (Figure 1). Peptide I is an 18-residue peptide containing the propeptide sequence of prothrombin (residues −18 to −1). Peptide II is identical with peptide I except for an added COOH-terminal tyrosine. Although residue +1 of prothrombin is Ala, not Tyr, two other vitamin K dependent proteins, factor IX and bone Gla protein, do have Tyr at this position. The tyrosine residue was useful in determination of peptide concentrations from the UV absorbance at 280 nm. Peptides I and II represent the unmutated propeptide sequence of prothrombin. Peptide III contains the propeptide sequence of prothrombin with Phe −16 changed to Ala. Peptide IV represents the 18-residue propeptide with Ala −10 changed to Glu. Peptides III and IV are analogous to mutations of the propeptide that significantly reduce or eliminate carboxylation in vivo (Jorgensen et al., 1987).

The peptides were synthesized by the solid-phase method with either Boc-amino acids or Fmoc-amino acids. After cleavage from the resin, the peptides were purified by reverse-phase HPLC. The purified peptides yielded a single symmetrical peak when analyzed by HPLC. Automated Edman degradation was employed to confirm the sequence of each peptide.

CD Spectra and the Effect of TFE. The CD spectrum of peptide I exhibited a small minimum at about 222 nm and a larger minimum at 208 nm at pH 7.7, 0.1 M NaCl, 23 °C (Figure 2). The spectra obtained at 4, 10, and 37 °C were not significantly different from the 23 °C spectrum. Variation of the pH from 2.0 to 10.0 and variation of NaCl concentration

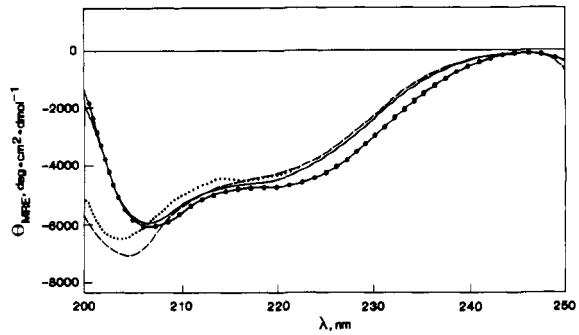

FIGURE 3: Comparison of the circular dichroism spectra of homologues of the prothrombin propeptide in 40% TFE. Peptide I, ---; peptide II, —; peptide III, ···; peptide IV, ●.

from 0 to 0.1 M made no detectable change in the CD spectrum of the prothrombin propeptide. The addition of TFE significantly altered the CD spectrum of the peptide in solution. TFE concentrations of 10% (TFE in water; by volume), 15%, 20%, 25%, 35%, 40%, and 50% led to deepening of the minima at 222 and 208 nm (Figure 2). A minimum at 222 nm reached a mean residue ellipticity of approximately −5000 deg cm^2 dmol^{-1}. At concentrations of 60% TFE or higher, precipitation of the peptide was observed. The magnitudes of the minima for the prothrombin propeptide in 40% TFE were reproducible to ±5% over a pH range of 2–8.5, NaCl concentration from 0 to 0.1 M, and peptide concentration from 15 to 100 μM.

CD spectra of the four peptides, recorded at 23 °C in 0.1 M NaCl and 40% TFE, are shown in Figure 3. CD spectra of the peptides were similar, suggesting that the four peptides share similar secondary structures. These results indicate that peptide II, with the added tyrosine on the COOH terminus, is structurally similar to peptide I and that the mutation of Phe −16 or Ala −10 does not disrupt the secondary structure observed under these conditions.

^{1}H NMR Resonance Assignments. Peptide II was studied by NMR to further characterize the secondary structure in 35% TFE. This peptide was readily soluble in 35% TFE at concentrations up to 100 mM peptide. The NMR line widths were not sensitive to concentration in the range of 0.1–20 mM, indicating negligible aggregation of the peptide.

Proton resonances assignments were made by standard 2D NMR procedures, involving identification of amino acid spin systems from analysis of DQF-COSY, DQF relayed COSY, and TOCSY experiments, followed by sequential resonance assignments from NOESY cross peaks connecting adjacent amino acids (Wüthrich, 1986). Figure 4 shows the regions of the COSY, relayed COSY, and TOCSY experiments that contain cross peaks between the backbone amide proton and the aliphatic proton resonances. There are 17 NH–C$^\alpha$H cross peaks in the COSY spectrum, corresponding to 17 of the 19 amino acids. No cross peak is observed for either the N-terminal histidine or the proline at −13. The relayed COSY spectrum contains the same NH–C$^\alpha$H cross peaks and the corresponding NH–C$^\beta$H cross peaks. Most of the side-chain resonances could be identified from the TOCSY spectrum. Confirmation of these spin system connectivities, and the Pro and His resonance assignments, were obtained from analysis of the aliphatic regions of these spectra (not shown).

Sequential connectivities were observed in the NOESY spectrum for both $d_{NN}(i, i+1)$ and $d_{\alpha N}(i, i+1)$ cross peaks. The $d_{NN}(i, i+1)$ connectivities were observed for residues −12 through −3 as shown in Figure 5. Connectivities for the entire sequence, with a single break at the Pro −13, were observed with the $d_{\alpha N}(i, i+1)$ cross peaks (Figure 6). These connectivities were compared to the spin system identification and the sequence of the peptide to make specific resonance assignments (Table I). Resonance assignments were made for the same peptide in H_2O (10% ^2H_2O) containing no TFE at 4 °C. Under these conditions the chemical shift distribution

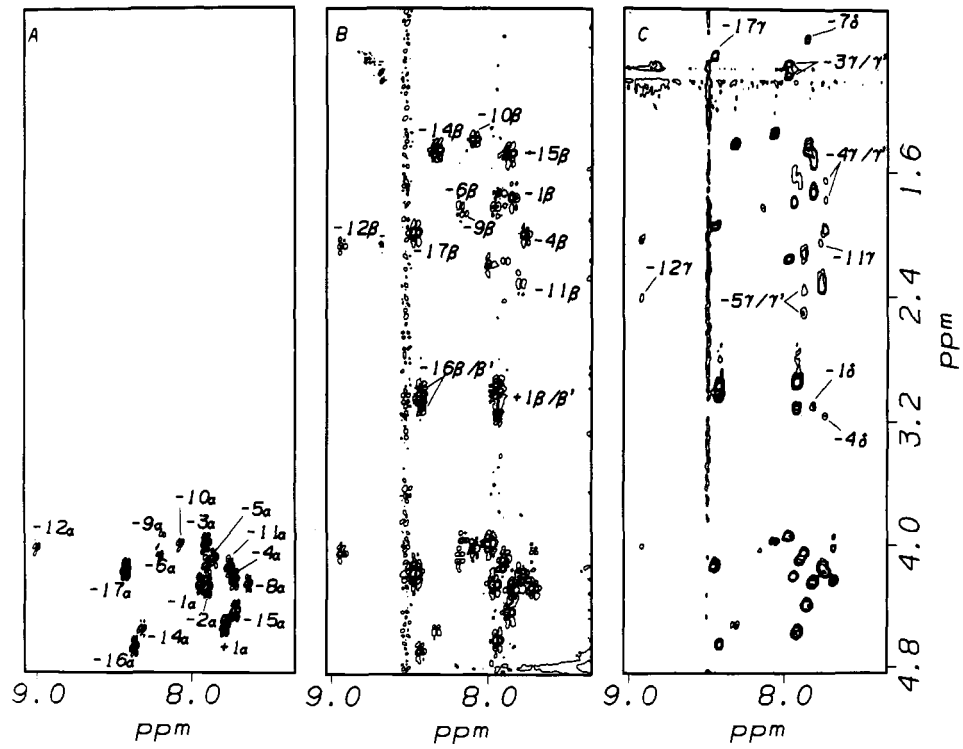

FIGURE 4: Contour plots of 400-MHz COSY, relayed COSY, and TOCSY spectra of peptide II in 35% TFE showing the cross peaks connecting the amide protons to the α and side-chain protons. (A)DQF-COSY spectrum showing the 17 amide–C$^\alpha$H cross peaks in the fingerprint region numbered according to their position in the sequence of the peptide. (B)DQF relayed COSY showing cross peaks to the corresponding β protons. (C) Additional peaks in the TOCSY spectrum connect the amide protons with C$^\gamma$H and C$^\delta$H protons as indicated.

Table I: Resonance Assignments for Peptide II[a]

	23 °C, pH 3.0, 35% TFE/65% H_2O					4 °C, pH 3.0, H_2O				
residue	NH	$C^{\alpha}H$	$C^{\beta}H$	$C^{\gamma}H$	other	NH	$C^{\alpha}H$	$C^{\beta}H$	C^{γ}	other
His −18		4.26	3.25		8.49, 7.01		4.28	3.31, 3.21		-
Val −17	8.44	4.18	1.99	0.91		8.63	4.17	1.99	0.91	-
Phe −16	8.38	4.69	3.03		7.32, 7.32, 7.20	8.84	4.63	3.06		-
Leu −15	7.72	4.45	1.47	1.70	0.78	8.36	4.37	-	-	-
Ala −14	8.32	4.56	1.47			8.58	4.30	1.41		
Pro −13		4.26	2.08, 2.39	2.20	3.82		4.39	1.91, 2.39	2.07	3.85, 3.71
Gln −12	9.01	4.03	2.07	2.44	6.66, 7.41	8.53	4.27	2.07	2.40	-
Gln −11	7.77	4.15	2.07	2.36	6.69, 7.28	8.74	4.26	2.04	2.40	-
Ala −10	8.09	4.00	1.40			8.48	4.46	2.06		
Arg −9	8.20	3.95	1.87	1.74	3.15, 7.20	-	-	-	-	-, -
Ser −8	7.64	4.26	3.97, 4.06			8.43	4.42	3.93, 3.88		
Leu −7	7.91	4.13	1.75	1.77	0.83	8.16	4.31	-	-	-
Leu −6	8.22	4.07	1.81	1.53	0.83	8.31	4.32	1.85, 1.69	1.65	0.84, 0.90
Gln −5	7.86	4.09	2.18	2.40, 2.53	6.80, 7.12	8.40	4.30	-	-, -	-, -
Arg −4	7.74	4.24	2.00	1.80, 1.71	3.06, 7.18	8.55	4.28	1.71	1.48	3.12, 7.13
Val −3	7.91	3.99	2.19	0.92, 1.00		8.31	4.08	2.06	0.89, 0.97	
Arg −2	7.97	4.27	1.81	1.60	3.12, 7.10	-	-	-	-	-, -
Arg −1	7.91	4.26	1.76	1.55	3.15, 7.16	8.51	4.28	-	-	-, -
Tyr +1	7.80	4.55	2.96, 3.13		6.82, 7.13	8.23	4.51	2.89, 3.12		-, -

[a] Chemical shifts are reported with respect to TSP. Some assignments in H_2O at 5 °C could not be made due to resonance overlap and are indicated by -.

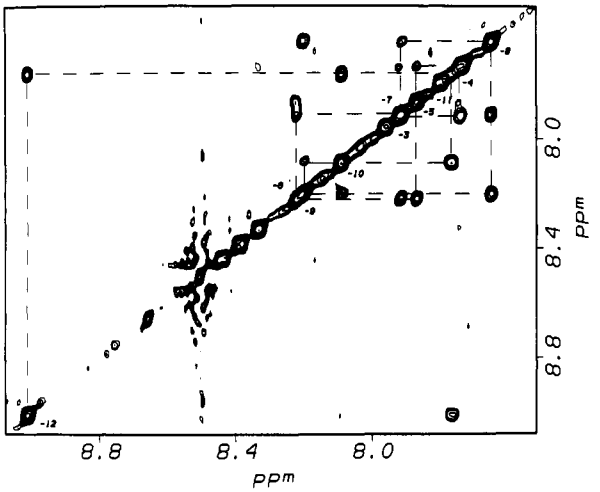

FIGURE 5: NOESY spectrum of peptide II in 35% TFE showing cross peaks between amide proton resonances. Sequential connectivities between adjacent amide protons [$d_{NN}(i, i + 1)$] are shown for residues −12 through −3.

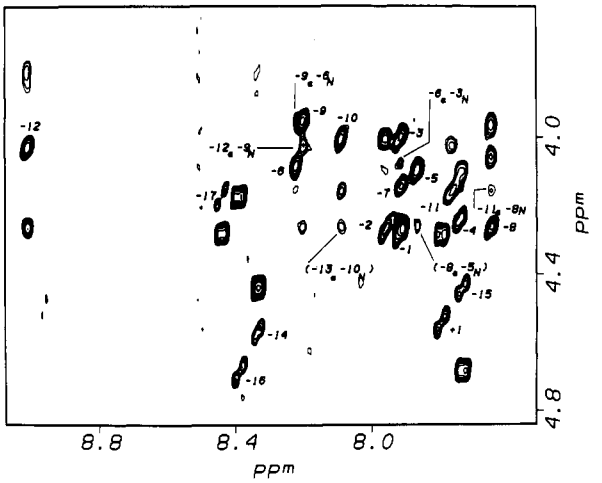

FIGURE 6: NOESY spectrum of peptide II in 35% TFE showing cross peaks between amide and α proton resonances. Intraresidue $d_{\alpha N}(i, i)$ cross peaks are numbered according to the residue position in the peptide sequence. The observed $d_{\alpha N}(i, i + 3)$ peaks are labeled. The parentheses around two of the labels indicate ambiguity in the cross-peak assignments due to overlap of the α proton resonances of Pro −13 and Ser −8, as discussed in the text.

for the NH and $C^{\alpha}H$ protons was narrow, allowing only a partial assignment of the resonances (Table I).

NMR Characterization of the Secondary Structure in 35% TFE. It is possible to interpret patterns of NOE effects in terms of peptide backbone conformations that are highly populated (Wright et al., 1988). As mentioned above, a number of short-range $d_{NN}(i, i + 1)$ NOE cross peaks were observed (Figure 5) in the region from residue −12 to −3. These cross peaks are not observed in disordered peptides and are consistent with an α-helical conformation for residues −12 to −3.

More definitive evidence of an α-helix comes from several medium-range NOE effects that connect the α proton of a residue with the amide or β proton three residues away. The $d_{\alpha N}(i, i + 3)$ connectivities are labeled in Figure 6. The cross peak between the α proton of Leu −6 and the amide proton of Val −3 is well resolved and unambiguous. A shoulder on the intraresidue $d_{\alpha N}(i, i)$ cross peak of Arg −9 corresponds to the NOE between the α proton of Arg −9 and the amide of Leu −6. A weak but well resolved cross peak is observed between the α proton of Gln −11 and the amide proton of Ser −8. The cross peak between the α proton of Gln −12 and the amide Arg −9 partially overlaps the $d_{\alpha N}(i, i + 1)$ cross peak between Ala −10 and Arg −9. The α protons of Pro −13 and Ser −8 have the same chemical shift, which complicates the assignment of the $d_{\alpha N}(i, i + 3)$ cross peaks. Two peaks were assigned to the connectivities between the α proton of Pro −13 and the amide proton of Ala −10 and between the α proton of Ser −8 and the amide of Gln −5. This assignment is consistent with an α-helical structure. The alternative assignments (e.g., connectivity between the α proton of Pro −13 and the amide proton of Gln −5) are inconsistent with the constraints placed on the the peptide backbone conformation by the unambiguous NOE connectivities. The $d_{\alpha N}(i, i + 3)$ cross peaks between the α proton of Ala −10 and the amide proton of Leu −7 and between the α proton of Leu −7 and the amide of Arg −4 would be obscured by overlap with strong $d_{\alpha N}(i, i)$ cross peaks. Three of the $d_{\alpha\beta}(i, i + 3)$ cross peaks are well resolved as indicated in Figure 7. These NOE cross peaks correspond to connectivities of the α protons of Pro −13, Gln −12, and Leu −7 to the β protons of Ala −10, Arg −9, and Arg −4, respectively.

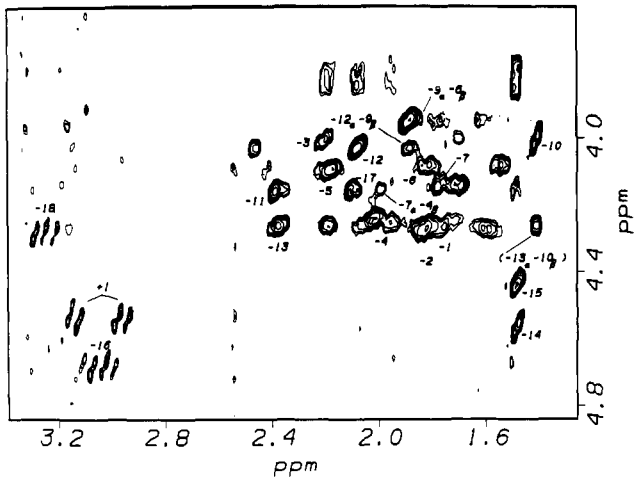

FIGURE 7: NOESY spectrum of peptide II in 35% TFE showing cross peaks between α and β proton resonances. Intraresidue $d_{\alpha\beta}(i, i)$ cross peaks are numbered according to the residue position in the peptide sequence. The observed $d_{\alpha\beta}(i, i + 3)$ peaks are labeled. The parentheses around one of the labels indicate ambiguity in the cross-peak assignment due to overlap of the α proton resonances of Pro −13 and Ser −8, as discussed in the text.

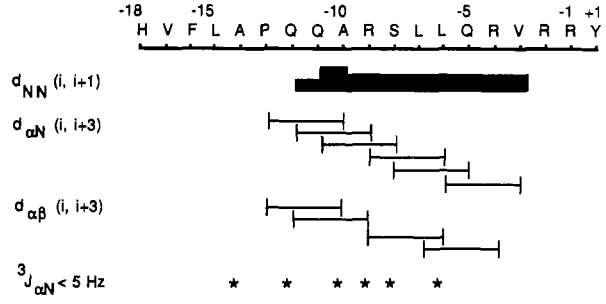

FIGURE 8: Summary of the NOE connectivities and $^3J_{HN\alpha}$ coupling constant data for peptide II in 35% TFE. Cross-peak intensity for the $d_{NN}(i, i + 1)$ connectivities is indicated by the thickness of the bar. The asterisks indicate residues for which $^3J_{HN\alpha}$ coupling constants were less than 5 Hz.

Additional information on the conformation of the peptide backbone can be obtained from the $^3J_{HN\alpha}$ coupling constants. The magnitude of a vicinal coupling constant is related to the value of the torsion angle between the two protons (Karplus, 1959). Observation of $^3J_{HN\alpha} < 6$ Hz correlates with an α-helical conformation, especially when 3–5 adjacent residues have small values of $^3J_{HN\alpha}$ (Wuthrich, 1986). Coupling constants were determined for the well resolved NH resonances in a 1D spectrum. The coupling constants for residues −17, −16, and +1 were >7.5 Hz, indicating significant amounts of extended structure at these positions. Residues −14, −12, −10, −9, −8, and −6 appeared as singlets with line widths of 10 Hz and, therefore, coupling constants of 5 Hz or less. The NOE and coupling constant data characterizing the secondary structure of the propeptide are summarized in Figure 8. These results are consistent with an α-helix that extends from Pro −13 to Val −3.

Stabilization of Secondary Structure in H_2O at Low Temperature. NOESY spectra recorded in H_2O at 23 °C showed no evidence of secondary structure, in agreement with the CD results. None of the NOE cross peaks indicative of an α-helix were observed under these conditions. At 5 °C, $d_{NN}(i, i + 1)$ cross peaks were observed that indicate the presence of an ordered conformation (Figure 9). With the partial assignments made under these conditions, it was possible to identify each of these cross peaks as indicated in Figure 9. A four-residue stretch of contiguous connectivities was made for

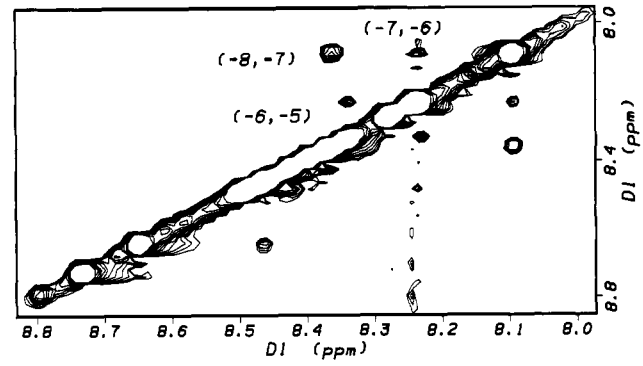

FIGURE 9: NOESY spectrum of peptide II in H_2O (10% ^2H_2O) at pH 3.0, 5 °C. The mixing time was 100 ms. Sequential connectivities between adjacent amide protons are shown.

residues −8, −7, −6, and −5. The ordered structure observed at low temperature is thus in the same region as the α-helix induced by TFE.

Discussion

The vitamin K dependent proteins contain a unique amino acid, γ-carboxyglutamic acid, that plays a major role in calcium ion binding by these proteins and in the interaction of these proteins with membrane surfaces (Furie & Furie, 1988). γ-Carboxyglutamic acid is formed posttranslationally by the carboxylation of specific glutamic acid residues by the vitamin K dependent carboxylase. These proteins share a signal element, known as the γ-carboxylation recognition site (Jorgensen et al., 1987), that designates the protein for carboxylation. The signal is located on the propeptide of the precursor forms of the blood coagulation proteins and bone Gla protein but has been observed as an internal signal within the sequence of the mature form of matrix Gla protein (Price & Williamson, 1985). The γ-carboxylation recognition site, defined by residues −18, −17, −16, −15, and −10 in prothrombin or factor IX (Jorgensen et al., 1987; Huber et al., 1990), binds directly to the vitamin K dependent carboxylase (Hubbard et al., 1989b).

The results presented here demonstrate that the prothrombin propeptide can form an α-helical structure that is stabilized by trifluoroethanol. The medium-range NOESY connectivities and $^3J_{HN\alpha}$ coupling constants indicate that the helix is located between residues −13 and −3. The observation of conformation-specific NOESY connectivities at low temperature, in the absence of TFE, suggests that this structure is present in aqueous solution. Residues −18 to −14 and −2 to +1 are not involved in the α-helix. The $^3J_{HN\alpha}$ coupling constants and lack of medium-range NOE contacts indicate that these two segments have significant populations of extended structure.

The secondary structural features determined by NMR were incorporated into a model of the propeptide of prothrombin by using the molecular modeling program Quanta (Figure 10). The ϕ and ψ torsion angles of the peptide backbone were set for a right-handed α-helix in the region between Pro −13 and Val −3, on the basis of the NMR data. Backbone conformations in the rest of the model were fully extended with $\phi = \psi = 180°$. Torsion angles in the side chains were arbitrarily set to 180°. The helical portion of this model is amphipathic with residues Gln −12, Gln −11, Arg −9, Ser −8, Gln −5, and Arg −4 making up the hydrophilic face and residues Pro −13, Ala −10, Leu −7, Leu −6, and Val −3 making up the hydrophobic face. The major elements of the γ-carboxylation recognition site are shown in red. This model provides a basis for comparison of the structural results to the results of the site-directed mutagenesis and in vitro carboxylation experi-

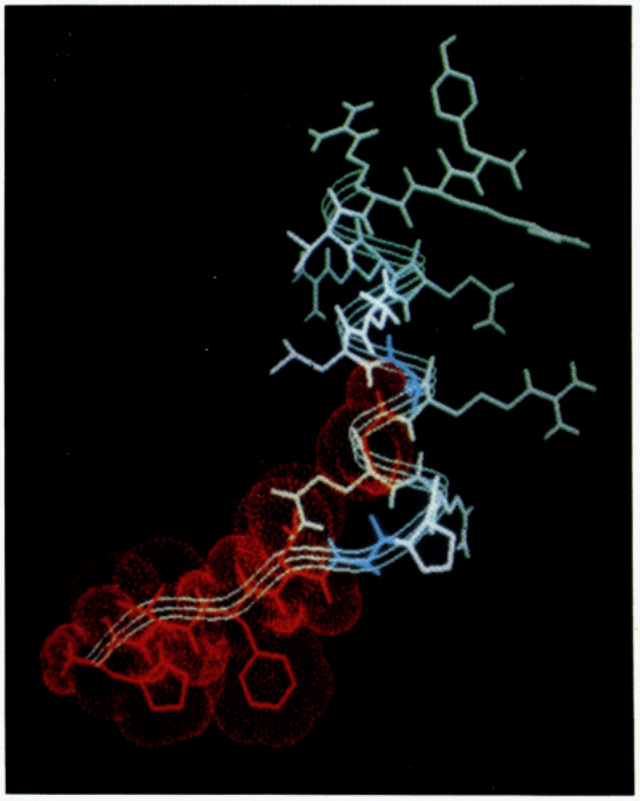

FIGURE 10: Molecular model of the prothrombin propeptide with an α-helical structure for residues −13 to −3. A ribbon has been drawn through the peptide backbone to illustrate the α-helix. The residues of the γ-carboxylation recognition site are in red and have dotted spheres at the van der Waals radius of each atom. The remaining residues of the hydrophobic face of the amphipathic α-helix, residues −13, −7, −6, and −3, are white. Residues −14 and −8, which are not involved in the γ-carboxylation recognition site, are colored blue.

ments that were used to define the carboxylation recognition site.

Residues −16 and −10 play an essential role in defining the carboxylation recognition site of the vitamin K dependent proteins (Jorgensen et al., 1987; Ulrich, et al., 1988). Recent studies also implicate residues −18, −17, and −15 in the carboxylation recognition site since point mutations at these positions in prothrombin disrupt carboxylation in vivo (Huber et al., 1990). Residue −10 resides on the hydrophobic face of the helical structure determined by NMR. The remaining residues of the γ-carboxylation recognition site, −18 to −15, are in the extended, amino-terminal region of the propeptide. In the synthetic peptides, the mutations at positions −16 and −10 did not alter the peptide secondary structure. If the helix is a valid model of the in vivo conformation of the propeptide, this suggests that the effect of these mutations on carboxylation is not due to a disruption of the secondary structure but results from direct interaction of the side chains of these residues with the γ-carboxylase. Mutations at positions −8, which is on the hydrophilic face of the α-helix, did not affect carboxylation. A helical conformation of the propeptide, which presents a hydrophobic binding site for the carboxylase, including the hydrophobic face of the helix and the residues of the N-terminus, is consistent with these mutagenesis results.

While the relationship of the structure determined in these experiments to the native conformation of the propeptide in vivo is uncertain, the model provides a basis for selecting additional residues for site-directed mutagenesis. Residues −7 and −6 are located adjacent to residue −10 on the hydrophobic face of the helix. Our model predicts that these residues may also interact with the enzyme. The results of Foster et al. (1987), in which a deletion mutant lacking residues −1 to −9 of the propeptide of protein C showed reduced carboxylation, are consistent with a carboxylation recognition site that includes residues −6 and −7. Furthermore, hydrophobic residues are found at position −6 (Leu, Ile, or Val) and −7 (all Leu except for protein C, which has Val) in all known propeptide sequences in vitamin K dependent proteins (Furie & Furie, 1988).

Little evidence of secondary structure in aqueous solution at 23 °C was detected by either CD or NMR. Most peptides of this size do not contain detectable amounts of secondary structure in aqueous solution (Epand & Sheraga, 1968; Taniuchi & Anfinsen, 1969), although several peptides have been shown to have partial or transient helical character that is stabilized by TFE or other alcohols (Brown & Klee, 1971; Chen & Sonenberg, 1977; Dyson et al., 1988; Green et al., 1987; Fry et al., 1989). The observation of NOE effects between neighboring amide protons of the prothrombin propeptide in H_2O, at low temperature, indicates that this peptide is not completely disordered in aqueous solution but contains a significant population of regular secondary structure.

In summary, we have presented conformational studies on peptide analogues of the propeptide of prothrombin which indicate that this peptide can adopt an amphipathic α-helix. A role for the helix in forming the γ-carboxylation recognition site is presented as a working hypothesis that is consistent with site-directed mutagenesis experiments and suggests further experimentation. The carboxylase is thought to be an integral membrane protein, and the lipid membrane itself may play a role in stabilizing the structure of the amphipathic α-helix within the propeptide. Experiments now in progress address the issue of the role of phospholipid membranes in stabilizing or inducing propeptide structure. Understanding the molecular basis of γ-carboxylation in terms of propeptide–carboxylase–membrane interaction should contribute to our knowledge of protein processing during protein biosynthesis.

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Mechanistic Studies on Thrombin Catalysis

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ABSTRACT: The kinetic mechanism of the cleavage of four *p*-nitroanilide (pNA) substrates by human α-thrombin has been investigated by using a number of steady-state kinetic techniques. Solvent isotope and viscosity effects were used to determine the stickiness of the substrates at the pH optimum of the reaction; a sticky substrate is defined as one that undergoes catalysis faster than it dissociates from the Michaelis complex. Whereas benzoyl-Arg-pNA could be classified as a nonsticky substrate, D-Phe-pipecolyl-Arg-pNA was very sticky. The other two substrates (tosyl-Gly-Pro-Arg-pNA and acetyl-D-Phe-pipecolyl-Arg-pNA) were slightly sticky. The pH profiles of k_{cat}/K_m were bell-shaped for all substrates. The pK_a values determined from the pH dependence of k_{cat}/K_m for benzoyl-Arg-pNA were about 7.5 and 9.1. Similar pK_a values were determined from the pH profiles of k_{cat}/K_m for tosyl-Gly-Pro-Arg-pNA and acetyl-D-Phe-pipecolyl-Arg-pNA and for the binding of the competitive inhibitor N^{α}-dansyl-L-arginine-4-methylpiperidine amide. The groups responsible for the observed pK_a values were proposed to be His57 and the α-amino group of Ile16. The temperature dependence of the pK_a values was consistent with this assignment. The pK_a values of 6.7 and 8.6 observed in the pH profile of k_{cat}/K_m for D-Phe-pipecolyl-Arg-pNA were displaced to lower values than those observed for the other substrates. The displacement of the acidic pK_a value could be attributed to the stickiness of this substrate. The basic pK_a value of 8.6 was caused by preferential binding of substrate molecules with protonated α-amino groups. The pH dependence of k_{cat} for benzoyl-Arg-pNA was consistent with the binding of the substrate decreasing the pK_a of His57 by more than 1 unit. The pH dependence of k_{cat} for the other three substrates was more complicated and suggested that the rate of catalysis was influenced by the ionization of groups not directly involved in catalysis.

Thrombin is a serine protease with considerable homology to chymotrypsin in trypsin in terms of both primary and tertiary structure (Bode et al., 1989). Like trypsin, thrombin shows a preference for cleaving substrates C-terminal to a basic residue, but the substrate specificity of thrombin is more restricted than that of trypsin (Chang, 1986; Lottenberg et al., 1981). In addition, thrombin appears to use sites that are distant from the active site in order to achieve specific interactions with macromolecular substrates, cofactors, and inhibitors (Fenton, 1981; Fenton & Bing, 1986). The recently determined crystal structures of D-Phe-Pro-ArgCH_2-thrombin (Bode et al., 1989) and hirudin-thrombin complexes (Rydel

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