Identification of Amino Acids in the \(\gamma\)-Carboxylation Recognition Site on the Propeptide of Prothrombin*

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A \(\gamma\)-carboxylation recognition site on the propeptide of the vitamin K-dependent blood coagulation proteins directs the carboxylation of glutamic acid residues by binding to the vitamin K-dependent carboxylase. To determine residues that define this site, we evaluated the effect of mutation of certain residues in the prothrombin propeptide on the extent of carboxylation. The prothrombin cDNA modified by site-specific mutagenesis was expressed in Chinese hamster ovary cells using a system that yields functionally fully carboxylated prothrombin. The cell supernatants containing recombinant prothrombin were evaluated for the extent of \(\gamma\)-carboxylation by immunoassay. Conformation-specific anti-prothrombin:Ca(II)-specific antibodies measure native completely carboxylated prothrombin; anti-prothrombin:total antibodies measure all forms of prothrombin, regardless of \(\gamma\)-carboxyglutamic acid content. Mutation of His\(^{16}\) to Gly, Val\(^{17}\) to Ser, Leu\(^{18}\) to Gly or Asp, or Ala\(^{-10}\) to Asp was associated with a partial (30–60\%) inhibition of \(\gamma\)-carboxylation. Mutation of Ala\(^{-14}\) to Ser or Ser\(^{-16}\) to Val did not inhibit \(\gamma\)-carboxylation. From this and earlier work, residues whose mutation leads to a significant impairment of carboxylation include His\(^{16}\), Val\(^{17}\), Phe\(^{19}\), Leu\(^{18}\), and Ala\(^{-10}\). Residues whose mutation does not alter the carboxylation recognition site include Ala\(^{-14}\), Ser\(^{-16}\), Arg\(^{-4}\), and Arg\(^{-1}\). To determine the size of the recognition site, the \textit{in vitro} carboxylation of propeptide-containing synthetic peptides was compared. A 28-residue peptide, based upon residues -18 to +10 of prothrombin, and a 54-residue peptide, based upon residues -18 to +36 of prothrombin, were carboxylated by partially purified bovine carboxylase with similar \(K_m\) values of 2–5 \(\mu\)M. These results indicate that the \(\gamma\)-carboxyglutamic acid-rich region of prothrombin makes a minimal contribution to carboxylase binding. A molecular surface of about five amino acids located within the propeptide appears to define the carboxylation recognition site on the precursor forms of the vitamin K-dependent proteins.

Prothrombin, a vitamin K-dependent blood coagulation protein, is the zymogen of the enzyme thrombin. This protein contains 10 \(\gamma\)-carboxyglutamic acid residues near the amino terminus of the mature protein (Stenflo et al., 1974; Nelsestuen et al., 1974). Prothrombin undergoes two metal-dependent conformational transitions that lead to the expression of a biologically active conformer that binds to membrane surfaces (Nelsestuen, 1976; Prendergast and Mann, 1977; Borowski et al., 1986). These transitions have an absolute requirement for most, if not all, of the \(\gamma\)-carboxyglutamic acid residues and for calcium ions. In the presence of calcium ions, a complex of Factor Xa, Factor Va, and membrane surfaces converts prothrombin to thrombin (Mann et al., 1982, 1988).

Vitamin K-dependent carboxylation is a post-translational process that takes place in the endoplasmic reticulum (Suttie, 1985, Furie and Furie, 1988, Carlisle and Suttie, 1990). The vitamin K-dependent carboxylase is a membrane protein that has recently been purified 10,000-fold (Hubbard et al., 1989b). This enzyme binds to a recognition element located on the propeptide of the precursor form of prothrombin, proprothrombin, and precursors of the other vitamin K-dependent proteins (Jorgensen, 1987a; Foster et al., 1987; Ulrich et al., 1988; Hubbard et al., 1989a). This recognition element, termed the \(\gamma\)-carboxylation recognition site (Jorgensen et al., 1987a), identifies the vitamin K-dependent proteins for post-translational carboxylation of specific glutamic acid residues to generate \(\gamma\)-carboxyglutamic acid residues. This reaction requires reduced vitamin K, molecular oxygen, carbon dioxide, the glutamyl-containing polypeptide substrate, and the carboxylase (Suttie, 1985).

Some of the residues that define the \(\gamma\)-carboxylation recognition site have been identified. From studies of the expression of Factor IX mutants prepared by site-specific mutagenesis, phenylalanine -16 and alanine -10 are critical residues in the 18-residue propeptide (Jorgensen et al., 1987a). Synthetic peptide substrates used in \textit{in vitro} carboxylation assays have confirmed the special role of phenylalanine -16 in peptides based upon the structure of proprothrombin (Ulrich et al., 1988). Another synthetic peptide based upon residues -10 to +10 of prothrombin is poorly carboxylated in this assay, suggesting the important role of the propeptide NH\(_2\)-terminal region in carboxylase recognition. Although naturally occurring Factor IX propeptide mutants, Factor IX Cambridge (Arg\(^{-2}\)→ Ser) and Factor IX San Dimas (Arg\(^{-2}\)→ Gln), are partially carboxylated (Diuguid et al., 1986; Ware et al., 1989), the carboxylation in \textit{in vitro} of synthetic peptides with primary structures based upon these mutants suggests that their carboxylation recognition sites are intact and that residues -1 and -4 are not part of the carboxylation recognition site (Hubbard et al., 1989a).

The extent of the carboxylation recognition site is unknown. Although the carboxylation of synthetic peptides re-
quires an intact propeptide (Ulrich et al., 1988), Price et al. (1987) have noted marked homology among the structures of the Gla regions in the vitamin K-dependent proteins. On this basis, Price has suggested that the carboxylation recognition site might extend into the Gia domain to include Glu-X-X-Glu-X-Cs (residues +16 to +22 in prothrombin) and may not be restricted to the propeptide as we have hypothesized (Jorgensen et al., 1987a).

To define the size and structure of the recognition site for the carboxylase, we have constructed a series of point mutations in the region of the prothrombin cDNA that encodes the propeptide. By using an expression system that yields fully carboxylated wild-type recombinant prothrombin (Jorgensen et al., 1987b), mutations of the amino-terminal propeptide amino acids were analyzed for the effect of mutation on the extent of carboxylation of thesecreted protein. We conclude that His, Val, Phe, Leu, and Ala compose part of the carboxylation recognition site. In addition, kinetic evidence comparing the binding of synthetic peptide mutagenesis (Oostra et al., 1987b). This fragment was inserted into the phage M13mp18, lacking nucleosides. When colonies became visible (15-30 colonies/dish) the colonies were pooled and the cells grown until confluent.

hamster ovary cell line CHODUKX-Bll (Chasin and Urlaub, 1980) characterized by DNA sequencing using the dideoxy chain termination method (Sanger et al., 1977). In each case the sequence of a 0.3-kilobase HindIII fragment containing the mutation was verified in its entirety and then inserted into the HindIII site of a plasmid PUC 12 containing a wild-type prothrombin cDNA lacking this fragment. The mutated prothrombin cDNA was excised from PUC 12 using EcoRI and inserted into the EcoRI site of an expression vector, pMT2 (Kaufman et al., 1986; Jorgensen et al., 1987b).

**EXPERIMENTAL PROCEDURES**

**Mutagenesis and Expression of the Mutated cDNAs**—The preparation of the 2-kilobase EcoRI fragment containing the human prothrombin coding sequence has been previously described (Jorgensen et al., 1987b). This fragment was inserted into the phage M13mp18, and the prothrombin cDNA was used as a template for site-directed mutagenesis (Oostra et al., 1989). Oligonucleotides used for the seven different mutations (Fig. 1) were synthesized on an Applied Biosystems 381A DNA synthesizer and purified prior to use. Phage containing the desired mutation were identified by colony hybridization using 32P-labeled mutagenic oligonucleotides. The mutants were finally purified by electroporation of the tissue culture supernatant for 1.5 h at 25°C to immunoprecipitate the radioactive prothrombin in the medium. After extensive washing, the protein A-Sepharose with antibody and prothrombin bound was incubated at 100°C in electrophoresis sample buffer. The eluted protein was then analyzed on a 7.5% sodium dodecyl sulfate-polyacrylamide gel. The proteins in the gel were visualized by autoradiography.

**Peptide Synthesis**—Peptides were synthesized using the solid-phase method using an Applied Biosystems model 430A synthesizer. proPT54 and proPT28 were synthesized using t-butoxycarbonyl/N-methylpyrrolidone chemistry (Oostra et al., 1988). Side chain protecting groups were benzyl (Ser and Thr), benzoxycarbonyl (Lys), 2-bromobenzoxycarbonyl (Tyr), 2,4-dinitrophenyl (His), mesitylene 2-sulfonyl (Arg), 4-methylbenzyl (Cys), and benzyl esters (Glu). Amine acids were coupled as 1-hydroxynaphthoic esters and sequentially coupled onto 0.1 mmol of OCH2phenylacetylmethylidemethyl resin. For proPT54, the coupling efficiency at each step was determined by ninhydrin analysis. Double coupling of amino acids was performed at selected positions within the first 43 amino acids and at all positions thereafter. Following each coupling step all uncoupled α-amine termini were acetylated. Sequential deprotection of the α-amine termini prior to coupling was achieved with 50% trifluoroacetic acid in methylene chloride. Prior to RP HPLC the 2,4-dinitrophenyl protecting group on histidine was removed from the resin-bound peptide using 20% β-mercaptoethanol. 10% disopropylthiophenyltriamine, dimethyformamide. A reaction mixture of 10 ml of 10% mercaptoethanol:disopropylthiophenyl triamineformamide/500 mg of resin was thrice mixed for 30 min at room temperature. The resin was then washed extensively with dimethylformamide followed by dichloromethane. The cleavage of the benzyl-protected peptide from the resin and simultaneous removal of the side chain protecting groups were performed using anhydrous hydrogen fluoride. The cleavage reactions were monitored in HF:anisole:resin (10:2:1) for proPT54. The coupling efficiency of each peptide was verified by automated Edman degradation using an ADolied Biosystems model 470 instrument (Hewick et al., 1982) and performed as described previously (Borowski et al., 1986).

**Immunoassays**—Fully carboxylated prothrombin was measured as native prothrombin antigen using anti-prothrombin:Ca(II)-specific antibodies, a conformation-specific antibody population that binds to the biologically active form of prothrombin (Borowski et al., 1986). The total prothrombin antigen was measured using anti-prothrombin:Ca(II)-specific antibodies. Both assays were performed as described previously (Borowski et al., 1986). Exogenous substrates were added as indicated. The

**Protein Reagents**—Human prothrombin was purchased from Enzyme Research Laboratories. The purification of rabbit anti-prothrombin:total or anti-prothrombin:Ca(II)-specific antibodies was performed as described previously (Borowski et al., 1986). The abbreviations used are: Gla, γ-carboxyglutamic acid; HPLC, high pressure liquid chromatography.
Preparation of Prothrombin Propeptide Mutations—Seven mutant forms of prothrombin were prepared using the wild-type prothrombin cDNA. Previously, phenylalanine -16 and alanine -10, residues that are highly conserved in the propeptides of vitamin K-dependent proteins, have been demonstrated to be part of the carboxylation recognition site (Jørgensen et al., 1987a; Rabiet et al., 1987; Ulrich et al., 1988). To define other residues that are also involved in defining this site, substitutions were made at residues -18, -17, -15, -14, -10, and -8 in the propeptide of prothrombin. These substitutions included histidine -18 to glycine (PT/HG-18), valine -17 to serine (PT/VS-17), leucine -15 to glycine (PT/LG-15) and to aspartic acid (PT/LD-15), alanine -14 to serine (PT/AS-14), alanine -10 to aspartic acid (PT/AD-10), and serine -8 to valine (PT/SV-8). Mutations were prepared in M13mp18 phage by site-directed mutagenesis using mutagenic oligonucleotides (Fig. 1) and the method of Oostra (1983). For each mutant the sequence of a fragment from the HindII site in the polylinker of M13mp18 to the HindIII site in prothrombin cDNA containing the mutation was verified by dideoxy sequencing and inserted into the wild-type prothrombin cDNA lacking this fragment in the PUC vector. The resulting mutated DNA was ligated into pMT2, a mammalian expression plasmid used in previous studies (Jorgensen et al., 1987a, 1987b) that is closely related to p91023 (Wong et al., 1985; Kaufman et al., 1986).

Expression of Mutated and Wild-type Prothrombin cDNAs in Chinese Hamster Ovary Cells—The wild-type and mutated prothrombin cDNAs were inserted into the EcoRI cloning site of pMT2. Introduction of the plasmids into dihydrofolate reductase-negative Chinese hamster ovary cells leads to the expression of a polycistronic message encoding both prothrombin and dihydrofolate reductase. The transfected cells were selected for their dihydrofolate reductase-positive phenotypes. Once the colonies were visible, the cells were dispersed and the polyclonal populations were grown until confluent. The conditioned media were harvested after 2 days at confluence and assayed by competition radioimmunoassay for total prothrombin antigen. The concentration of prothrombin expressed by these primary transfectants varied from 0.07 to 2.2 ng/ml (about 0.035-1.1 ng/10⁶ cells/24 h).

Post-translational Cleavage of Mutant Prothrombins To assure that normal post-translational propeptide cleavage had occurred during the synthesis of the mutant prothrombins, the molecular size of each prothrombin form was determined and compared with plasma-derived prothrombin and wild-type recombinant prothrombin. The proteins synthesized by Chinese hamster ovary cells expressing each of the different prothrombin species were metabolically labeled with [35S] cysteine, leading to the production of 35S-labeled prothrombin in the tissue culture supernatant. The mutant and wild-type labeled prothrombins were immunoprecipitated with anti-prothrombin:total antibodies complexed to protein A-Sepharose. After elution from the matrix the proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. As shown in Fig. 2, autoradiography demonstrated co-migration of the mutant prothrombins and the wild-type prothrombin. These results indicate that the mutation of these specific residues within the propeptide does not interfere with either propeptide cleavage or signal peptide cleavage. Thus, the lack of immunoreactivity of any prothrombin species to the conformation-specific antibodies would be due to decreased carboxylation and not to the presence of an attached propeptide.

Effects of Propeptide Mutations on Prothrombin Carboxylation—The extent of carboxylation of prothrombin derived from each mutant cDNA was determined by comparison of the concentrations of the native prothrombin antigen and the total prothrombin antigen. The native prothrombin antigen in the tissue culture supernatant is the fully carboxylated form of prothrombin. Fully carboxylated prothrombin was quantitated using a competition radioimmunoassay with anti-prothrombin:Ca(II)-specific antibodies which bind to fully carboxylated prothrombin in the presence of metal ions. These antibodies recognize prothrombin only when the protein is sufficiently y-carboxylated to undergo the two metal-induced conformational transitions. The concentration of native prothrombin antigen correlates closely with the coagulant activity (Blanchard et al., 1983). The total prothrombin antigen in the culture supernatants was measured by a competitive radioimmunoassay using anti-prothrombin:total antibodies that bind to all forms of prothrombin regardless of the state of carboxylation. The extent of carboxylation of the different prothrombin species is expressed as the ratio of the native prothrombin antigen concentration to the total prothrombin antigen concentration. Under the conditions used for protein expression, wild-type prothrombin appears fully carboxylated, with a native prothrombin antigen to total prothrombin antigen ratio of 0.89 ± 0.15 (Fig. 3). By comparison, plasma-derived prothrombin that is known to be fully carboxylated had a native prothrombin antigen to total pro-

![Figure 1. Oligonucleotides for the preparation of prothrombin propeptide mutants.](image-url)
FIG. 2. Immunoprecipitation of 36S-labeled prothrombin and mutant prothrombin forms. Lane 1, 35S-labeled prothrombin standard; lane 2, 35S-labeled prothrombin immunoprecipitated from tissue culture supernatant; lane 3, wild-type prothrombin; lane 4, PT/ SV-8; lane 5, PT/AD-10; lane 6, PT/AS-14; lane 7, PT/LD-15; lane 8, PT/LG-15; lane 9, PT/VS-17; lane 10, PT/HG-18.

Thrombin antigen ratio of 1.00 ± 0.17. The prothrombin expressed by the plasmids pMT2-PT/AS-14 and pMT2-PT/ SV-8 yielded species with a native prothrombin antigen to total prothrombin antigen ratio of 0.87 ± 0.12 and 1.00 ± 0.16, respectively. These results indicate that the mutation of alanine -14 to serine or serine -8 to valine does not alter the extent of carboxylation. Thus, alanine -14 and serine -8 are not located within the γ-carboxylation recognition site. In contrast, the prothrombin expressed by the plasmids pMT2-PT/HG-18, pMT2-PT/VS-17, pMT2-PT/LG-15, pMT2-PT/ LD-15, and pMT2-PT/AD-10 yielded species with a native prothrombin antigen to total prothrombin antigen ratio of 0.63 ± 0.11, 0.31 ± 0.04, 0.44 ± 0.12, 0.62 ± 0.07, and 0.53 ± 0.11, respectively. These results indicate that the mutation of histidine -18 to glycine, valine -17 to serine, leucine -15 to glycine or aspartic acid, or alanine -10 to aspartic acid partially impairs γ-carboxylation. The degree of inhibition is less for these amino acid substitutions than that of the mutation of phenylalanine -16 to alanine (Jorgensen et al., 1987a; Rabiet et al., 1987; Ulrich et al., 1988). These results indicate that the γ-carboxylation recognition site is partially disrupted by mutations at residues -18, -17, -15, and -10 and, based upon previous results, is almost completely disrupted by mutation at residue -16.

The observation of partially carboxylated prothrombin forms in the tissue culture supernatants could be due to the presence of some cells that can fully carboxylate prothrombin and other cells that are defective in carboxylation. To demonstrate that the protein products of all cells transfected with a given plasmid generated prothrombin that was carboxylated to the same extent, four stable transfectants were subcloned. Five to eight single colonies were isolated from cells transfected with pMT2-PT/AD-10, pMT2-PT/LG-15, pMT2-PT/ LD-15, and pMT2-PT. The supernatants were analyzed as described above (Fig. 4). Although the expression level of prothrombin varied in each subclone, the extent of carboxylation was identical, within experimental error, among subclones derived from the same primary transfectant.

Extent of the γ-Carboxylation Recognition Site; in Vitro Carboxylation of Peptides Containing Part of or the Entire Gla Domain—To determine the extent to which amino acids within the Gla domain contribute to the recognition of prothrombin by the vitamin K-dependent carboxylase, we studied the in vitro carboxylation kinetics of synthetic peptide homologs based upon the amino acid sequence of prothrombin. proPT28, previously described and extensively studied (Ulrich et al., 1988; Hubbard et al., 1989), contains residues -18 to +10 of prothrombin including two glutamic acid residues at positions +6 and +7. proPT54, spanning prothrombin from residues -18 to +36, includes all of the amino acids encoded by Exon II in the prothrombin gene (Friezner

FIG. 3. Carboxylation of prothrombin derived from forms of prothrombin containing point mutations in the propeptide. Cell culture supernatants from Chinese hamster ovary cells expressing prothrombin derived from various mutant forms of prothrombin were analyzed for the extent of γ-carboxylation by immunoblot. The concentration of total prothrombin antigen was measured using anti-prothrombin:total antibodies. This antibody population binds to all species of prothrombin regardless of the extent of carboxylation. The concentration of native prothrombin antigen was measured using anti-prothrombin:Ca(II)-specific antibodies. This population binds only to fully carboxylated prothrombin species that are able to undergo the metal-dependent conformational transition and bind to phospholipid membranes. The ratio of the native prothrombin antigen to the total prothrombin antigen, presented on the y-axis as relative native prothrombin antigen, is shown for each position tested. Error bars indicate the standard deviation of five independent measurements. The x-axis indicates the propeptide sequence of the wild-type prothrombin. The boxed residues are highly conserved among the propeptides of vitamin K-dependent proteins. The substituted amino acid is shown above the bar. Recombinant prothrombin (WT) and plasma-purified prothrombin are shown for comparison.
The y-carboxylation recognition site is a recognition sequence within the vitamin K-dependent proteins that directs γ-carboxylation during the final stages of the biosynthesis of these proteins. This recognition element in the vitamin K-dependent blood coagulation proteins immediately follows the signal peptide that destines the protein for translocation to the endoplasmic reticulum and precedes the glutamic acid-rich region that becomes γ carboxylated. The carboxylation recognition site binds directly to the carboxylase. Synthetic peptides that contain the carboxylation recognition site competitively inhibit γ-carboxylation of large peptide substrates (Ulrich et al., 1988), stimulate the carboxylation of small glutamate-containing substrates (Kuocho and Suttie, 1987), and form a complex with the vitamin K-dependent carboxylase (Hubbard et al., 1989b). This region shows marked sequence homology among the vitamin K-dependent proteins (Pan and Price, 1985). Previously, we demonstrated that phenylalanine –16 and alanine –10, both highly conserved amino acids in the vitamin K-dependent proteins, play a critical role in defining the carboxylation recognition site (Jorgensen et al., 1987a; Rabiet et al., 1987; Ulrich et al., 1988). In the current study, we have extended the analysis of the amino acids that define the carboxylation recognition site by exploring the role of other residues in the propeptide of prothrombin.

In contrast to Factor IX (Kaufman et al., 1986; Jorgensen et al., 1987a), prothrombin is fully carboxylated in the expression system that we have employed (Jorgensen et al., 1987b). Therefore, we have used prothrombin rather than Factor IX to determine the effect of point mutations in the propeptide on the extent of γ-carboxylation. Our choice of mutations remains somewhat arbitrary. Independent circular dichroism analyses indicate that mutations at residues –16 and at –10 do not disrupt the secondary structure of synthetic peptides containing the propeptide of prothrombin (Huber et al., 1989).

The extent of γ-carboxylation was estimated using an immunohemiac approach. We have previously used conformation-specific antibodies to monitor the extent of carboxylation of various forms of prothrombin (Tai et al., 1980; Blanchard et al., 1981; Blanchard et al., 1983). Since the conformational transition that is monitored is dependent upon full or nearly full carboxylation of prothrombin, the expression of native prothrombin antigen correlates closely with 5 μM for proPT28 and 10 mM for the pentapeptide substrate FLEEL. The Vmax values for proPT28 and proPT54 were the same within experimental error. These results indicate that the affinities of proPT28 and proPT54 for the carboxylase are similar when measured by the indirect yet physiologically relevant kinetic parameter Km. Furthermore, a synthetic peptide proPT18, from –18 to –1, is a competitive inhibitor of proPT28, with a K of 3.5 μM (Ulrich et al., 1988).

Thus, lengthening the substrate to include the entire Gla domain does not enhance binding of these peptides to the carboxylase. Under typical carboxylation reaction conditions using 1 μM substrate, it was found that approximately 45% of the glutamic acid residues in proPT28 were carboxylated, and approximately 40% of the glutamic acid residues in proPT54 were carboxylated (Table II). These data reveal that more CO2 molecules were incorporated into proPT54 than proPT28 on a mole to mole basis. However, these data alone are insufficient to distinguish the extent of carboxylation at any individual glutamic acid position, e.g. whether 40% of the proPT54 molecules were fully carboxylated or whether all proPT54 molecules were 40% carboxylated.

**DISCUSSION**

The γ-carboxylation recognition site is a recognition sequence within the vitamin K-dependent proteins that directs γ-carboxylation during the final stages of the biosynthesis of these proteins. This recognition element in the vitamin K-dependent blood coagulation proteins immediately follows the signal peptide that destines the protein for translocation to the endoplasmic reticulum and precedes the glutamic acid-rich region that becomes γ-carboxylated. The carboxylation recognition site binds directly to the carboxylase. Synthetic peptides that contain the carboxylation recognition site competitively inhibit γ-carboxylation of large peptide substrates (Ulrich et al., 1988), stimulate the carboxylation of small glutamate-containing substrates (Kuocho and Suttie, 1987), and form a complex with the vitamin K-dependent carboxylase (Hubbard et al., 1989b). This region shows marked sequence homology among the vitamin K-dependent proteins (Pan and Price, 1985). Previously, we demonstrated that phenylalanine –16 and alanine –10, both highly conserved amino acids in the vitamin K-dependent proteins, play a critical role in defining the carboxylation recognition site (Jorgensen et al., 1987a; Rabiet et al., 1987; Ulrich et al., 1988). In the current study, we have extended the analysis of the amino acids that define the carboxylation recognition site by exploring the role of other residues in the propeptide of prothrombin.

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with the amount of fully carboxylated prothrombin. Since the same observations have been made for Factor IX (Liebman et al., 1985; Liebman et al., 1987), we previously employed conformation-specific antibodies to estimate the amount of fully carboxylated Factor IX in the tissue culture supernatants of cells expressing Factor IX from plasmids containing Factor IX cDNA with propeptide-based mutations (Jorgensen et al., 1987a). When these Factor IX species were previously purified and subjected to direct γ-carboxyglutamic acid analysis, we confirmed excellent correlation between the γ-carboxyglutamic acid content of each species and its immunoreactivity with the conformation-specific antibodies (Rabiet et al., 1987). The immunochemical analysis obviate the need for large scale expression, protein purification, alkaline hydrolysis, and chemical analysis. Thus, in the current work we were able to examine 6 residues in the prothrombin propeptide by site-specific mutagenesis.

Taken with earlier data, our results indicate that histidine -18, valine -17, phenylalanine -16, leucine -15, and alanine -10 are components of the carboxylation recognition site (Fig. 6). The substitution of alanine -10 by aspartic acid decreased carboxylation of prothrombin by about 50%. The substitution of alanine by glutamic acid decreased immunoactivity in Factor IX by about 80% (Jorgensen et al., 1987a). Although both results clearly demonstrate a role for alanine -10 in carboxylation recognition, it remains uncertain whether these differences reflect variations between Factor IX and prothrombin propeptides or whether the substitution of an aspartic acid or glutamic acid yields different results.

Alanine -14 and serine -8 are not part of the site. These amino acids were mutated to substantially alter the character of the amino acid side chain. Despite conversion of the hydrophobic alanine to a hydrophilic serine and a hydrophilic serine to a hydrophobic valine, no effect on the extent of γ-carboxylation was noted. Neither of these residues is well conserved in the propeptides of the vitamin K-dependent proteins (Pan and Price, 1985).

We have previously hypothesized that the γ-carboxylation recognition site is restricted to the propeptide region of the vitamin K-dependent blood coagulation proteins (Jorgensen et al., 1987a). Price et al. (1987) identified a consensus sequence within the vitamin K-dependent proteins, Glu-X-X-Glu-X-Cys (located at residues 16-22 in human prothrombin), and suggested that the carboxylation recognition site might include this region of the Gla domain in addition to sites located within the propeptide. Pottorf and co-workers (1987) prepared a synthetic 17-residue substrate corresponding to residues 13-29 of bovine prothrombin and found that it was not an effective substrate for in vitro carboxylation (Pottorf et al., 1987). Our in vitro carboxylation studies utilized synthetic peptides that contain the intact propeptide of human prothrombin and varying amounts of the 36-residue Gla domain of prothrombin. proPT54 includes and proPT28 does not include the consensus sequence at positions +16 to +22. That proPT28 and proPT54 exhibit similar K_m values in in vitro carboxylation leads us to conclude that the carboxylase binds similarly to these substrates and that peptide sequences +11 to +36 within the Gla domain do not contribute to substrate recognition. Additional findings that the prothrombin propeptide, proPT18 (residues -18 to -1), is a competitive inhibitor of the carboxylase with a K_i of 3.5 μM (Ulrich et al., 1988) and that placement of the propeptide sequence in front of the first 14 residues of tissue plasminogen activator results in carboxylation of the glutamyl residue at position +12 (a residue not carboxylated in proTPA32) further support the argument that the carboxylation recognition site may be the sole determinant for carboxylation of accessible and adjacent glutamic acid residues. Our studies, including successful synthesis, purification, and efficient in vitro carboxylation of proPT54, will allow us to address questions of carboxylation directionality and processivity by determining the kinetic course of 14CO_2 fixation at each of the glutamyl residues.

Huber et al. (1989) have demonstrated that synthetic peptides that contain the carboxylation recognition site can be induced to assume α-helical character under conditions that are known to lead to secondary structure in short peptides that are helical within the context of the native protein of which they are a component (Kaiser and Kezdy, 1987). This α-helical structure suggests that the carboxylation recognition site is located within or adjacent to this helix. The examination of additional amino acids in the propeptide for their possible role in γ-carboxylation and the determination of the three-dimensional structure of the propeptide by two-dimensional NMR, currently in progress, should facilitate understanding of the structural correlates required for carboxylase recognition.

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2 M. J. Jorgensen, B. R., Hubbard, M. Jacobs, and D. DiMichele, unpublished data.
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