Amino Acids Responsible for Reduced Affinities of Vitamin K-Dependent Propeptides for the Carboxylase[†]

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ABSTRACT: The binding of the γ-glutamyl carboxylase to its protein substrates is mediated by a conserved 18 amino acid propeptide sequence found in all vitamin K-dependent proteins. We recently found that the apparent affinities of the naturally occurring propeptides for the carboxylase vary over a 100-fold range and that the propeptide of bone Gla protein has severely impaired affinity for the carboxylase [Stanley, T. B., et al. (1999) *J. Biol. Chem. 274*, 16940−16944 (*1*)]. Here we report a consensus propeptide sequence that binds tighter ($K_i = 0.43$ nM) to the carboxylase than any known propeptide sequence. Comparing the factor IX propeptide to the propeptides of protein C, bone Gla protein, and prothrombin, the weakest binding propeptides, allowed us to predict which residues might be responsible for these substrates' relatively weak binding to the carboxylase. We then made propeptides with the predicted amino acid changes and determined their binding affinities. The reduced binding affinity of these propeptides relative to that of FIX is due to residues −15 in protein C, −10 and −6 in bone Gla protein, and −9 in prothrombin. A role for the −9 position was not previously recognized but is further shown by our identification of a new, naturally occurring mutation at this position in factor IX which causes a warfarin-sensitive hemophilia B phenotype. In addition, we find that propeptides with mutations found in warfarin-sensitive patients have reduced affinity for the carboxylase, suggesting a physiological relevance of propeptide binding affinity.

The vitamin K-dependent γ-glutamyl carboxylase catalyzes the posttranslational modification of specific glutamic acid residues to γ-carboxyglutamic acid (Gla)[1] residues. This modification is essential for more than a dozen proteins involved in blood coagulation, bone metabolism, and calcium homeostasis (*2*−*6*). In all known vitamin K-dependent proteins, the mechanism by which the carboxylase recognizes its substrate is believed to be through initial binding to a conserved 18 amino acid propeptide. This propeptide is proteolytically removed before formation of the mature protein (*7*) except in the matrix Gla protein, where the propeptide-like sequence is part of the mature form of the protein (*8*). The importance of the propeptide sequence for carboxylation is demonstrated by studies which show that removal of the propeptide abolishes carboxylation of factor IX or protein C in tissue culture (*9*, *10*). In addition, attaching the propeptide to a glutamate-containing protein that is normally uncarboxylated can result in its carboxylation both in vitro and in tissue culture (*11*, *12*). Therefore, the conserved propeptide sequence appears to be essential for directing carboxylation of most vitamin K-dependent proteins.

The importance of the propeptide in vitamin K-dependent carboxylation was first suggested by Pan and Price (*13*), who noticed a number of highly conserved positions within the propeptide sequence. A number of studies confirmed this suggestion. For example, mutations at positions −18, −17, −16, −15, −10, and −6 impair carboxylation in tissue culture (*9*, *14*, *15*), while mutations at −14 and −8 have little or no effect (*14*). Comparison of the propeptide-like sequences of the known vitamin K-dependent propeptides reveals that the most conserved amino acids in the propeptide are phenylalanine at −16, alanine at −10, leucine at −6, and arginines at −4 and −1. Mutations at −4 and −1 are not believed to affect carboxylation but rather impair propeptide cleavage and processing (*14*, *16*).

We have recently determined that the relative affinities of the vitamin K-dependent propeptides vary over a ∼100-fold range, with the propeptide of bone Gla protein having no apparent affinity for the carboxylase in competitive inhibition assays (*1*). The propeptides of factors VII, IX, and

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[1] Abbreviations: Gla, γ-carboxyglutamic acid; CHAPS, 3-[(3-cholamidopropyl)]dimethylammonio-1-propanesulfonate; MOPS, 3-(*N*-morpholino)propanesulfonic acid; FIXproGLA, 59 amino acid peptide containing the human factor IX propeptide and first 41 residues of the factor IX Gla domain (sequence −18 to 41).

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X, protein S, matrix Gla protein, and proline-rich Gla protein (PRGP1) have K_i values between 2 and 40 nM. The propeptides of protein C and prothrombin have apparent affinities 2 orders of magnitude weaker than that of factor X and 1 order of magnitude weaker than that of factor IX. No inhibition was observed with the bone Gla protein propeptide, suggesting its affinity is at least 10 000-fold weaker than that of factor IX.

Further insight into the role of specific residues of the propeptide in binding to the carboxylase has been gained by the analysis of naturally occurring mutations in the propeptide. In previous work (*17*), we reported a patient with a substitution of threonine for alanine at position −10 in the propeptide of FIX which results in a syndrome of warfarin sensitivity, with FIX levels declining to <1% on exposure to therapeutic levels of warfarin. In vitro assays disclosed that the mutant propeptide results in a 30-fold increase in the apparent K_m of the variant propeptide for the carboxylase (compared to the wild-type FIX propeptide/Gla domain sequence). Subsequent patient studies identified a second mutation, A-10V, in the factor IX propeptide resulting in warfarin sensitivity (*18*).

Previous studies on the role of specific amino acids within the vitamin K-dependent propeptides for carboxylase recognition assessed the qualitative effects of propeptide mutations on proteins expressed in tissue culture (*9*, *14*, *15*). In this work, we have determined the relative affinities of the propeptides for the carboxylase by competitive inhibition of a propeptide/Gla domain substrate. With this assay, the quantitative effects of specific amino acid differences in the propeptide could be determined. The variation in affinities of the conserved propeptide sequences (*1*) provides an opportunity to understand the relative importance and contribution of individual amino acids within the propeptide for recognition by the carboxylase. Protein C and prothrombin propeptides both contain the highly conserved residues phenylalanine at −16, alanine at −10, and leucine at −6; nevertheless they have approximately 100-fold weaker affinity for the carboxylase (89- and 108-fold, respectively) than the factor X propeptides and 10-fold weaker than the factor IX propeptide (7- and 8-fold, respectively). Therefore, additional amino acids must be important in carboxylase recognition. To identify the amino acid(s) responsible for the reduced affinity of the protein C, bone Gla protein, and prothrombin propeptides relative to that of FIX, we changed specific amino acids to those found in factor IX or in a vitamin K-dependent propeptide consensus sequence and then determined the inhibition constants for each of these propeptides. We find that a single amino acid substitution in the protein C and prothrombin propeptides and a double substitution in the bone Gla protein propeptide suffice to increase their affinity to approximately that of the wild-type factor IX propeptide. In addition we have examined the relative affinities of three propeptides containing changes found in patients who acquired a syndrome similar to hemophilia B while on warfarin therapy; they all have significantly reduced affinity for the carboxylase.

EXPERIMENTAL PROCEDURES

Materials. All chemicals were reagent grade. Peptides based on the propeptide sequences (Figure 1) of the human vitamin K-dependent proteins were chemically synthesized, purified by reverse-phase HPLC, and verified to be correct by ion spray mass spectrometry by Chiron Technologies (Clayton Victoria, Australia). The wild-type FIXproGLA and FIXproGLA (R-4Q,R-1S) peptides based on amino acids −18 to 41 of human factor IX were prepared as described previously (*19*). FIXproGla peptides with the A-10T and N-9K mutations were expressed in *E. coli* and purified by reverse-phase HPLC as described previously (*17*). The human recombinant vitamin K-dependent carboxylase was expressed in baculovirus-infected insect cells and purified by immunoaffinity chromatography to an engineered metal ion-dependent epitope at the C-terminal end of the carboxylase as detailed previously (*1*).

Carboxylase Assays. All carboxylase assays were conducted using purified recombinant carboxylase (~40 nM final concentration) mixed on ice with a final concentration of 25 mM MOPS, pH 7.4, 0.5 M NaCl, 0.28% CHAPS, 0.12% phosphatidylcholine, 222 μM vitamin K hydroquinone, 6 mM dithiothreitol, and 5 μCi of $NaH^{14}CO_3$ (specific activity, 54 mCi/mmol; ICN Corp). Aliquots of this mixture were added to an indicated final concentration of the FIXproGLA peptide and/or propeptide, transferred to a 20 °C water bath, and incubated for 1 h. Reactions were stopped by the addition of 75 μL of 1 N NaOH, and the total amount of $^{14}CO_2$ incorporation was determined as described previously (*17*). Inhibition constants (K_i) were determined by the ability of the synthetic propeptides to inhibit the carboxylation of 0.5 μM FIXproGLA (R-4Q,R-1S) substrate. Data were fit by nonlinear regression to the equation for tight binding competitive inhibition (eq 1):

$$\frac{v_i}{v_0} = \frac{\sqrt{(K_i^* + I_t - E_t)^2 + 4K_i^* E_t} - (K_i^* + I_t - E_t)}{2E_t} \quad (1)$$

$$K_i = \frac{K_i^*}{\left(1 + \frac{[\text{FIXproGLA}]}{K_m}\right)}$$

where v_i is the initial velocity in the presence of inhibitor, v_o is the initial velocity in the absence of inhibitor, I_t is the total propeptide concentration, E_t is the total carboxylase concentration, K_m is the Michaelis−Menten constant for carboxylation of FIXproGLA, and K_i^* is the apparent inhibition constant. For K_m determinations of the FIXproGLA peptides, the peptides varied between 10-fold above and below their respective K_m values, and data were fit by nonlinear regression to the Michaelis−Menten equation. All kinetic constants are the average of at least two independent experiments ± the standard deviation.

Patient History. A patient with a mutation in the factor IX propeptide was evaluated as a part of this work. The patient is a 52-year-old male who was referred to the hematology service for evaluation of unusual coagulation studies while on warfarin. The patient had been in good health until 1 year prior to presentation when he underwent mitral valve replacement. The patient did well postoperatively and was discharged on warfarin. He had an uneventful course, but 12 months after surgery presented with hemoptysis and pleuritic chest pain. Coagulation studies on warfarin showed PT 19.7 s (11.5−13.5), INR 2.9, aPTT 85

s (22.7−35.5), mixing studies aPTT 29.6 s (immediate mix), aPTT 30.2 s (1 h mix), factor VII activity 22%, factor IX 1.3%, factor XI 116%. The patient underwent bronchoscopy, but no lesions were identified. Warfarin was stopped, and symptoms resolved. One week after stopping warfarin, the PT was 12.1 s, aPTT 27.7 s, and Factor IX level 86%. Warfarin was restarted at a lower dose; 1 month later coagulation studies showed PT 19.1 s, aPTT 61.2 s, and FIX level 1.8%.

Mutation Analysis. All eight exons of the factor IX gene were amplified by PCR using primer pairs previously described (*17*). Nucleotide sequences for all coding regions and intron−exon junctions were determined using an ABI automated sequencer. All nucleotide sequences were normal except exon 2 which contained a C to A transversion that changes the amino acid at position −9 from Asn (AAC) to Lys (AAA).

RESULTS

Affinity of a Consensus Vitamin K-Dependent Propeptide Sequence. To examine the relative role of individual amino acids in the recognition of the propeptide by the carboxylase, we created a vitamin K-dependent propeptide consensus sequence (Figure 1) based on the most commonly occurring amino acids at each position within the known vitamin K-dependent propeptides. As can be seen in Figure 2, the K_i of the vitamin K-dependent propeptide consensus sequence, 0.43 ± 0.2 nM, is at least 8-fold tighter than the affinity of the tightest known vitamin K-dependent propeptide, factor X, greater than 80-fold tighter than the factor IX propeptide, and 500-fold tighter than the propeptides of protein C and prothrombin. Because the consensus sequence binds significantly more tightly to the carboxylase than any known propeptide, we assumed that the varied reduced-affinity propeptides bound less tightly because of deviations from the consensus sequence.

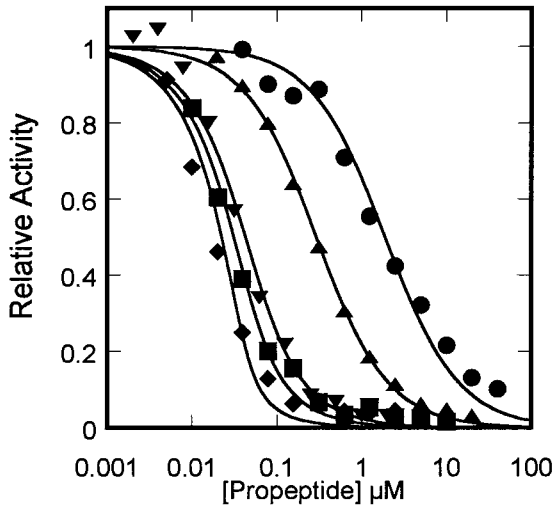
Amino Acid(s) Responsible for Reduced Affinity of Protein C Propeptide. We previously observed that the protein C propeptide binds to the γ-glutamyl carboxylase with 7-fold weaker affinity than the factor IX propeptide and an 88-fold weaker affinity than the factor X propeptide (*1*). The protein C propeptide contains the highly conserved sequences Phe −16, Ala −10, and Leu −6; however, residue −15 is serine rather than the consensus hydrophobic amino acid. Therefore, we determined whether this substitution is responsible for the increased K_i of the protein C propeptide relative to that of FIX. When leucine was substituted for serine at the −15 position in human protein C, its K_i was decreased 153-fold to 1.5 nM (Figure 2). Thus, this single substitution in protein C's propeptide makes its affinity for the carboxylase tighter than factor IX's propeptide and only 3-fold weaker than the consensus sequence.

Amino Acids Responsible for Reduced Affinity of Bone Gla Protein Propeptide. Our previous work indicates that the propeptide from bone Gla protein does not inhibit carboxylation of the factor IX propeptide/Gla domain peptide even at 400 μM propeptide. All known vitamin K-dependent proteins contain an alanine in the propeptide at the −10 position (Figure 1), except the bone Gla protein propeptide in which glycine is found at −10 in most species. Therefore, we substituted the glycine in the human bone Gla protein propeptide with alanine. As is seen in Figure 3, the G-10A substitution increased the affinity of bone Gla protein at least 45-fold; nevertheless, it is still 360-fold weaker than the factor IX propeptide. Another residue common only to bone Gla protein propeptides is valine at −6, where leucine or isoleucine is always found in the other known propeptides. Substitution of leucine for valine at −6 in bone Gla's propeptide increases the affinity ~100-fold, but it still binds to the γ-glutamyl carboxylase 170-fold weaker than factor IX's propeptide. However, when both G-10A and V-6L are substituted into bone Gla protein's propeptide, its apparent affinity is 60 nM, 80−200-fold tighter than either substitution alone and in the same range observed for the wild-type factor IX propeptide. Therefore, the reduced affinity of bone Gla protein's propeptide relative to that of FIX's is due to the glycine at −10 and valine at −6.

	-18-16	-10	-6	-1
human PT	HVFLAPQQARSLLQRVRR			
bovine PT	HVFLAHQQASSLLQRARR			
rat PT	HVFLAPQQALSLLQRVRR			
mouse PT	HVFLAPQQALSLLQRVRR			
human VII	RVFVTQEEAHGVLHRRRR			
mouse VII	AVFITQEEAHGVLHRQRR			
rabbit VII	AVFITQEEAHSVLRRQRR			
chick X	GVFIKKESADKFLERTKR			
human X	SLFIRREQANNILARVTR			
bovine X	SVFLPRDQAHRVLQRARR			
human IX	TVFLDHENANKILNRPKR			
dog IX	AVFLDRENATKILSRPKR			
mouse IX	AVFLDRENATKILTRPKR			
human PC	SVFSSSERAHQVLRIRKR			
bovine PC	SVFSSSQRAHQVLRIRKR			
rat PC	PVFSSSEGAHQVLRVR-R			
mouse PC	PVFSSSEHAHQVLRVR-R			
human PS	ANFLSKQQASQVLVRKRR			
bovine PS	ANFLSRQHASQVLIRRRR			
mouse PS	TNFLSKERASQVLVRKRR			
rat PS	TNFLSKERASQVLVRKRR			
human PZ	SVFLPASKANDVLVRWKR			
human PRGP1	RVFLTGEKANSILKRYPR			
human gas6	AALLPAREATQFLRRQRR			
human MGP	NPFINRRNANTFISPQQR			
bovine MGP	NPFINRRNANSFISPQQR			
mouse MGP	SPFINRRNANTFMSPQQR			
rabbit MGP	NPFINRRNANTFMSPQQR			
rat MGP	SPFTTRRNANTFISPQQR			
human BGP	AAFVSKQEGSEVVKRPRR			
bovine BGP	AAFVSKQEGSEVVKRLRR			
rat BGP	KAFMSKQEGSKVVNRLRR			
mouse BGP	KAFMSKQEGNKVVNRLRR			
chick BGP	KAFISHRQRAEMVRRQKR			
mouse BGP	KAFMSKQEGNKVVNRLRR			
consensus	AVFLSREQANQVLQRRRR			

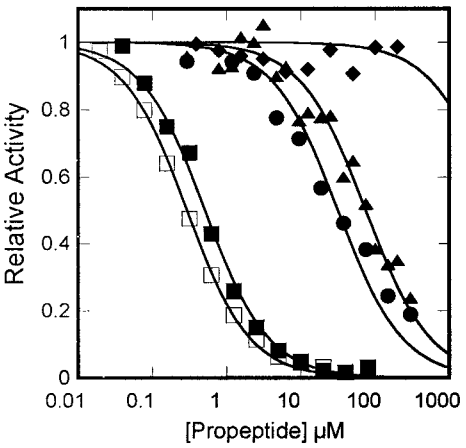
FIGURE 1: Alignment sequences of the vitamin K-dependent propeptides. The sequences of the propeptides or propeptide-like sequence found in 35 vitamin K-dependent proteins are shown. All sequences were obtained from the Prosite database (*23*) except for PRGP1 which is from Kuhlman et al. (*24*). The consensus sequence is based on the most common amino acid occurring at each position.

Amino Acids Responsible for Reduced Affinity of Prothrombin Propeptide. The prothrombin propeptide has an 8-fold weaker K_i than the factor IX propeptide. Two sequence differences in the prothrombin propeptide compared to the



		K_i (nM)
PC	SVFSSSERAHQVLRIRKR	230 ± 20
PC S-15L	SVF**L**SSERAHQVLRIRKR	1.5 ± 0.2
FIX	TVFLDHENANKILNRPKR	33.6 ± 5
FX	SLFIRREQANNILARVTR	2.6 ± 0.1
Consensus	AVFLSREQANQVLQRRRR	0.43 ± 0.2

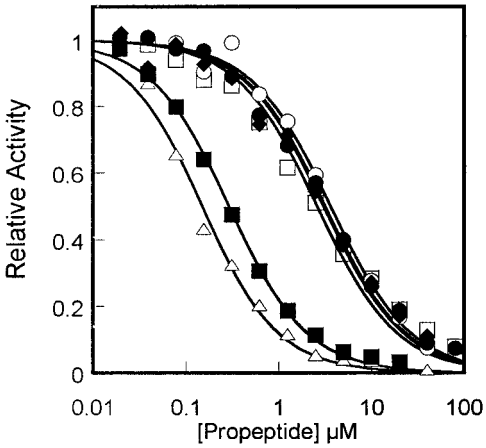
FIGURE 2: Inhibition of FIXproGLA carboxylation by protein C-derived propeptides. Inhibition of 0.5 μM FIXproGLA carboxylation by the wild-type protein C propeptide (closed circles), wild-type factor IX (closed triangles), protein C S-15L (closed squares), wild-type factor X (inverted triangles), and the consensus sequence (diamonds). Inhibition constants were determined by fitting the data to eq 1.



		K_i (μM)
BGP wt	KAFVSKQEGSEVVKRPRR	>500
BGPG-10A	KAFVSKQE**A**SEVVKRPRR	11 ± 0.8
BGPV-6L	KAFVSKQEASEV**L**KRPRR	5.1 ± 2.3
BGP A-10G V-6L	KAFVSKQE**A**SEV**L**KRPRR	0.06 ±.01

FIGURE 3: Inhibition of FIXproGLA carboxylation by bone Gla protein-derived propeptides. Inhibition of 0.5 μM FIXproGLA carboxylation by the wild-type bone Gla propeptide (closed diamonds), bone Gla protein V-6L (closed circles), bone Gla protein G-10A (closed triangles), bone Gla protein G-10A,V-6L (closed squares), and wild-type factor IX (open squares). Inhibition constants were determined by fitting the data to eq 1.

other vitamin K-dependent propeptides are candidates for this reduced affinity. The first, a proline at −13, is not found in any of the other propeptides and would be expected to disrupt the helical structure of the propeptide postulated by



		K_i (nM)
PT	HVFLAPQQARSLLQRVRR	280 ± 120
PT P-13H	HVFLA**H**QQARSLLQRVRR	440 ± 30
PT R-9N	HVFLAPQQA**N**SLLQRVRR	16 ± 2.7
FIX	TVFLDHENANKILNRPKR	33.6 ± 5
FIX N-9K	TVFLDHENA**K**KILNRPKR	370 ± 30
FIX N-9L	TVFLDHENA**L**KILNRPKR	460 ± 120

FIGURE 4: Inhibition of FIXproGLA carboxylation by prothrombin-derived propeptides. Inhibition of 0.5 μM FIXproGLA carboxylation by the wild-type prothrombin propeptide (open squares), prothrombin propeptide P-13H (open circles), prothrombin propeptide R-9N (open triangles), wild-type factor IX propeptide (closed squares), FIX propeptide N-9K (closed diamonds), and FIX propeptide N-9L (closed circles). Inhibition constants were determined by fitting the data to eq 1.

NMR studies (*20*). Substitution of the proline at −13 with histidine had little effect on the K_i of the propeptide (Figure 4). The second candidate is the arginine at the −9 position. This residue is unique to human prothrombin (Figure 1). While the −9 position is not strongly conserved, 77% of the 35 sequences shown in Figure 1 are histidine, serine, or asparagine. In addition, as is discussed below, we had found that substituting another basic residue, lysine, for asparagine at −9 in factor IX results in a propeptide affinity nearly identical to prothrombin. Therefore, we postulated that arginine at −9 in prothrombin may result in the reduced affinity of the propeptide of prothrombin for the γ-glutamyl carboxylase. This hypothesis was substantiated by the observation that substituting asparagine for arginine at −9 increased the affinity of the prothrombin propeptide 17-fold; this is somewhat tighter than the affinity of factor IX's propeptide. To further examine the role of this position, we changed FIX's −9 asparagine to leucine, the residue found at this position in the mouse and rat prothrombin propeptides. This factor IX N-9L substitution reduces the relative affinity of the factor IX propeptide 14-fold, a value in the same range as prothrombin's propeptide. Therefore, the −9 position is important for the propeptide's interaction with the carboxylase, and the prothrombin propeptide's reduced affinity for the carboxylase compared to that of factor IX can be attributed to the −9 position.

Naturally Occurring Mutation at −9 Causes Warfarin Sensitivity. The identification of the −9 position of prothrombin as the cause of its reduced affinity for the carboxylase was surprising due to the poor sequence conservation at this residue. But further evidence for an

Table 1: Kinetic Parameters for Factor IX Propeptides and Substrates with Warfarin-Senistive Hemophilia B Mutations

peptide	V_{max}[a] (pmol/h)	K_m (nM)[a]	*x*-fold	K_i (nM)[b]	*x*-fold
wild-type	84 ± 13	179 ± 44	1.0	33 ± 5.0	14.0
N-9K	163 ± 13	597 ± 194	3.3	370 ± 30	11.0
A-10T	188 ± 10	2212 ± 662	12	20000 ± 3200	606
A-10V	ND	ND	ND	6050 ± 200	183

[a] Kinetic parameters for carboxylation of FIXproGLA peptides with the indicated mutations. Parameters were determined by fitting data to the Michaelis−Menten equation by nonlinear regression. [b] Inhibition constants for inhibition of FIXproGLA(R-4Q,R-1S) by synthetic factor IX propeptides with the indicated mutations. Parameters were determined by nonlinear regression to eq 1.

important role of this position is provided by a naturally occurring mutation. A patient undergoing warfarin therapy demonstrated severely reduced factor IX activity (1.3%) compared to normal activity (86%) upon removal of warfarin. This phenotype is similar to the warfarin-sensitive phenotype demonstrated previously for mutations at the −10 position in factor IX (*17*, *18*). Sequencing of the patient factor IX gene revealed a single transversion mutation (C to A) resulting in a lysine for arginine at position −9 within the factor IX propeptide. Therefore, we have identified a novel naturally occurring mutation at the −9 position within the propeptide that results in a pronounced bleeding disorder on exposure to warfarin.

Effect of Naturally Occurring Propeptide Mutations on Propeptide Affinity for Carboxylase. Insights into the physiologic consequences of the varied propeptide affinities is gained by studies of patients with naturally occurring mutations at the residues we have identified as determinants of carboxylase affinity. We (*17*) and others (*18*) have previously reported the existence of a syndrome of warfarin sensitivity in patients with mutations at alanine −10 (A-10T and A-10V) of the FIX propeptide. In this report, we identify another mutation, N-9K, also in the FIX propeptide, with a similar phenotype. Kinetic parameters for carboxylation of propeptides containing these mutations are shown in Table 1. Note that substitution of the highly conserved alanine −10 with threonine or valine severely impairs the binding of the propeptides to the carboxylase with 600- and 180-fold higher K_i values, respectively. The lysine substitution for asparagine has a much less dramatic effect but nevertheless reduces the affinity 12-fold. Of interest is the correlation in vivo of the affinities observed in the in vitro assay. Thus, the patient with the N-9K mutation had factor IX levels in the range of 1−2%, while on therapeutic levels of warfarin, while the patient with the A-10T mutation had factor IX levels of <1% in the same setting (*17*).

To evaluate how variations in propeptide affinity impact the binding of substrates of the carboxylase in vivo, we examined the effect of the A-10T and N-9K mutations on the kinetics of carboxylation of a factor IX propeptide/Gla (FIXproGla) peptide. This peptide resembles the native substrates of the enzyme (*12*, *19*). The A-10T mutation increases the K_i of the factor IX propeptide 600-fold, but only a 10-fold increase in the K_m of the FIXproGla is observed. Similarly, with the N-9K mutation, the K_i for the factor IX propeptide is increased 12-fold while only a 3-fold increase in K_m is observed. Therefore, point mutations in the factor IX propeptide which cause a bleeding diathesis also cause a reduced propeptide binding affinity which in turn results in impaired substrate binding.

DISCUSSION

The purpose of this study was to identify amino acids of the propeptides of the vitamin K-dependent proteins bone Gla protein, protein C, and prothrombin responsible for their decreased affinity, relative to FIX, for the vitamin K-dependent γ-glutamyl carboxylase. In addition, we have identified a new naturally occurring mutation within the factor IX propeptide that results in warfarin-sensitive hemophilia B and show how warfarin-sensitive hemophilia B mutations affect both the propeptide affinity and binding of vitamin K-dependent protein substrates.

The −10 position is alanine in the propeptides of all known vitamin K-dependent proteins except for bone Gla protein (Figure 1). This position is well established as a critical residue in carboxylase recognition since it is one of the most highly conserved residues within the propeptide and since mutations at this position impair carboxylation in tissue culture (*9*, *14*) or cause a bleeding diathesis (*17*, *18*). We find that a substitution of threonine or a conservative change to valine severely impaired the factor IX propeptide's affinity for the carboxylase as evidenced by their 200−600-fold increased K_i values. In addition, a glycine at the −10 position is partly responsible for the unmeasurable affinity of the bone Gla protein propeptide. Therefore, alanine at the −10 position is required for optimal binding to the γ-glutamyl carboxylase.

All the known vitamin K-dependent blood proteins have a leucine at the −6 position except matrix Gla and bone Gla proteins. Therefore, −6 is one of the most highly conserved positions in the vitamin K-dependent propeptides. The only reported in vitro mutation, an aspartate for leucine in the FIX propeptide, results in impaired carboxylation in tissue culture (*15*). In this study, we find that a valine for leucine substitution is partially responsible for bone Gla protein's undetectable carboxylase binding and causes at least a 100-fold reduction in affinity. The G-10A and V-6L substitutions in combination are sufficient to convert the bone Gla propeptide from undetectable binding to one whose binding constant is in the same range as that of FIX. It is interesting to note that even a conservative change at the −6 position (i.e., valine for leucine) dramatically impairs carboxylase recognition. This suggests that a leucine at −6, like the alanine at −10, is highly favorable for binding of the carboxylase and that these residues likely make intimate contact with the carboxylase.

The importance of a hydrophobic residue at −15 is demonstrated by the increased affinity of the protein C propeptide when serine at −15 is replaced by leucine. As can be seen in Figure 1, the −15 position is almost always hydrophobic, with 77% of the propeptides containing valine, leucine, or isoleucine at this position. Mutations at −15 from leucine to glycine or aspartate have previously been shown to impair carboxylation of prothrombin in tissue culture (*14*). We find that changing the serine in the protein C propeptide to leucine, which is found both in the consensus propeptide sequence and in the factor IX propeptide, increases its affinity 150-fold. Curiously, this substitution alone changes the affinity of the protein C propeptide from one of the weakest to one of the tightest binding propeptides and demonstrates

the significant contribution of the hydrophobic residues at the −15 position to carboxylase recognition.

We have identified a previously unrecognized role of the −9 position in carboxylase recognition by its effect on the affinity of the prothrombin propeptide and by the discovery of a naturally occurring mutation which results in a significant bleeding diathesis. Although the −9 position is not well conserved, 77% of the 35 sequences shown in Figure 1 have histidine, serine, or asparagine at this position. In this work, we have identified three amino acid substitutions at the −9 position which result in modest, ∼10-fold alterations in apparent binding affinity. Therefore, the poor sequence identity and modest affinity changes suggest that this residue likely does not make a direct contact with the carboxylase, but is important for maintaining proper propeptide structure.

The physiological relevance of the propeptide binding affinities is suggested by our findings that patients with warfarin sensitivity have reduced affinity for the carboxylase. We are aware of three factor IX mutations which cause warfarin sensitivity: Ala-10 to Thr, Ala-10 to Val (*17*, *18*), and now Asn −9 to Lys. Patients with these mutations possess normal or near-normal levels of factor IX before anticoagulant therapy with warfarin, but during therapy their factor IX levels are depressed to approximately 1% of normal at doses of warfarin that decrease the other vitamin K-dependent clotting factor levels to the 30−40% range (*17*). We find that the mutations at the −10 position severely impair binding (200−600-fold) of the propeptide while the mutation at −9 reduces affinity about 10-fold compared to the wild-type propeptide. When we examined the kinetics of carboxylation of factor IX propeptide/Gla domain peptides with the same mutations, we found less dramatic increases (3−10-fold) in their K_m parameters for carboxylation. This phenomenon could be explained by the presence of another significant binding site in the vitamin K-dependent proteins which contributes to substrate affinity. But this seems unlikely since propeptides attached to irrelevant glutamate-containing proteins have similar K_m values in vitro (*12*) and are carboxylated in tissue culture (*11*). We believe it is instead likely that the more modest effects of propeptide mutations on the K_m of the factor IX propeptide/Gla domain peptides are a consequence of the composite nature of the K_m in these assays. Vitamin K-dependent carboxylation is a multistep process in which binding of other substrates and catalytic steps after the initial interaction of the carboxylase with the propeptide may contribute to both composite kinetic parameters K_m and $V_{max.}$ Therefore, large differences in affinity of the propeptide may result in only modest changes in the apparent affinity of the vitamin K-dependent substrate suggested by the K_m kinetic parameter.

Although severely reduced propeptide binding can have a modest effect on the perceived binding of the entire substrate (i.e., K_m), these modest changes can have significant physiological impact. For example, the N-9K mutation in the factor IX propeptide increases the K_i of the factor IX propeptide 11-fold, while the K_m for carboxylation of a factor IX propeptide/Gla domain substrate is increased only 3-fold. Nevertheless, this modest reduction in substrate binding can have profound clinical consequences as evidenced by the warfarin-sensitive hemophilia B phenotype of a patient with the N-9K mutation.

One of the proposed explanations for the demonstrated reduction of factor IX levels in this warfarin-sensitive syndrome is that the factor IX precursors with reduced affinities are out-competed in the endoplasmic reticulum by the other normal affinity vitamin K-dependent precursors. Evidence for competition among vitamin K-dependent precursors with different affinities is also seen with microsomes isolated from warfarin-treated cows (*21*) or warfarin-treated HepG2 cells (*22*). In both of these studies, the majority of the carboxylase is found complexed with the factor X precursors rather than the prothrombin precursors. Our recent study indicates that the factor X propeptide has a ∼100-fold greater affinity than the prothrombin propeptide (*1*). Therefore, vitamin K-dependent precursors with higher affinity propeptides out-compete those with weaker binding during warfarin-induced accumulation of vitamin K-dependent precursors in the endoplasmic reticulum. Such a competition effect could be overcome by having a much higher concentration of the weaker affinity substrate. It is curious that the wild-type prothrombin propeptide and the factor IX N-9K mutation have almost identical affinities for the carboxylase. Nevertheless, the patient with the factor IX N-9K mutation has reduced factor IX levels on warfarin administration while the prothrombin level is in the therapeutic range. Therefore, the higher expression levels and concentration of the prothrombin propeptide may permit it to overcome its somewhat poorer affinity for the carboxylase, while the lower concentration of the mutant factor IX precursor cannot.

While this explains the observations mentioned above, it is not simply a matter of abundance relative to affinity because factor X, which has a tighter binding constant than FIX, is present at higher concentrations in the blood plasma. It is possible that some of the most crucial vitamin K-dependent factors, such as matrix Gla protein and factor X, have evolved tight binding for the organism's survival when vitamin K is low. However, in the absence of additional data, such as the levels of mRNA of the different coagulation factors, it is not possible to do anything more than speculate. Evolution, after all, only selects for survival, not optimal survival.

This work has for the first time identified the quantitative importance of specific amino acids within the vitamin K-dependent propeptides for binding by the carboxylase. The lower binding affinities of the protein C and prothrombin propeptides have been ascribed in each case to a single amino acid difference from the consensus or factor IX propeptide sequences, while the almost imperceptible affinity of the bone Gla protein propeptide results from two differences. Amino acid differences at the −15, −10, and −6 positions result in greater than 100-fold reductions in affinity. In addition, a previously unidentified propeptide residue, −9, was found to be important for the recognition of the propeptide by the γ-glutamyl carboxylase by its role in the reduced affinity of the prothrombin propeptide and by the discovery of a naturally occurring mutation at this position. Finally, reduced propeptide affinity results in reduced vitamin K-dependent substrate binding which in turn can have profound clinical consequences, indicating the importance of propeptide affinity in maintaining levels of vitamin K-dependent proteins.

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