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# GGCX mutants that impair hemostasis reveal the importance of processivity and full carboxylation to VKD protein function

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**Abstract:**
The gamma-glutamyl carboxylase (GGCX) generates multiple carboxylated Glus (Glas) in vitamin K-dependent (VKD) proteins that are required for their functions. GGCX is processive, remaining bound to VKD proteins throughout the multiple Glu carboxylations, and this study reveals the essentiality of processivity to VKD protein function. GGCX mutants (V255M, S300F) whose combined heterozygosity causes defective clotting and calcification were studied using a novel assay that mimics in vivo carboxylation: complexes between variant carboxylases and VKD proteins important to hemostasis (factor IX (fIX)) or calcification (Matrix Gla Protein (MGP)) were reacted in the presence of a challenge VKD protein that could potentially interfere with carboxylation of VKD protein in the complex. The VKD protein in the complex with wild type carboxylase was carboxylated before challenge protein carboxylation occurred, and became fully carboxylated. In contrast, the V255M mutant carboxylated both forms at the same time, and did not completely carboxylate fIX in the complex. S300F carboxylation was poor with both fIX and MGP. Additional studies analyzed fIX and MGP-derived peptides containing the Gla domain linked to sequences that mediate carboxylase binding. The V255M mutant generated more carboxylated peptide than wild type GGCX, however the peptides were partially carboxylated. Analysis of the V255M mutant in fIX-HEK293 cells lacking endogenous GGCX revealed poor fIX clotting activity. The studies show that disrupted processivity causes disease, and explain the defect in the patient. The kinetic analyses also suggest that disrupted processivity may occur in wild type carboxylase under some conditions, e.g. warfarin therapy or vitamin K deficiency.

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# GGCX mutants that impair hemostasis reveal the importance of processivity and full carboxylation to VKD protein function

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Impaired processivity in a carboxylase mutant causes partial carboxylation that explains reduced vitamin K-dependent protein function.

The results suggest that incomplete carboxylation may also occur under other conditions, e.g. low vitamin K or during warfarin therapy.

The gamma-glutamyl carboxylase (GGCX) generates multiple carboxylated Glus (Glas) in vitamin K-dependent (VKD) proteins that are required for their functions. GGCX is processive, remaining bound to VKD proteins throughout the multiple Glu carboxylations, and this study reveals the essentiality of processivity to VKD protein function. GGCX mutants (V255M, S300F) whose combined heterozygosity causes defective clotting and calcification were studied using a novel assay that mimics in vivo carboxylation: complexes between variant carboxylases and VKD proteins important to hemostasis (factor IX (fIX)) or calcification (Matrix Gla Protein (MGP)) were reacted in the presence of a challenge VKD protein that could potentially interfere with carboxylation of VKD protein in the complex. The VKD protein in the complex with wild type carboxylase was carboxylated before challenge protein carboxylation occurred, and became fully carboxylated. In contrast, the V255M mutant carboxylated both forms at the same time, and did not completely carboxylate fIX in the complex. S300F carboxylation was poor with both fIX and MGP. Additional studies analyzed fIX and MGP-derived peptides containing the Gla domain linked to sequences that mediate carboxylase binding. The V255M mutant generated more carboxylated peptide than wild type GGCX, however the peptides were partially carboxylated. Analysis of the V255M mutant in fIX-HEK293 cells lacking endogenous GGCX revealed poor fIX clotting activity. The studies show that disrupted processivity causes disease, and explain the defect in the patient. The kinetic analyses also suggest that disrupted processivity may occur in wild type carboxylase under some conditions, e.g. warfarin therapy or vitamin K deficiency.

## INTRODUCTION

Vitamin K-dependent (VKD) proteins are essential to hemostasis, as several of these proteins function in coagulation and platelet activation[1-3]. The major site of synthesis of VKD hemostatic factors is liver; however, many of these proteins are also expressed in extrahepatic tissues where they have roles beyond coagulation, e.g. inflammation, barrier function, and phagocytosis[4-6]. Nonhemostatic VKD proteins have also been identified, which have diverse roles that include regulation of calcification[7]. VKD proteins contain carboxylated Glu (Gla) residues required for activity, which are generated by a single gamma-glutamyl carboxylase[8]. The importance of VKD proteins to human health makes it essential to understand how they become activated by the carboxylase, and how disruption of normal VKD protein carboxylation leads to disease.

The carboxylase uses vitamin K epoxidation to drive Glu carboxylation to Gla (Fig. 1A). The vitamin K epoxide product must be recycled for continuous carboxylation, which is accomplished by the vitamin K epoxide reductase (VKORC1)[9,10]. VKORC1 and the carboxylase reside in the endoplasmic reticulum, where VKD proteins are modified during their secretion. The VKD proteins are selectively modified because they contain a sequence that mediates high affinity binding to an exosite in the carboxylase (Fig. 1B). In most cases, this exosite-binding domain (EBD) is a propeptide that is adjacent to the Gla domain and is cleaved after carboxylation. The mechanism by which the carboxylase modifies VKD proteins is only partly

understood. Functional regions important for catalysis and VKD protein interaction have been identified (Fig. 1C)[11-23]; however, carboxylase residues that regulate VKD protein carboxylation are essentially unknown.

Several regulatory mechanisms impact the efficiency of VKD protein carboxylation. For example, binding of the EBD in VKD proteins to the carboxylase stimulates Glu catalysis (Fig. 1B)[24], and binding of both the EBD and Glu increases the affinity of the carboxylase for vitamin K[25]. The carboxylase is also regulated by substrate-assisted catalysis, where vitamin K epoxidation only occurs in the presence of Glu residues[22,26], which is important in preventing a highly reactive vitamin K intermediate from modifying other molecules when Glu is not present. Finally, carboxylation is regulated through carboxylase processivity, in which VKD proteins remain bound to the carboxylase throughout the multiple Glu to Gla conversions[27,28]. Processivity is indispensable for the function of other enzymes that perform repetitive reactions, e.g. DNA polymerases[29]. While other processive enzymes have been extensively studied, the processive mechanism for the carboxylase is poorly understood.

Disruption of VKD protein carboxylation causes disease. Naturally occurring mutations in VKORC1 or the carboxylase cause bleeding defects[30-32], and mice lacking either gene die around birth from hemorrhaging[33,34]. Some patients with carboxylase mutations also show defects in skin, i.e. excessive calcification of elastic fibers in the dermis associated with significant folding and sagging of skin[35-39]. The

phenotype is due at least in part to defective carboxylation of Matrix Gla Protein (MGP), an inhibitor of calcification, and is referred to as pseudoxanthoma elasticum (PXE)-like because of similarity to the PXE disease caused by mutations in a different gene, ABCC6[40]. The mechanisms by which carboxylase mutations cause the PXE-like phenotype are not well understood.

We studied the consequence of two carboxylase mutants, V255M and S300F, identified in a PXE-like patient with defective clotting and aberrant calcification[36]. Both mutations are in regions of unknown function in the carboxylase (Fig. 1C), and analysis using small peptides derived from VKD proteins did not explain the PXE-like phenotype[36]. We therefore developed approaches to study the mutants using more natural VKD substrates that can assess the complex carboxylase reaction. These biochemical assays revealed impaired processivity that resulted in partially carboxylated VKD product. Cellular analysis showed defective factor IX activity. This study linking impaired carboxylase processivity with disease explains the patient phenotype, and reveals the essentiality of carboxylase processivity to normal physiology.

## METHODS

*Functional assays.* FLAG-tagged V255M and S300F mutants were generated and subcloned into a vector for insect cell expression (Supplemental Fig. 1), and then expressed in SF21 cells and purified and quantitated as previously reported[41,42].

Mutant and wild type carboxylases were tested in three different assays. One assay monitored the overall reaction, i.e. binding of VKD protein to the carboxylase, catalysis of Glus to Glas, and VKD protein release. Carboxylase variants were reacted with fIX or MGP-derived peptides that contained the EBD and Gla domains ($fIX_{-18/+41}$and $MGP_{1-64}$, respectively, Supplemental Table). Individual peptides (1 μM) were incubated at 25°C in a reaction cocktail (160 μl) containing 500 mM NaCl, 50 mM BES pH 6.9, 2.5 mM DTT, 0.16% CHAPS, 0.16 phosphatidyl choline, 200 μM reduced vitamin K ($KH_2$), and 1.3 mM [$^{14}$C]-$CO_2$. Aliquots withdrawn at timed intervals were quenched in SDS-PAGE loading dye, followed by gel electrophoresis and PhosphorImager analysis. [$^{14}$C]-BSA and [$^{14}$C]-peptide standards were analyzed in parallel to quantitate [$^{14}$C]-$CO_2$ counts, which were converted to pmol Gla as previously described[20]. The [$^{14}$C]-peptide standard was generated by carboxylation of $fIX_{-18/+41}$ with wild type carboxylase, followed by precipitation with chloroform/methanol[17], HPLC purification, and quantitation by scintillation counting.

A second assay studied the specific Glu to Gla catalytic reaction by monitoring the carboxylation of VKD protein-carboxylase complexes. Complexes were generated in SF21 cells coexpressing full-length VKD protein and carboxylase variants as previously described[28], followed by immunopurification. The fIX-carboxylase$_{FLAG}$ complex was isolated using an antibody against the fIX heavy chain (ESN1, American Diagnostica) immobilized to Sepharose. $MGP_{FLAG}$-carboxylase complexes were isolated from cells coexpressing $MGP_{FLAG}$ and untagged carboxylase variants, using

anti-FLAG agarose (Sigma). $MGP_{FLAG}$ was generated using an MGP cDNA (Open Biosystems) and overlap PCR, which added an $Ala_3$-FLAG epitope at the C-terminus. The amount of complex bound to resin was quantitated by assaying aliquots for epoxidation of reduced vitamin K, as before[20]. Aliquots were also analyzed in a Western using anti-carboxylase antibody[16], after elution from resin in SDS-PAGE loading buffer. Complex on the remaining resin was monitored for carboxylation by incubation in the reaction cocktail described above, followed by quenching of timed aliquots with SDS loading dye. After heating at 70°C and centrifugation to pellet resin, the samples were subjected to gel electrophoresis and PhosphorImager analysis.

The third assay monitored carboxylase processivity by using a challenge assay in which VKD protein-carboxylase complexes were reacted in the presence of a second VKD substrate[28]. FIX-$carboxylase_{FLAG}$ and $MGP_{FLAG}$-carboxylase complexes isolated as described above were reacted in the presence of $fIX_{-18/+41}$ or $MGP_{1-64}$ peptides, respectively. The reaction cocktail was the same as described above except for the addition of peptide (1μM) and less $KH_2$ (10 μM), which was used to mimic low vitamin K levels that exist in vivo[43]. [$^{14}$C]-$CO_2$ incorporation into challenge VKD substrate and VKD protein in the complexes was quantitated using SDS-PAGE and PhosphorImager analysis.

RESULTS

*Analysis of an EBD-Gla domain linked substrate reveals surprisingly higher levels of carboxylation by V255M than wild type carboxylase.* S300F and V255M mutants identified in a patient with defective clotting and aberrant calcification were initially analyzed in an activation assay. A Glu-containing peptide derived from the Gla domain was coincubated with a separate peptide containing an EBD, which stimulates Glu catalysis[44] (Fig. 1). When a range of EBD peptide concentrations was tested, half-maximal activity required higher peptide concentrations for the V255M mutant than wild type carboxylase (Supplemental Fig. 1). The results suggested mildly weakened EBD interaction with the V255M carboxylase. S300F carboxylase was essentially inactive.

Subsequent analysis used a fIX-derived substrate ($fIX_{-18/+41}$) containing the EBD covalently linked to the Gla domain (Fig. 2A, Supplemental Table). Linkage of the two domains is significant because carboxylase affinity for the Gla domain is orders of magnitude lower than for the EBD; however, linkage of the EBD to the Gla domain results in a high local concentration of Glu residues for efficient carboxylation. $FIX_{-18/+41}$ was incubated in reactions containing $[^{14}C]$-$CO_2$, $KH_2$ and equivalent amounts of purified carboxylase variants. Aliquots withdrawn at timed intervals were quenched by SDS-PAGE loading dye, followed by gel electrophoresis and PhosphorImager analysis. $[^{14}C]$-$CO_2$ incorporation into the peptides was quantitated by comparison to $[^{14}C]$-standards, and $[^{14}C]$-$CO_2$ counts were converted to pmol Gla as previously described[20]. The results revealed surprisingly higher levels of carboxylated $fIX_{-18/+41}$ generated by V255M than wild type carboxylase

(400%, Fig. 2B). In contrast, S300F activity was low, i.e. ~10% that of wild type carboxylase.

The assay in Fig. 2B monitors multiple steps in carboxylation (i.e. binding, catalysis and release, Fig. 2A), and further analysis specifically monitored catalysis (Figs. 2C-E). This assay is possible because fIX remains bound to wild type carboxylase long after it becomes fully carboxylated[45]. Preformed complexes between carboxylase variants and full-length fIX were generated in cells, which were cultured in the absence of vitamin K so that fIX was uncarboxylated. The complexes were isolated on anti-fIX antibody resin to remove free carboxylase that would interfere with analysis, using anti-fIX antibody that reacts to a region of fIX several hundred amino acids away from the Gla domain. Some fIX not in a complex with the carboxylase was also captured on the resin, which control experiments showed did not interfere with the assay (Supplemental Fig. 2). Specifically, the uncomplexed fIX was not carboxylated, presumably because its concentration was far below the $K_m$ for fIX binding by the carboxylase, and because immobilization prevented interaction with carboxylase in the fIX-carboxylase complexes.

To monitor carboxylation, equivalent amounts of complex containing mutant or wild type carboxylase and fIX were incubated in reactions containing $KH_2$ and [$^{14}$C]-$CO_2$, and timed aliquots quenched with SDS gel loading buffer were subjected to gel electrophoresis and PhosphorImager analysis to monitor [$^{14}$C]-$CO_2$ incorporation into fIX. [$^{14}$C]-$CO_2$ standards analyzed in parallel allowed the conversion of [$^{14}$C]-

$CO_2$ counts to pmol Gla, as previously described[20]. The values were compared to the amount of complex as quantitated in anti-carboxylase Westerns (Fig. 2D) and by an epoxidase assay that gave similar carboxylase levels. The results indicated full carboxylation of fIX by wild type carboxylase, consistent with previous studies[28]. Gla production by the V255M and S300F mutants was lower than with wild type carboxylase (Fig. 2E). Higher V255M activity in the overall reaction (Fig. 2B) was therefore not due to the catalytic step.

A comparison of the rates of catalysis versus the overall reaction was of interest to the V255M carboxylase defect. Wild type carboxylase catalysis was complete in ~15 min (Fig. 2E, F), and this rate for fIX-carboxylase complexes immobilized on resin was similar to that observed with free complex (Supplemental Fig. 3). The overall reaction for $IX_{-18/+41}$ carboxylation took 75 min (Fig. 2F), similar to the value previously determined for full-length propeptide-containing fIX[28]. FIX catalysis was therefore ~5 times faster than the overall reaction with wild type carboxylase. In contrast, the overall reaction with V255M carboxylase was faster than wild type carboxylase, while catalysis was slower (Figs. 2B, E). These differences raised the question of whether the V255 mutant fully carboxylates fIX.

Isoelectric focusing was performed to assess the $fIX_{-18/+41}$ carboxylation products. This approach separates proteins based on charge, and the conversion of Glus to Glas adds negative charge to VKD proteins. $FIX_{-18/+41}$ was carboxylated, precipitated to remove [$^{14}$C]-$CO_2$, and an aliquot of resuspended sample was subjected to

scintillation counting. The S300F sample did not show detectable carboxylation, and was not processed any further. Isoelectric focusing revealed a more basic product generated by V255M than wild type carboxylase (Fig. 2G), indicating undercarboxylation. Thus, the V255M mutant generates more Gla in peptides than wild type carboxylase (Fig. 2B); however, the individual peptides are not completely carboxylated (Fig. 2G). The results suggested impaired processivity in the V255M mutant, which was subsequently tested.

*Processive fIX carboxylation is impaired in the V255M mutant.* Carboxylase processivity was tested using a challenge assay in which carboxylase-VKD protein complexes are reacted in the presence of excess challenge VKD substrate, and carboxylation of both VKD forms is monitored (Fig. 3A). Our previous development of this assay showed that wild type carboxylase is processive, resulting in full carboxylation of fIX in the complex in the presence of the challenge VKD protein[28]. The challenge substrate used in the current studies was $fIX_{-18/+41}$, which has kinetic parameters (i.e. $K_m$, $k_{cat}$) similar to propeptide-containing full-length fIX[28] and therefore could potentially compete with fIX in the complex for carboxylation.

Preformed fIX-carboxylase complexes generated in cells were isolated on anti-fIX antibody resin to remove free carboxylase. The anti-fIX antibody reacts to a region distinct from the $fIX_{-18/+41}$ sequences, and did not cross-react with the $fIX_{-18/+41}$ peptide (data not shown). Equivalent amounts of fIX-carboxylase complexes were incubated in reactions containing [$^{14}$C]-$CO_2$, and $fIX_{-18/+41}$ at a concentration (1 μM)

50-fold in excess over the complex (0.02 μM). The $fIX_{-18/+41}$ peptide concentration was 100-fold higher than uncomplexed fIX also captured on the anti-fIX resin, which as described in the previous section is not carboxylated. Carboxylation was initiated by the addition of $KH_2$, and timed aliquots were quenched with SDS loading buffer and subjected to gel electrophoresis and PhosphorImager analysis. [$^{14}$C]-standards were processed in parallel to quantitate [$^{14}$C]-$CO_2$ incorporation into fIX in the complex and $fIX_{-18/+41}$, which was converted to pmol Gla as previously described[20]. FIX in the complex with wild type carboxylase showed a mobility shift as the 12 Glus underwent conversion to Glas (Fig. 3B). [$^{14}$C]-$CO_2$ incorporation into the discrete slower-migrating fIX form observed at the end of the reaction was compared to the amount of complex determined by an anti-carboxylase Western and an epoxidase assay, which indicated full fIX carboxylation, similar to previous results[45]. Carboxylation of $fIX_{-18/+41}$ occurred subsequent to that of fIX in the complex (Fig. 3F). Some carboxylated $fIX_{-18/+41}$ was observed before the carboxylation of fIX in the complex was complete, possibly due to nonsynchronous carboxylation of individual complexes. A control experiment analyzing carboxylation with free carboxylase showed simultaneous carboxylation of both proteins (Supplemental Fig. 4), indicating that the delay in $fIX_{-18/+41}$ carboxylation was specific to the fIX-carboxylase complex.

The results with V255M-fIX complexes were strikingly different. $FIX_{-18/+41}$ carboxylation occurred at the same time as fIX in the complex (Figs. 3D,E,G). Carboxylation of fIX in the V255M-fIX complex was 8-fold lower than observed with

wild type carboxylase (Figs. 3F,G), and the heterogeneous mixture of carboxylated fIX forms did not transition into the slow migrating form observed with wild type carboxylase (Figs. 3B,D). A similar pattern of fIX carboxylation was observed even in the absence of $fIX_{-18/+41}$ (Supplemental Fig. 5). Decreased V255M carboxylation of fIX was not due to inactivation, as indicated by $fIX_{-18/+41}$ carboxylation (Fig. 3G) and an epoxidase assay (data not shown). By the end of the reaction, V255M carboxylase produced significantly more carboxylated challenge protein that carboxylated fIX compared with wild type carboxylase. The results indicate impaired processivity in the V255M carboxylase.

Carboxylation of fIX in the S300F-fIX complex was barely detectable (Fig. 3H). Carboxylation was lower than observed in the assay for catalysis (Fig. 2E), possibly because of the lower vitamin K concentration used in the processivity assay. $FIX_{-18/+41}$ carboxylation by the S300F mutant was also poor, and low levels precluded quantitation.

*Cellular V255M carboxylation results in decreased fIX clotting activity.* cDNAs encoding V255M and wild type carboxylases were subcloned into pCMV6-AC (Origene) and then stably expressed in fIX 293 cells (Fig. 4A), which were previously edited to eliminate endogenous carboxylase[18]. To assess multiple fIX turnovers (i.e. the overall reaction) by the carboxylase as in the biochemical studies, a pool of uncarboxylated fIX was generated in cells lacking vitamin K, and secreted fIX was analyzed after exchanging the cells into media containing vitamin K (Fig. 4B).

Serum free media was used throughout so that bovine serum VKD proteins did not interfere with analysis. Carboxylation of secreted fIX was monitored in a Western using a pan-specific anti-Gla antibody generated against a consensus sequence in VKD proteins[46]. Antibody reactivity was observed in fIX 293 cells but not the progenitor 293 cells (Fig. 4C), which allowed the specific analysis of fIX carboxylation. FIX secreted from the fIX 293 cells migrated differently from the purified plasma control (Fig. 4C, D) due to differences in post-translational modifications that do not impact activity[47,48].

The anti-Gla signal was 5-fold higher in V255M 293 cells versus cells expressing wild type carboxylase (Fig. 4D, E), consistent with the higher levels of modified fIX observed in the biochemical assay (Fig. 2B). Media was also analyzed using an anti-fIX antibody that detects both carboxylated and uncarboxylated fIX[18]. The anti-fIX signal in V255M 293 cells was only slightly higher than that observed in wild type carboxylase expressing cells, due to only some of the fIX pool undergoing carboxylation. Thus, FIX was secreted from cells lacking vitamin K or the carboxylase (Fig. 4D), indicating that fIX carboxylation was not obligatory for secretion and that fIX in cells containing vitamin K can therefore be a mixture of carboxylated and uncarboxylated forms. The anti-Gla and anti-fIX signals were converted to pmol using fIX standards, and adjusted for 12 Gla residues in fIX (Fig. 4E), which indicated that 6% of the fIX was carboxylated in cells expressing wild type carboxylase.

FIX activity was determined by monitoring the ability of samples to restore clotting in fIX-deficient serum. The same media samples analyzed in the Westerns were tested for activity, which revealed 2-fold lower clotting activity for fIX carboxylated by V255M than wild type carboxylase (Fig. 4E). A comparison of the amount of clotting activity versus Gla in secreted fIX indicated a 10-fold lower level in cells expressing V255M carboxylase (Fig. 4F). The V255M mutant therefore generates defective fIX, consistent with decreased fIX activity in the patient[36]. Analysis of carboxylation by isoelectric focusing as in the biochemical studies was not possible, because fIX undergoes other modifications that impact charge (e.g. sulfation and Asp β-hydroxylation).

*Processive MGP carboxylation by the V255M and S300F carboxylases is impaired.* The patient with the V255M and S300F mutations shows aberrant calcification, and MGP that regulates calcification was therefore also studied. Processivity was tested in the challenge assay using complexes between $MGP_{FLAG}$ and variant carboxylases and a challenge substrate ($MGP_{1-64}$) that contains the EBD and Gla domain (Fig. 5A, Supplemental Table). The organization of the EBD and Gla domains in MGP is distinct from most VKD proteins, as the EBD is part of the mature protein. $MGP_{FLAG}$-carboxylase complexes were separated from free carboxylase by isolation on anti-FLAG resin, and equivalent amounts of complexes containing variant carboxylases were then incubated with [$^{14}$C]-$CO_2$, and $MGP_{1-64}$ in 50-fold excess over the complexes. After the addition of $KH_2$, timed aliquots were subjected to gel electrophoreses and PhosphorImager analysis, along with a [$^{14}$C]-standard that

allowed [$^{14}$C]-$CO_2$ quantitation and conversion to pmol Gla, as before[20]. $MGP_{FLAG}$ in the complex with wild type carboxylase was carboxylated before $MGP_{1-64}$, while V255M carboxylase modified both MGP forms at the same time (Figs. 5E,F). The V255M mutant also differed from wild type carboxylase in producing substantially more carboxylated $MGP_{1-64}$ than $MGP_{FLAG}$ in the complex (Figs. C,F). The results indicate impaired processivity. $MGP_{FLAG}$ carboxylation by the S300F mutant was poor, but produced significant amounts of carboxylated $MGP_{1-64}$ (Figs. 5D,G).

MGP carboxylation was also tested in the assay that monitors the overall reaction (Fig. 6A), using the $MGP_{1-64}$ peptide. Purified carboxylase variants were incubated with peptide in reactions containing $KH_2$ and [$^{14}$C]-$CO_2$, and timed aliquots were subjected to gel electrophoresis and PhosphorImager analysis. A [$^{14}$C]-standard was included to quantitate [$^{14}$C]-$CO_2$ incorporation into the peptide and consequent pmol Gla. $MGP_{1-64}$ reacted with wild type carboxylase at a rate similar to that of $fIX_{-18/+41}$ despite their having different EBD-Gla domain organizations (Fig. 6B). V255M carboxylation of $MGP_{1-64}$ was higher than that of wild type carboxylase (Fig. 6C), similar to the results with $fIX_{-18/+41}$ (Fig. 2B), while S300F turnover was poor. MGP catalysis was also analyzed (Fig. 6D-F), revealing slower $MGP_{FLAG}$ catalysis by the V255M mutant than wild type carboxylase (Fig. 6F). A comparison of the rates for the overall reaction versus catalysis showed that the time for wild type carboxylase to modify the MGP Gla domain (10 min) was 6-fold faster than the time for the overall reaction (60 min). A faster overall reaction and slower catalysis with V255M carboxylase suggested the potential for undercarboxylation of $MGP_{FLAG}$. Several

attempts were made to analyze MGP carboxylation by isoelectric focusing, which were unsuccessful due to solubility issues (as observed by others[49-52]).

## DISCUSSION

This study reveals the importance of processive carboxylation in generating the multiple Gla residues in VKD proteins that form a calcium-binding module required for activity. Processivity was studied with V255M and S300F carboxylase mutants present in a patient with defective clotting and aberrant calcification[36]. VKD proteins important to hemostasis (fIX) or calcification (MGP) were investigated using a novel challenge assay in which a VKD protein-carboxylase complex is reacted in the presence of exogenous VKD substrate that is in large excess over the complex. FIX and MGP in a complex with wild type carboxylase were carboxylated before the challenge substrates, and became fully carboxylated (Figs. 3, 5). S300F activity was poor with MGP, and barely detectable with fIX. In the case of the V255M mutant, fIX and MGP in the complexes were carboxylated at the same time as challenge protein, the challenge VKD protein was carboxylated at higher levels than VKD protein in the complex, and fIX carboxylation in the complex was poor. Wild type and V255M carboxylases also showed significant differences in the relative rates of catalysis versus the overall reaction. Processive, complete carboxylation results from the Gla domain becoming fully carboxylated before VKD proteins are released from the carboxylase (Fig. 7A). Wild type carboxylase completed carboxylation of the fIX and MGP Gla domains at rates 5 and 6-fold faster

than the overall reaction, respectively. In contrast, the two rates were similar for the V255M mutant, which generated partially carboxylated fIX (Fig. 2). When V255M carboxylase was expressed in fIX 293 cells edited by CRISPR/Cas9 to eliminate endogenous carboxylase, fIX with defective clotting activity was produced (Fig. 4), as in the patient[36]. Impaired processivity can therefore explain the phenotype of the patient.

Multiple defects could explain partial VKD protein carboxylation by the V255M mutant. The affinity of the V255M mutant for the EBD of VKD proteins is weaker than that of wild type carboxylase (Supplemental Fig. 1), and VKD proteins may not remain bound to the mutant for a sufficient time to achieve full carboxylation. Alternatively, a conformational change in the V255M mutant could impact the normal process by which carboxylated proteins are released. During wild type carboxylation, VKD proteins are maintained in a closed carboxylase conformation that blocks the access of exogenous substrate and allow the carboxylation of multiple Glu residues (Fig. 7B). The model is consistent with the delay in carboxylation of challenge peptide reacted with complexes between VKD proteins and wild type carboxylase (Figs. 3, 5), which was not observed with free carboxylase (Supplemental Fig. 4). The model is also supported by previous studies showing that even a small Glu-peptide derived from the Gla domain is not carboxylated until after carboxylation of fIX in a fIX-carboxylase complex[28]. The results implicate a transition at the end of the catalytic reaction, i.e. from a closed to an open carboxylase conformation that allows the exit of carboxylated VKD protein and

entrance of new uncarboxylated protein (Fig. 7B). Carboxylation alone is not sufficient for the release of VKD proteins, as we found that fully carboxylated fIX either isolated from mammalian cells or generated in vitro remained bound to the carboxylase[45]. Interestingly, the presence of exogenous VKD protein accelerates release of carboxylated VKD protein, indicating a release mechanism more complicated than previously appreciated. The V255M mutant, then, may disrupt the normal process of release.

The observation that wild type carboxylase only shows a small difference in the time to fully carboxylate the Gla domain versus the length of time the carboxylase remains bound to VKD proteins is significant because it suggests that wild type carboxylase may sometimes generate partially carboxylated VKD proteins. The overall reaction was only 5-6 fold slower than full carboxylation of the Gla domain (Fig. 2); however the relative rates may vary in vivo. For example, vitamin K levels that control the catalytic rate are low in vivo and vary significantly with diet[53], which could impact processivity. Warfarin therapy that targets VKORC1 to decrease reduced vitamin K levels could also disrupt processivity, explaining previous studies suggesting undercarboxylation with warfarin treatment[54,55]. Partially carboxylated VKD forms may therefore be present more frequently than previously appreciated. We note that previous studies reported a much larger difference between the rates of Glu catalysis and release (3000-fold)[56,57], which should result in full carboxylation. However, the 3000-fold value was obtained studying small, unlinked peptides, i.e. release of an EBD peptide from the carboxylase was compared to

catalysis of a separate peptide containing a few Glus. Our results with more natural VKD substrates provide a substantially revised view of carboxylase processivity and consequent extent of VKD protein carboxylation in vivo.

This study illustrates the value of using both biochemical and cellular approaches to understand how carboxylase mutations cause disease. Biochemical studies are important because some analyses are not possible in cells (e.g. studying processivity). In addition, biochemical analyses are direct, while the cellular approach that analyzes the carboxylation status of secreted VKD proteins is indirect and impacted by the secretory process, e.g. quality control mechanisms that eliminate poorly-carboxylated VKD proteins[58-60]. While the biochemical analyses have a potential limitation, i.e. high salt conditions used in the assays as required for activity, the results predicted consequences consistent with what was observed in the cellular studies. The value of biochemical approaches is underscored by two recent cellular studies on carboxylase mutations that included V255M[61,62]. Both studies assessed carboxylation using antibodies that did not reveal partial carboxylation and indicated wild type levels of carboxylation by V255M. One of the studies also performed functional analysis, which showed that factor VII modified by the mutant had activity (85%) within the normal range[61]. We observed decreased fIX clotting activity in cells expressing V255M carboxylase, similar to the patient[36], whose phenotype can be explained by biochemical analyses that revealed impaired processivity. Combined biochemical and cellular approaches are therefore highly informative for determining how carboxylase mutations cause disease.

The V255M mutant generated fIX that retained some clotting activity (Fig. 4E), and future studies that define the type of partially carboxylated fIX generated by V255M carboxylase will be of interest. The number of Gla residues required for VKD protein function is unknown. In addition, some Glas are likely to be more important than others, as indicated by previous mutagenesis studies where Glu to Asp substitutions in the Gla domains of protein C, factor X and prothrombin showed that individual Gla residues contribute differently to function[63-66]. A second area of interest will be to determine whether processive carboxylation is the same for all VKD proteins. Widely different affinities have been reported for the EBDs that mediate VKD protein binding to the carboxylase[67], which could impact the length of time a VKD protein remains bound to the carboxylase and consequent processivity. Interestingly, the patient with the V255M and S300F mutations showed 33% and 64% clotting activities for factor X and prothrombin, respectively[36], despite 100-fold reported differences in their EBD affinities[67]. The patient phenotype suggests that processivity is not solely due to EBD affinity and likely involves additional mechanisms, e.g. the transition after Glu carboxylation that allows the exchange of carboxylated and uncarboxylated VKD proteins (Fig. 7B).

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## AUTHORSHIP AND CONFLICT-OF-INTEREST STATEMENTS

Contribution: MAR, KWH, LAW, and JMH performed the experiments. MAR and KLB designed the research and analyzed the results. MAR, KLB and KWR prepared the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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FIGURE LEGENDS

**Figure 1. Carboxylase function and disruption in the pseudoxanthoma elasticum-like disease.** A. The carboxylase performs two reactions: epoxidation of reduced vitamin K ($KH_2$) to vitamin K epoxide (KO), and carboxylation where $CO_2$ addition to Glu generates carboxylated Glu (Gla). B. Vitamin K-dependent (VKD) proteins contain an exosite-binding domain (EBD) that mediates high affinity binding through the carboxylase exosite, and also activates Glu catalysis (red arrow). Multiple Glu residues are converted to Gla by a processive mechanism in which VKD proteins remain bound to the carboxylase until the Gla domain is fully carboxylated[27,28]. C. Known functional regions of the carboxylase include those facilitating catalysis (CAT), VKD protein binding (VKS, EXO), Glu binding (Glu), and the carboxylase Gla domain (Gla). Most of the residues whose mutations cause disease reside in regions where the function of the carboxylase is unknown. This study shows that impaired processivity in the V255M mutant results in the pseudoxanthoma elasticum-like disease.

**Figure 2. V255M carboxylase generates higher levels of modified fIX than wild type carboxylase.** A. Carboxylation of Glu (E) to Gla (Y) residues in factor IX (fIX) occurs in three steps, i.e. binding, catalysis and release. The overall reaction was studied using $fIX_{-18/+41}$, which contains the 18 amino acid exosite binding domain (EBD) covalently linked to the 41 amino acid Gla domain with 12 Glu residues. The EBD in fIX is a propeptide that is cleaved subsequent to carboxylation. B. FLAG-

tagged wild type and mutant carboxylases were immunopurified using anti-FLAG antibody, and quantitated in Westerns with anti-FLAG and anti-carboxylase antibodies. Equivalent amounts of variant carboxylases were then reacted with $fIX_{-18/+41}$, followed by SDS-PAGE and PhosphorImager analysis. C-E. The specific catalytic step was monitored using fIX-carboxylase complexes generated in cells expressing fIX and individual variant carboxylases. After isolation on anti-fIX resin, aliquots were monitored for the amount of fIX-carboxylase complex by an epoxidation assay[20], which was confirmed by Western analysis. Equivalent amounts of complex (6 pmol) were then monitored for pmol Gla incorporation into fIX using gel electrophoresis and PhosphorImager analysis. F. The amounts of carboxylated $fIX_{-18/+41}$ or fIX in the complex in panels B and E were compared to the amount of wild type $carboxylase_{FLAG}$ to determine the times to complete the reactions. The time for an individual fIX-carboxylase complex to become fully carboxylated was ~15 min (lower panel), while $fIX_{-18/+41}$ was carboxylated in ~75 min (as indicated by a ratio of 1 for $fIX_{-18/+41}$: carboxylase levels in the upper panel). G. $FIX_{-18/+41}$ was reacted with wild type (WT) or mutant carboxylases as in panel B, and the products were precipitated with chloroform/methanol[17] and resuspended in 25 mM ammonium bicarbonate. Aliquots were quantitated by scintillation counting, and similar levels of counts were subjected to isoelectric focusing using a pH 3-10 Criterion IEF gel (Bio-Rad) and the buffers recommended by the manufacturer. This analysis revealed a more basic $fIX_{-18/+41}$ product with V255M than wild type carboxylase, indicating partial carboxylation.

**Figure 3. Processive carboxylation of factor IX is impaired in the V255M mutant.** A. A complex between the carboxylase and factor IX (fIX) was reacted in the presence of a challenge fIX ($fIX_{-18/+41}$), and carboxylation of both fIX forms was monitored. B-E. Independent gels were used to monitor $fIX_{-18/+41}$ and fIX in the wild type (WT) or V255M complexes because of differences in fIX size, and [$^{14}$C]-standards were included on the gels to allow quantitation of carboxylation products. F-G. $FIX_{-18/+41}$ was carboxylated after fIX in the complex with wild type carboxylase, but occurred simultaneously with fIX in the complex with V255M carboxylase. FIX carboxylation was much lower in the complex with V255M than wild type carboxylase. It was also lower than observed in the reaction monitoring catalysis (Fig. 2E), which may be due to the lower $KH_2$ concentrations used in the processivity assay. H. S300F carboxylation of both fIX in the complex and $fIX_{-18/+41}$ was poor.

**Figure 4. V255M carboxylation in cells generates factor IX with decreased clotting activity.** A. FLAG-tagged wild type (WT) and V255M carboxylases were individually expressed in factor IX (fIX) 293 cells edited to eliminate endogenous carboxylase (-). M indicates molecular weight markers. B. Cells cultured in the absence of vitamin K resulted in an uncarboxylated intracellular pool of fIX in ~50-fold excess over the carboxylase, as determined by Western analysis with anti-fIX and anti-FLAG antibodies and fIX and FLAG standards. Cells were then exchanged into serum free media containing vitamin K (5 ng/ml), and harvested after 18 hr. During secretion, fIX is carboxylated in the endoplasmic reticulum (ER), and additional modifications occur in the Golgi (e.g. propeptide processing, sulfation,

and aspartyl β-hydroxylation). Carboxylation is not obligatory for secretion, and secreted fIX can therefore be a mixture of forms with different degrees of carboxylation. C. A pan-specific anti-Gla antibody[46] detects VKD protein in cell-spent media from fIX 293 cells but not the progenitor 293 cells, allowing the carboxylation of fIX expressed in 293 cells to be specifically analyzed. The fIX control is purified human plasma fIX (Enzyme Research Laboratories). D. Media from cells cultured in the presence or absence of vitamin K (vit K) were analyzed in Westerns using antibody against Gla[46], or anti-fIX antibody that detects both carboxylated and uncarboxylated fIX[18]. Lysates analyzed with anti-GAPDH antibody indicated similar amounts of cellular material. E. Media was quantitated for Gla and fIX content by comparison with purified plasma fIX (shown in panel D). Aliquots from the same media samples analyzed in the Westerns were assayed for fIX activity in a clotting assay, as previously described[47,68]. F. Clotting activity and Gla content were compared and normalized to a ratio of one for fIX secreted from 293 cells expressing wild type carboxylase. The ratio of clotting activity to Gla content reveals defective clotting in fIX carboxylated by the V255M mutant.

**Figure 5. Processive MGP carboxylation is impaired in the V255M and S300F mutants.** A. Complexes between $MGP_{FLAG}$ and variant carboxylases were reacted in the presence of a challenge peptide ($MGP_{1-64}$) that contains both the exosite binding domain (EBD) and the entire Gla domain. B-D. $MGP_{FLAG}$-carboxylase complexes were isolated on anti-FLAG resin, and the amount of complex was determined by an epoxidase assay, as before[20]. Equivalent amounts of complex were then analyzed in

the challenge assay. Carboxylation of $MGP_{FLAG}$ in the complexes and $MGP_{1-64}$ was quantitated by monitoring [$^{14}$C]-$CO_2$ incorporation into each form, using gel electrophoresis and PhosphorImager analysis. E-G. $MGP_{1-64}$ carboxylation occurred after $MGP_{FLAG}$ in the complex with wild type carboxylase. Both MGP forms were carboxylated at the same time by mutant carboxylases, which produced higher levels of $MGP_{1-64}$ than $MGP_{FLAG}$ in the complex.

**Figure 6. The V255M mutant shows higher levels of modified MGP than wild type carboxylase.** A. The overall reaction for MGP carboxylation comprises binding, catalysis and release, which produces MGP containing 5 Glas (indicated by Ys). B. $MGP_{1-64}$ and $fIX_{-18/+41}$ were incubated with the same amount of wild type carboxylase, followed by SDS-PAGE and PhosphorImager analysis to quantitate [$^{14}$C]-$CO_2$ incorporation into the peptides. C. The same assay was used to measure $MGP_{1-64}$ carboxylation by wild type and mutant carboxylases, using equivalent amounts of enzyme as determined by Western analysis with an anti-carboxylase antibody. Higher carboxylase concentrations were used than in panel B, and the duplicate samples that were assayed gave almost identical results and consequent small error bars. The overall reaction for wild type carboxylase took 60 minutes, as determined by comparing pmol Gla in MGP to the amount of carboxylase. D-F. The specific catalytic step was monitored using preformed $MGP_{FLAG}$-carboxylase complexes generated in cells, which were adsorbed to anti-FLAG resin to remove free carboxylase. The amount of carboxylase was quantitated by an epoxidase assay[20], and equivalent amounts of complex were then incubated in reaction

containing $KH_2$ and [$^{14}$C]-$CO_2$, followed by gel electrophoresis and PhosphorImager analysis to monitor [$^{14}$C]-$CO_2$ incorporation into $MGP_{FLAG}$.

**Figure 7. Carboxylase processivity and disruption in disease.** A. Processivity depends upon the relative rates of catalysis and release. Wild type (WT) carboxylase completed carboxylation of the factor IX Gla domain at a rate 5-fold faster than the overall reaction. The rates of catalysis and the overall reaction were similar with the V255M mutant, which generated partially carboxylated factor IX. B. Wild type carboxylase shields a VKD protein undergoing carboxylation from external VKD proteins, which allows full carboxylation. Shielding implicates a transition after Glu carboxylation to an open conformation that allows exit of the carboxylated VKD protein and entrance of an uncarboxylated substrate. A conformational change in the V255M mutant may disrupt this normal process to result in partially carboxylated VKD proteins. Alternatively, weakened interaction between the V255M mutant and VKD proteins could lead to premature release that accounts for partial carboxylation.

Figure 1

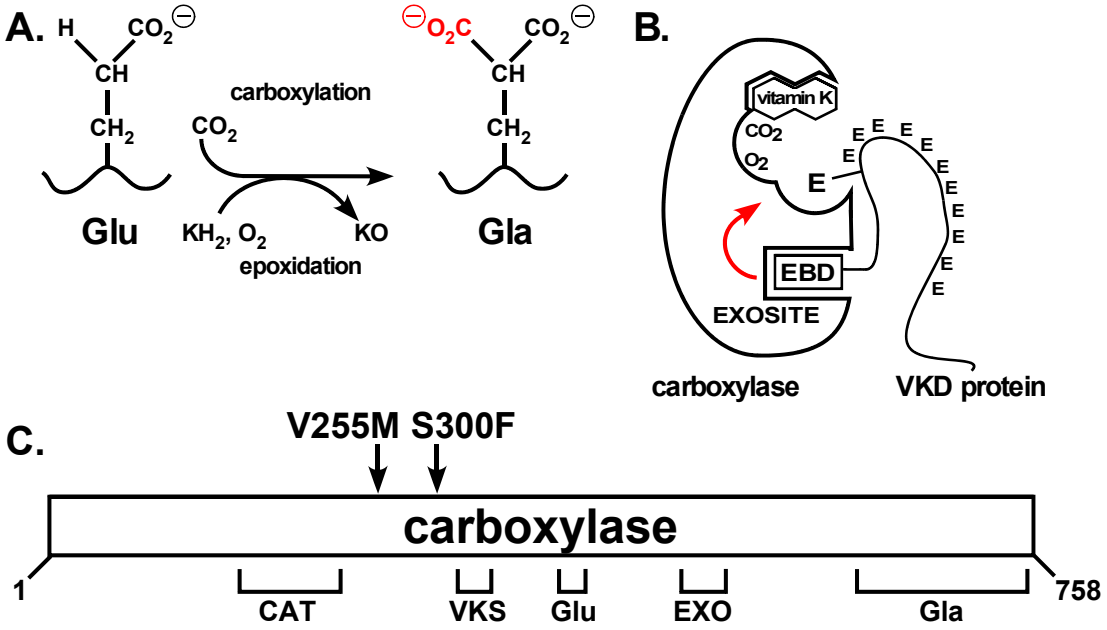

A.
H
CO2
CH
CH2
Glu
carboxylation
CO2
KH2, O2
KO
epoxidation
O2C
CO2
CH
CH2
Gla
B.
vitamin K
CO2
O2
E
EBD
EXOSITE
carboxylase
VKD protein
C.
V255M S300F
carboxylase
1
CAT
VKS
Glu
EXO
Gla
758

Figure 2

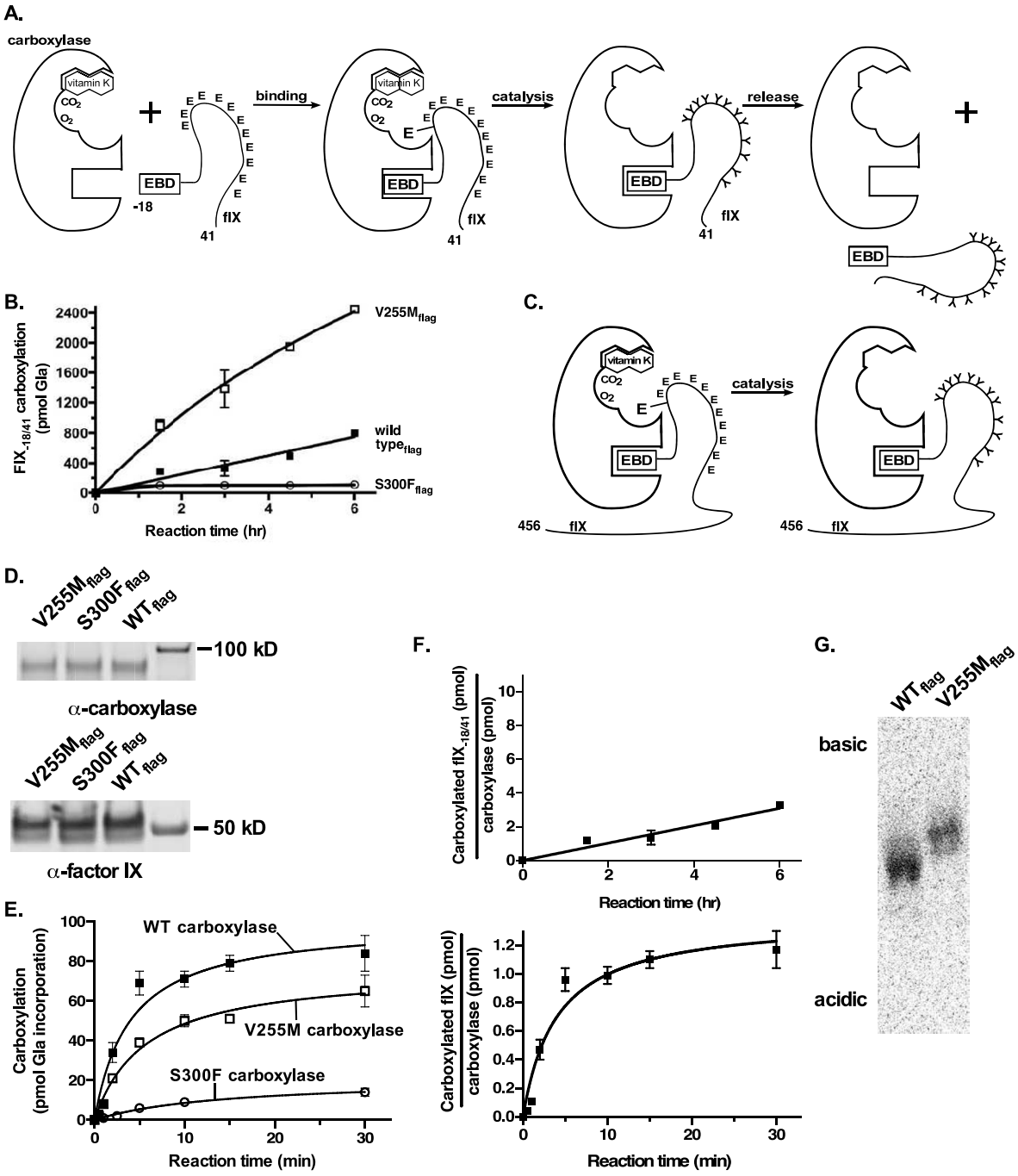
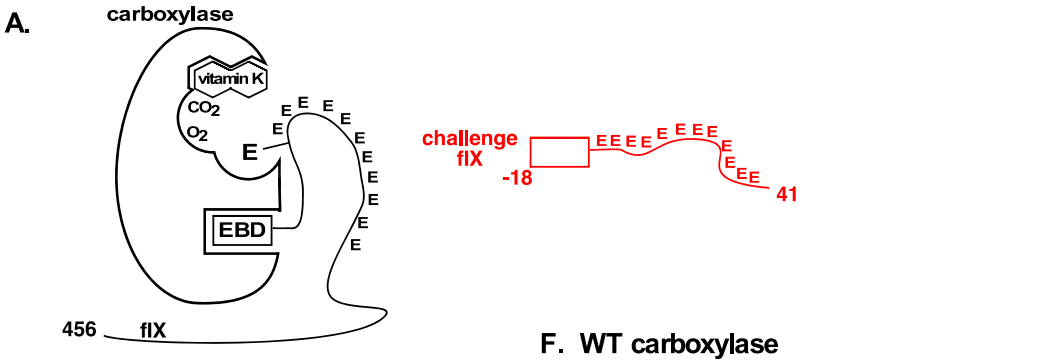
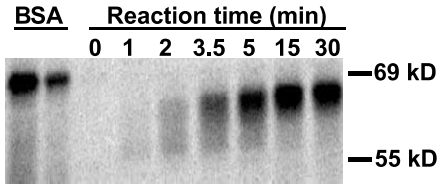

A.
carboxylase
vitamin K
$CO_2$
$O_2$
EBD
-18
41
fIX
binding
catalysis
release
B.
$FIX_{-18/41}$ carboxylation (pmol Gla)
$V255M_{flag}$
wild $type_{flag}$
$S300F_{flag}$
Reaction time (hr)
C.
456
D.
$V255M_{flag}$
$S300F_{flag}$
$WT_{flag}$
100 kD
α-carboxylase
50 kD
α-factor IX
E.
WT carboxylase
V255M carboxylase
S300F carboxylase
Carboxylation (pmol Gla incorporation)
Reaction time (min)
F.
Carboxylated $fIX_{-18/41}$ (pmol) / carboxylase (pmol)
Carboxylated fIX (pmol) / carboxylase (pmol)
G.
basic
acidic

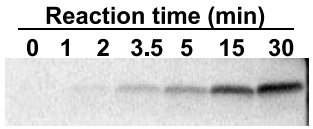
Figure 3



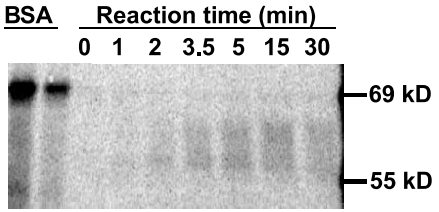

B. flX in complex - WT carboxylase



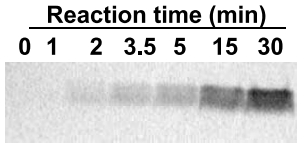

C. challenge flX - WT carboxylase



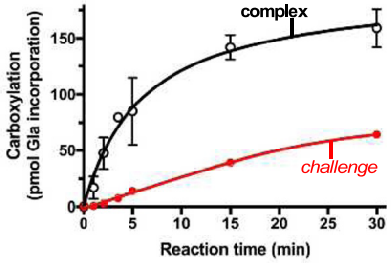

D. flX in complex - V255M carboxylase



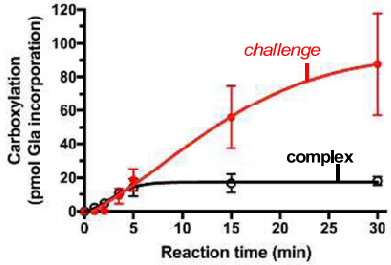

E. challenge flX - V255M carboxylase




F. WT carboxylase




G. V255M carboxylase




H. S300F carboxylase

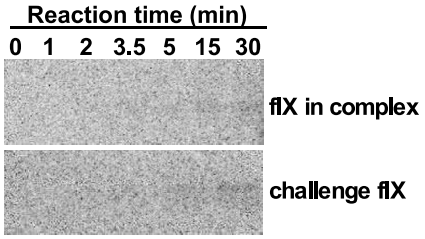


Figure 4

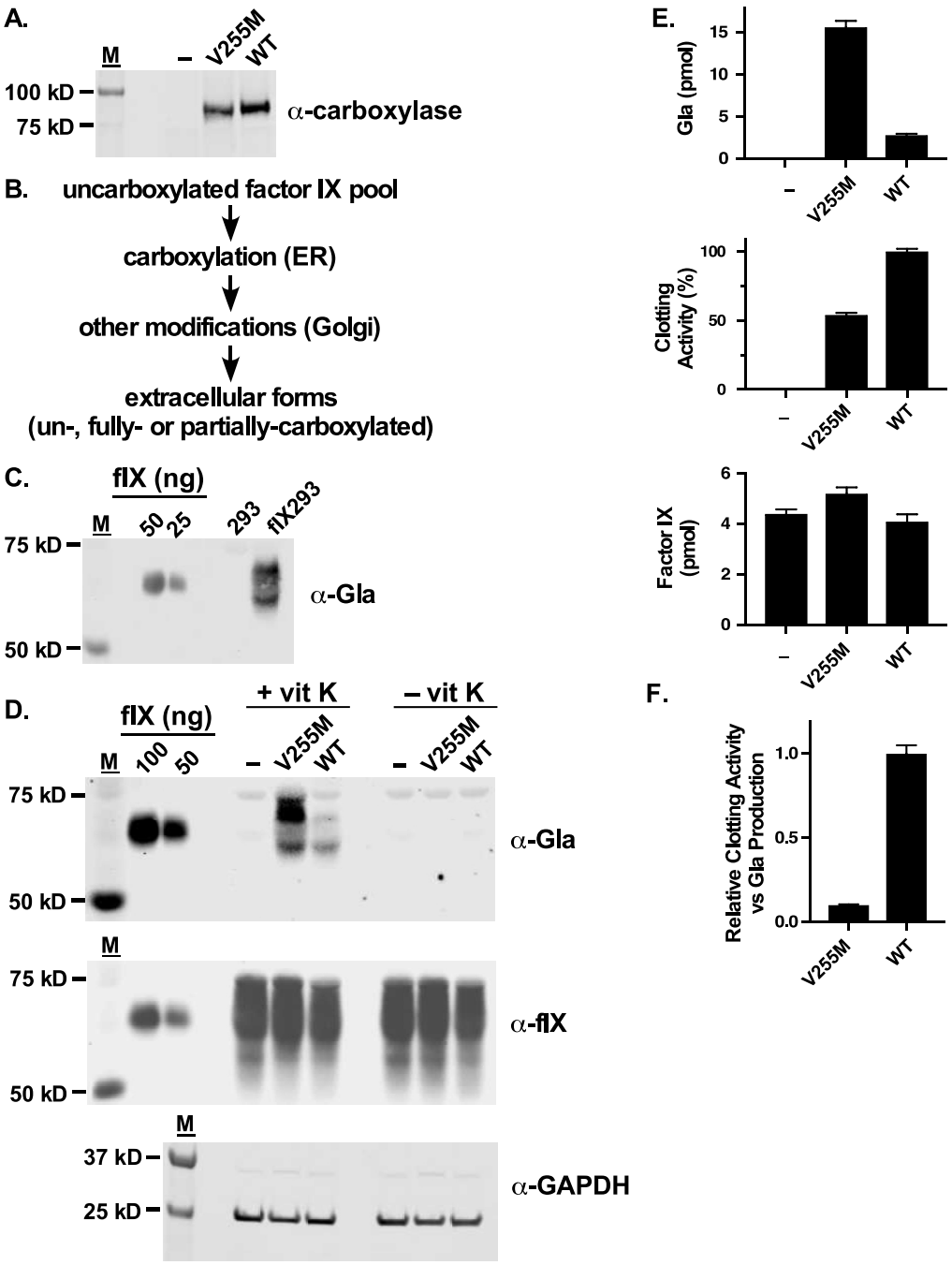
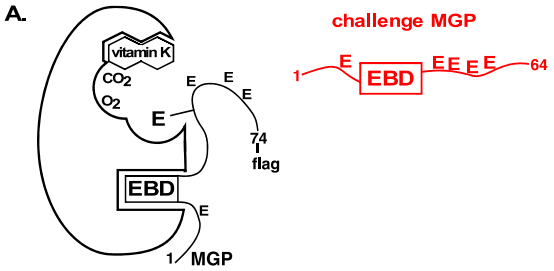
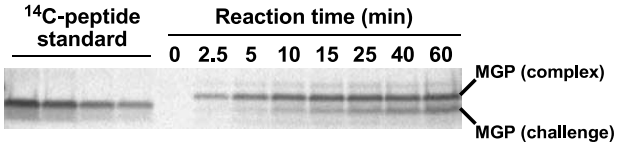

A.
M
–
V255M
WT
100 kD
75 kD
α-carboxylase
B.
uncarboxylated factor IX pool
carboxylation (ER)
other modifications (Golgi)
extracellular forms
(un-, fully- or partially-carboxylated)
C.
fIX (ng)
M
50
25
293
fIX293
75 kD
50 kD
α-Gla
D.
fIX (ng)
+ vit K
– vit K
M
100
50
–
V255M
WT
–
V255M
WT
75 kD
50 kD
α-Gla
M
75 kD
50 kD
α-fIX
M
37 kD
25 kD
α-GAPDH
E.
Gla (pmol)
15
10
5
0
–
V255M
WT
Clotting Activity (%)
100
50
0
–
V255M
WT
Factor IX (pmol)
6
4
2
0
–
V255M
WT
F.
Relative Clotting Activity vs Gla Production
1.0
0.5
0.0
V255M
WT

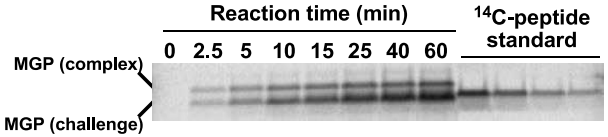
Figure 5



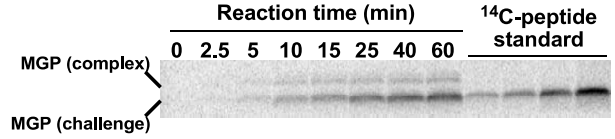

B. Wild type carboxylase



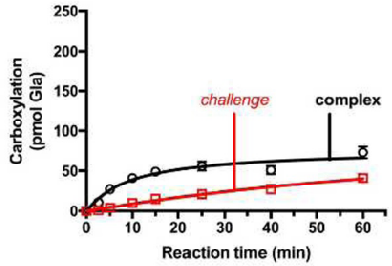

C. V255M carboxylase



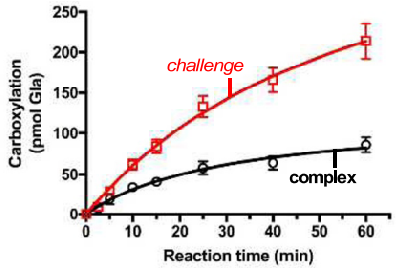

D. S300F carboxylase




E. Wild type carboxylase




F. V255M carboxylase




G. S300F carboxylase

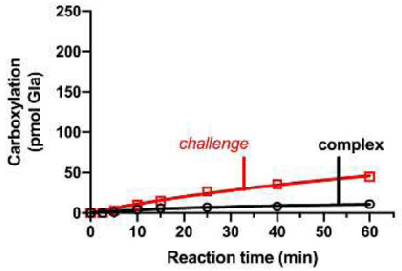


Figure 6

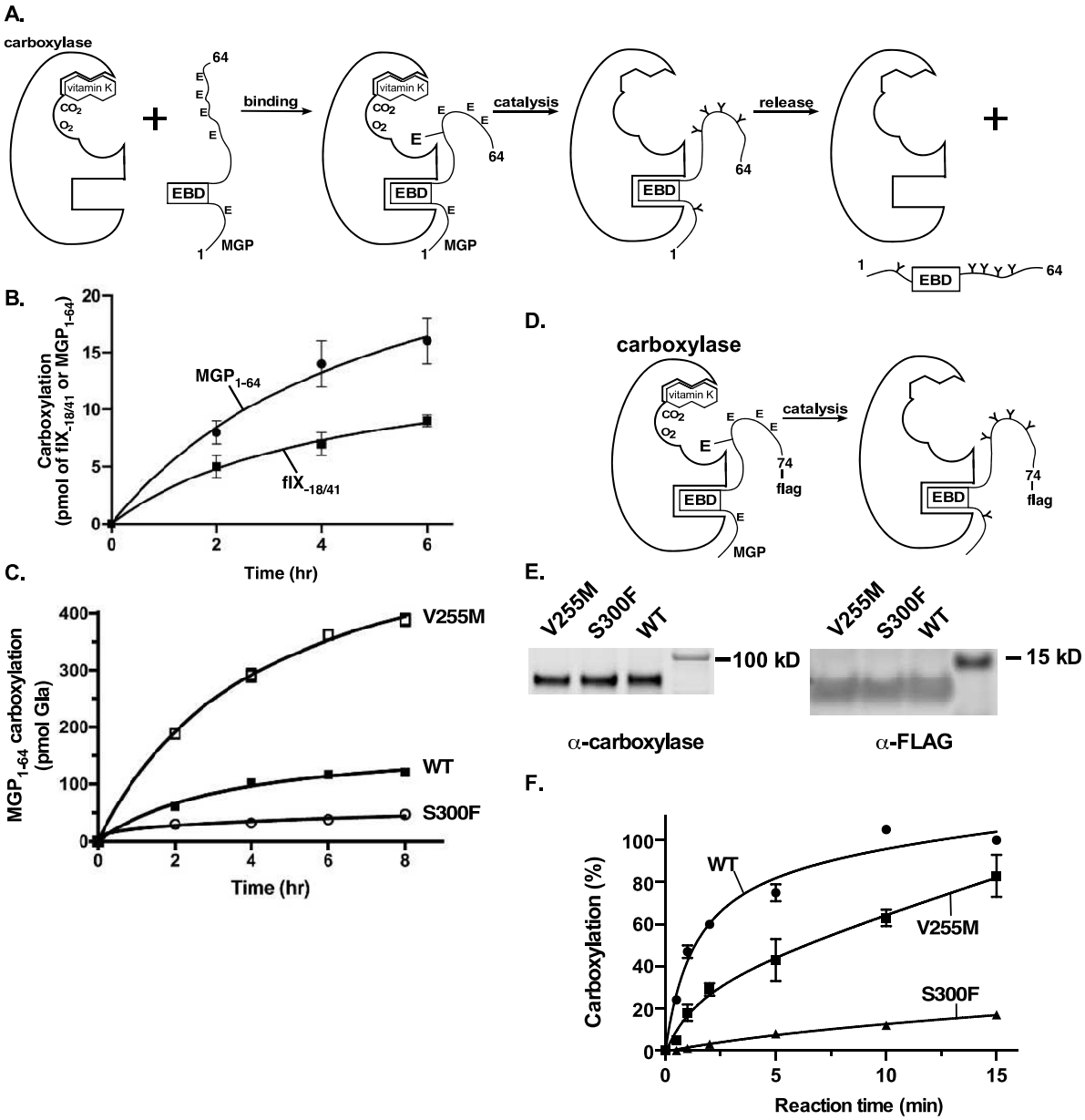
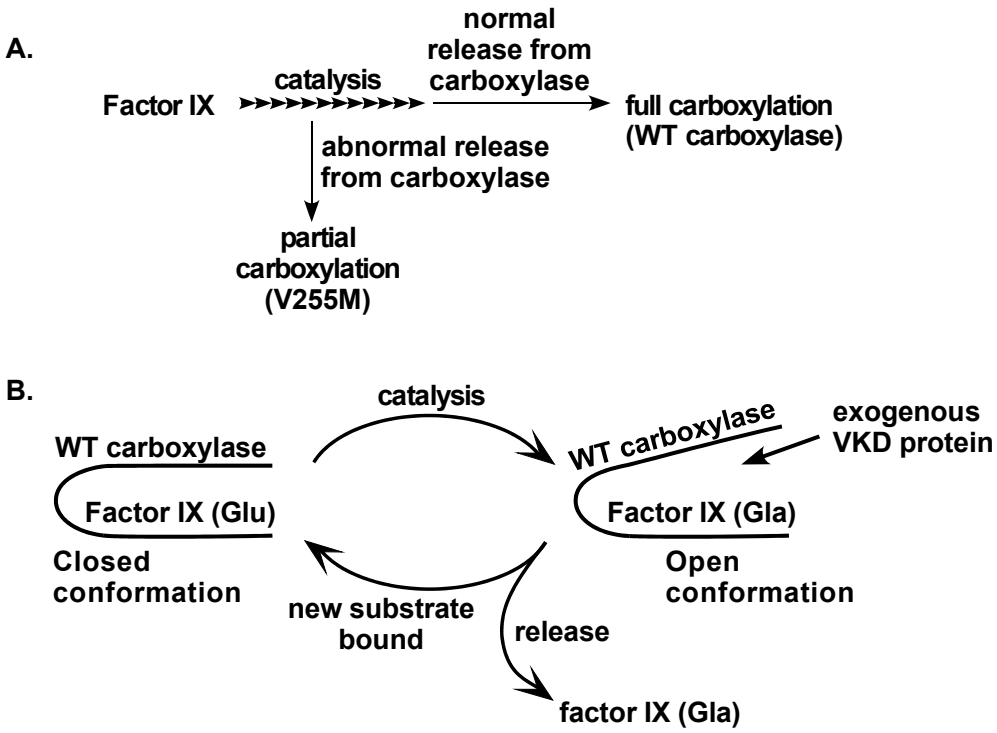

A.
carboxylase
vitamin K
$CO_2$
$O_2$
64
E
EBD
MGP
1
binding
catalysis
release
B.
Carboxylation (pmol of $fIX_{-18/41}$ or $MGP_{1-64}$)
$MGP_{1-64}$
$fIX_{-18/41}$
Time (hr)
C.
$MGP_{1-64}$ carboxylation (pmol Gla)
V255M
WT
S300F
Time (hr)
D.
carboxylase
catalysis
74
flag
E.
V255M
S300F
WT
100 kD
15 kD
α-carboxylase
α-FLAG
F.
Carboxylation (%)
WT
V255M
S300F
Reaction time (min)

Figure 7


A.
normal
release from
carboxylase
catalysis
Factor IX
full carboxylation
(WT carboxylase)
abnormal release
from carboxylase
partial
carboxylation
(V255M)
B.
catalysis
WT carboxylase
exogenous
VKD protein
WT carboxylase
Factor IX (Glu)
Factor IX (Gla)
Closed
conformation
Open
conformation
new substrate
bound
release
factor IX (Gla)