Formation Microequilibria of Proton, Calcium and Magnesium Complexes of the γ-Carboxyglutamate Ion and Related Compounds

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Abstract

The formation microequilibria of the proton, calcium and magnesium complexes of γ-carboxyglutamic acid (GLA) and some related compounds were studied via pH-metric titration. The inductive effects of differently protonated or protected donor groups are discussed. The distribution curves of the differently protonated microspecies and the probabilities of the different protonation pathways are presented. The formation constants of differently protonated metal complexes are given. The calcium ion binding constant for *N*-acetyl-γ-carboxyglutamic acid α-methylamide (which functionally models a single GLA residue in polypeptide chains) was found to be greater than that for GLA, but much smaller than that for the natural GLA-containing polypeptides.

Introduction

The role of γ-carboxyglutamic acid (GLA) in the calcium ion coordination processes of vitamin K-dependent blood coagulation factors and other calcium ion-dependent polypeptides was recognized shortly after the discovery of GLA in 1974 [1]. The GLA moieties were found to participate in the calcium ion binding of natural GLA-containing polypeptides, and relatively stable calcium complexes were detected [2–9]. It was suggested that the secondary structure of polypeptides could be changed by complex formation, and that polypeptide–polypeptide connections or polypeptide–membrane phospholipid mixed-ligand complexes could be formed via calcium bridges. Several reviews have been published on this subject [10–16]. The macroscopic protonation constants of GLA have been determined via pH-metric titration, electrophoresis [17] and ^{13}C NMR spectroscopy [18], but the data showed considerable differences, especially in the lower pH region.

The stability constants of the binary Ca–GLA complex was found to be relatively small ($\log K$ = 1.3) [17, 18]. In the binary calcium complexes of GLA and small peptides containing one GLA moiety, the geminal carboxylate groups were found to be coordinated to one side of the primary coordination sphere of the metal ion. The free coordination sites are available to interact with another GLA residue or with the hydrophilic part of the membrane phospholipid [18, 21, 22]. The above results were verified by X-ray studies [23] and theoretical calculations [24] on suitable model systems. Small peptides containing two neighbouring GLA moieties were reported to bind calcium ions with the same affinity [17, 19, 20] as the vitamin K-dependent blood coagulation factor prothrombin ($\log K$ = 3.5) [9]. On the other hand, structural similarities and very comparable formation constants were found for the europium(III) complexes of small peptides containing one or two GLA moieties [22]. In view of these results, the question arises as to whether the neighbouring GLA moieties of the protein or some other effects are responsible for the increased stability of the calcium complex of the protein.

Our aim was therefore to study the protonation microequilibria of GLA and related compounds, to reveal the inductive effects of the polypeptide chain influencing the basicity and metal ion coordination ability of the donor groups of the GLA residues. The following compounds were investigated (Scheme 1): DL-γ-carboxyglutamic acid (GLA); *N*-acetyl-DL-γ-carboxyglutamic acid α-methylamide (PGLA) (modelling a single GLA moiety built into the polypeptide chain); *N*-acetyl-DL-γ-carboxyglutamic acid (PNGLA); DL-glutamic acid α-methylamide (PCGLU); DL-glutamic acid (GLU); malonic acid (MA); glutaric acid (GA).

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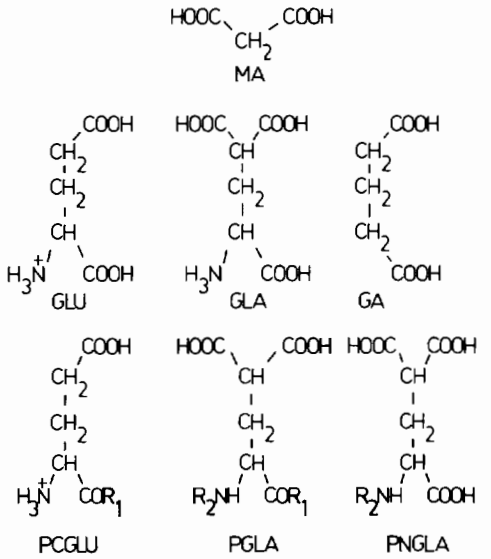


Scheme 1. The investigated compounds ($R_2 = CH_3-\overset{\overset{O}{\|}}{C}-$, $R_1 = CH_3-NH-$).

The measurements were carried out via pH-metric titration. The results are presented below.

Experimental

Materials

To prepare DL-γ-carboxyglutamic acid and its derivatives, γ,γ-di-t-butyl benzyloxycarbonyl-DL-γ-carboxyglutamate was hydrogenated according to Juhász and Bajusz [25], giving γ,γ-di-t-butyl DL-γ-carboxyglutamate [26], which was acetylated with acetic anhydride in pyridine solution.

The γ,γ-di-t-butyl *N*-acetyl-DL-γ-carboxyglutamate [27] thus obtained and γ,γ-di-t-butyl DL-γ-carboxyglutamate were transformed by trifluoracetic acid treatment to *N*-acetyl-DL-γ-carboxyglutamic acid [25, 26], respectively.

The synthesis of *N*-acetyl-DL-γ-carboxyglutamic acid α-methylamide started with the condensation of γ,γ-di-t-butyl benzyloxycarbonyl-DL-γ-carboxyglutamate and methylammonium pentafluorophenolate with dicyclohexylcarbodiimide [28]; hydrogenation, acetylation and subsequent trifluoroacetic acid treatment led to the crystalline product (m.p. 142–144 °C).

Repetition of the latter sequence of reactions without acetylation yielded DL-glutamic acid α-methylamide (amorphous, FAB-MS: $M - H^+ = 161$) instead of the expected DL-γ-carboxyglutamic acid derivative.

All crystalline compounds gave satisfactory microanalyses. The ^1H NMR spectra (Varian EM 360) of all derivatives conformed with the postulated structures.

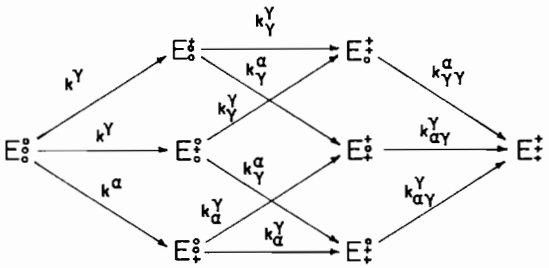
All the other chemicals used were of analytical grade. The solutions were prepared with doubly distilled water. The measurements were performed at 25.0 ± 0.1 °C.

The pH-metric titrations were carried out as previously described [29], with a Radiometer 62028 glass electrode and a Radelkis OP 08303 double-junction reference electrode at 2.5×10^{-3} mol dm^{-3} ligand concentration and constant (1.0 mol dm^{-3} NaCl) ionic strength.

For study of the metal ion coordination equilibria, the ionic strength was made up with $CaCl_2$ or $MgCl_2$. Only data below the region of the hydrolysis of metal ions were considered in the evaluation. For the measurements, a computer-controlled on-line automatic titrator was constructed according to ref. 30.

Evaluation Method for Determination of Microscopic Protonation Constants

Several combined pH-metric and spectroscopic methods have been elaborated for the determination of protonation microconstants [31, 32]. These methods could not be used for GLA and GLA-type compounds, however, because of the similar spectroscopic properties of the carboxylate groups in question and the small number of C atoms separating the donor groups [33]. The microscopic constants therefore had to be determined by a deductive method [34]. The protonation scheme for GLA and PNGLA (taking into account that the protonation state of the α-amino group in GLA remains unchanged in the acidic region) is as follows:



In the symbol $E^{\circ}_{\circ}$ the two upper circles denote the two equivalent γ-carboxylate groups and the lower one the α-carboxylate group. The upper index of a microconstant refers to the group to be protonated, and the lower index that which is already protonated.

The quantitative connections between macro- and microconstants are

$$\beta_1 = 2k^{\gamma} + k^{\alpha} \quad (1)$$

$$\beta_2 = k^{\gamma}k^{\gamma}_{\gamma} + k^{\alpha}k^{\gamma}_{\alpha} + k^{\gamma}k^{\alpha}_{\gamma} \quad (2)$$

$$\beta_3 = k^{\alpha}k^{\gamma}_{\alpha}k^{\gamma}_{\alpha\gamma} = k^{\gamma}k^{\alpha}_{\gamma}k^{\gamma}_{\alpha\gamma} = k^{\gamma}k^{\gamma}_{\gamma}k^{\alpha}_{\gamma\gamma} \quad (3)$$

The interaction between two carboxylate groups can be characterized by the ratio of the corresponding microconstants:

$$\frac{k_j^i}{k^i} = \frac{k_i^j}{k^j} = E_{ij} \tag{4}$$

where E_{ij} is the spreading factor (interactivity constant) between groups i and j. For the symmetric dicarboxylic acids (GA and MA), E_{ij} can be determined from the macroscopic constants in the following way:

$$E_{ij} = \frac{K_1}{4K_2} \tag{5}$$

The protonation microconstants with a lower index in eqns. (1)–(3) can be expressed via the E_{ij} values in the following way:

$$k_\gamma^\gamma = k^\gamma E_{\gamma\gamma} \tag{6}$$

$$k_\alpha^\gamma = k^\gamma E_{\alpha\gamma} \tag{7}$$

$$k_\gamma^\alpha = k^\alpha E_{\alpha\gamma} \tag{8}$$

$$k_{\alpha\gamma}^\gamma = k_\alpha^\gamma E_{\gamma\gamma} = k^\gamma E_{\alpha\gamma} E_{\gamma\gamma} \tag{9}$$

$$k_{\gamma\gamma}^\alpha = k_\gamma^\alpha E_{\alpha\gamma} = k^\alpha E_{\alpha\gamma}^2 \tag{10}$$

Substituting eqns. (6)–(10) into eqns. (1)–(3):

$$\beta_1 = 2k^\gamma + k^\alpha \tag{11}$$

$$\beta_2 = (k^\gamma)^2 E_{\gamma\gamma} + 2k^\alpha k^\gamma E_{\alpha\gamma} \tag{12}$$

$$\beta_3 = (k^\gamma)^2 k^\alpha E_{\alpha\gamma} E_{\gamma\gamma}^2 \tag{13}$$

The microconstants k^α and k^γ can be determined from eqns. (11)–(13) from knowledge of the macroconstants* β_1, β_2 and β_3 and the spreading factors $E_{\gamma\gamma}$ and $E_{\alpha\gamma}$ of MA and GA. The values of k^α and k^γ best satisfying each equation of (11)–(13) were determined for GLA and PNGLA.

From knowledge of the protonation microconstants, the distribution curves of the different microspecies can be determined:

$$\alpha_{E_\circ^\circ} = \frac{1}{A} \tag{14}$$

$$\alpha_{E_\circ^\ddagger} = \frac{2k^\gamma[H^+]}{A} \tag{15}$$

$$\alpha_{E_+^\circ} = \frac{k^\alpha[H^+]}{A} \tag{16}$$

$$\alpha_{E_\circ^{\ddagger\ddagger}} = \frac{k^\gamma k_\gamma^\gamma[H^+]^2}{A} \tag{17}$$

$$\alpha_{E_+^\ddagger} = \frac{2k^\alpha k_\alpha^\gamma[H^+]^2}{A} \tag{18}$$

$$\alpha_{E_+^+} = \frac{k^\gamma k_\gamma^\gamma k_{\gamma\gamma}^\alpha[H^+]^3}{A} \tag{19}$$

*For GLA: $\beta_1 = K_2$, $\beta_2 = K_2K_3$, $\beta_3 = K_2K_3K_4$, because K_1 is the protonation constant of the α-amino group.

$$A = \sum_{i=1}^{3} \beta_i[H^+]^i \tag{20}$$

The expression

$$\frac{\alpha_{E_+^\circ}}{\alpha_{E_+^\circ} + \alpha_{E_\circ^\ddagger}} = \frac{[E_+^\circ]}{[E_+^\circ] + [E_\circ^\ddagger]} = \frac{k^\alpha}{\beta_1} \tag{21}$$

gives the ratio of the concentration of the microspecies with a protonated α-carboxylate group to the sum of the concentrations of the species with one protonated carboxylate group. The analogous concentration ratios can be calculated for diprotonated microspecies in a similar manner. From these ratios, the probabilities of the three different protonation pathways can be determined.

Protonation microconstants and α *versus* pH functions for compounds containing two non-symmetric carboxylate groups (see, for example, GLU) can be calculated in an analogous manner.

To determine the stability constants of the calcium and magnesium complexes, the Z_H functions of the ligands in the presence of the complex-forming metal ions were analysed. The general form of the model function is:

$$Z_H = \frac{\text{concentration of } H^+ \text{ bound to ligand}}{\text{analytical concentration of ligand}}$$

$$= \frac{\sum_{i=1}^{N} i\beta_i[H^+]^i + \sum_{i=1}^{N-1} ik_i^M K_i^c[H^+]^i[M^{2+}]}{\sum_{i=0}^{N} \beta_i[H^+]^i + \sum_{i=0}^{N-1} k_i^M K_i^c[H^+]^i[M^{2+}]} \tag{22}$$

where N is the maximum possible number of bound protons, k_i^M is the product of the corresponding protonation microconstants referring to i-times protonated species, and K_i^c is the formation constant of the metal complex of an i-times protonated species, characterized by the k_i^M product of the corresponding microconstants. The products $k_i^M K_i^c$ were determined by a computer program using least-squares refinement. These calculations were performed for each ligand containing two geminal carboxylate groups.

Results

Protonation Processes

The macroscopic protonation constants of the studied ligands are given in Table I. The reproducibilities of the macroconstants are: 0.05 in the $\log K > 3$ region; 0.10 in the $3 > \log K > 2$ region; and 0.20 in the $2 > \log K$ region. The corresponding

TABLE I. Protonation Macroconstants of the Investigated Ligands

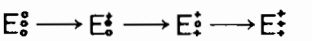
	$\log K_1$	$\log K_2$	$\log K_3$	$\log K_4$
MA	4.97	2.71		
GA	4.82	4.11		
GLU	9.50	4.07	2.39	
PCGLU	7.87	3.94		
GLA	9.60	4.33	2.66	1.80
PNGLA	4.82	3.32	2.45	
PGLA	4.68	2.38		

spreading factors calculated in the knowledge of the protonation macroconstants of MA† and GA are: $\log E_{\gamma\gamma} = -1.68$, $\log E_{\alpha\gamma} = -0.11$. These values are in good agreement with data presented in the literature [35].

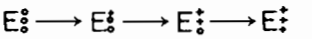
The microscopic protonation constants of the carboxylate groups are shown in Table III. The differences between the two sides of eqns. (11)–(13) were found to be smaller than 0.02 log units in the case of GLA and PNGLA. This good agreement seems to verify the value of $\log K_4 = 1.8$ for GLA. This value is intermediate between the data determined by electrophoresis ($\log K_4 = 1.6$ [17]) and ^{13}C NMR spectroscopy ($\log K_4 = 2.0$ [18]).

From knowledge of the protonation microconstants, the concentration distributions of differently protonated GLA, PNGLA and GLU microspecies were calculated. The distribution curves are shown in Fig. 1.

The mole fractions of the singly or doubly protonated microspecies referring to the sums of all singly or doubly protonated species, respectively, were also determined. On the basis of these calculations, 98% of the singly protonated carboxylate group-containing GLA species carry the proton in the γ-position and 2% in the α-position, while 76% of the diprotonated carboxylate-containing GLA species carry the protons in the α- and γ-positions, and 24% in the γ,γ-position. The corresponding data for PNGLA are: for the singly protonated species, 96% γ and 4% α; and for the diprotonated carboxylate-containing species, 84% αγ and 16% γγ. Thus the probability of the pathway



is 0.74 for GLA and 0.80 for PNGLA. This is the 'main-route' of the protonation process. The probability of the pathway



is 0.24 for GLA and 0.16 for PNGLA, and this is the 'side-route' of the protonation process.

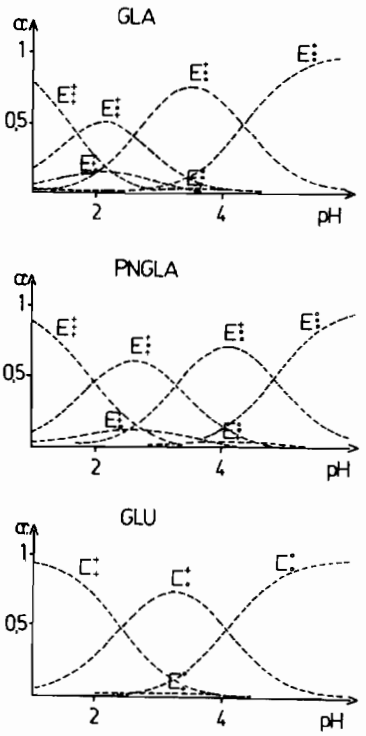


Fig. 1. Distribution curves of microspecies of GLA, PNGLA and GLU.

†The γ-carboxylate groups in GLA are structurally similar to the MA carboxylate groups; this is why the latter groups are denoted by γ.

TABLE II. Protonation Microconstants Relating to the Carboxylate Groups of the Investigated Compounds[a]

	$\log k^{\gamma}$	$\log k^{\alpha}$	$\log k^{\gamma}_{\gamma}$	$\log k^{\alpha}_{\gamma}$	$\log k^{\gamma}_{\alpha}$	$\log k^{\alpha}_{\gamma\gamma}$	$\log k^{\gamma}_{\alpha\gamma}$
MA	4.67		3.01				
GA	4.52			4.41			
GLU	4.05	2.51		2.40	3.95		
PCGLU	3.94				3.94*		
GLA	4.02	2.66	2.34	2.55	3.91	2.44	2.23
PNGLA	4.52	3.39	2.84	3.28	4.41	3.17	2.73
PGLA	4.38		2.68		4.38*		2.68*

[a]The explanation concerning values marked by an asterisk is given in the text.

TABLE III. Stability Constants of Calcium and Magnesium Complexes[a]

	Protonation state of the ligand	Ca^{2+} complex log K_1		Mg^{2+} complex log K_1	
		γ-position	α-position	γ-position	α-position
MA	A^{2-}	1.15		1.73	
PGLA	A^{2-}	0.84		1.03	
GLU	A^{2-}		0.60		1.33
PNGLA	$H_\alpha A^{2-}$	0.84*		1.03*	
	A^{3-}	1.06		1.15	
GLA	$H_\alpha A^{-}$	0.4*		0.7*	
	A^{2-}	0.60		0.92	
	A^{3-}		0.64		1.42

[a]The data marked by an asterisk are estimated values; for the corresponding explanation, see text.

The pathway

$$E_{\bullet\bullet}^{\bullet\bullet} \longrightarrow E_{\bullet}^{\bullet} \longrightarrow E_{\bullet}^{\bullet} \longrightarrow E_{\bullet}^{\bullet}$$

has the least probability for both GLA (0.02) and PNGLA (0.04).

Calcium and Magnesium Complex Formation Processes

The stability constants of the metal complexes of ligands containing two geminal donor groups are presented in Table III.

For MA and PGLA, the following equations were used to model the Z_H function, which was experimentally determined in the presence of a 120–140-fold excess of calcium or magnesium ions:

$$Z_H = \frac{\sum_{i=1}^{2} i\beta_i[H^+]^i + K_{MHA}k^{\gamma}2[H^+][M^{2+}]}{\sum_{i=0}^{2} \beta_i[H^+]^i + K_{MA}[M^{2+}] + K_{MHA}k^{\gamma}2[H^+][M^{2+}]} \quad (23)$$

where

$$K_{MA} = \frac{[MA]}{[M^{2+}][A]} \quad (24)$$

$$K_{MHA} = \frac{[MHA]}{[M^{2+}][HA]} \quad (25)$$

The products $k^{\gamma}K_{MHA}$ became zero in the least-squares refinement of the data, while real values were found for K_{MA}. Consequently, the formation of metal complexes containing a protonated carboxylate group in a γ-position can be neglected under the given experimental conditions. Since analogous complex formation behaviour could also be expected for the γ-carboxylate groups in GLA and PNGLA, only species with a protonated α-carboxylate group were considered besides the fully deprotonated ligands. The corresponding model function is:

$$Z_H = \frac{\sum_{i=1}^{3} i\beta_i[H^+]^i + K_{MH_\alpha A}k^{\alpha}[H^+][M^{2+}]}{\sum_{i=0}^{3} \beta_i[H^+]^i + K_{MA}[M^{2+}] + K_{MH_\alpha A}k^{\alpha}[H^+][M^{2+}]} \quad (26)$$

where

$$K_{MH_\alpha A} = \frac{[MH_\alpha A]}{[M^{2+}][H_\alpha A]} \quad (27)$$

and $H_\alpha A$ denotes the ligand containing a protonated carboxylate in the α-position.

The products $k^{\alpha}K_{MH_\alpha A}$ also became zero in the least-squares refinement of the data, and real values were found for K_{MA}. Consequently, the formation of metal complexes of GLA and PGLA in which the metal ion is bound by the γ-carboxylate groups and there is a protonated carboxylate in the α-position can be neglected.

Given knowledge of the low probability of formation of $H_\alpha A$ species, it is not surprising that

$$Z_H = \frac{K_1[H^+]}{1 + K_1[H^+] + K_{MA}[M^{2+}]} \quad (28)$$

(where K_1 is the protonation constant of the α-amino group) is a suitable model for metal ion binding to the α-carboxylate group in the case of GLU. Surprisingly, this simple model results in the same stability constants for GLA as for GLU. These data suggest that the metal ion coordination by the α-donor groups is not disturbed by the state of the γ-carboxylate groups (bound to metal ion or not).

On the basis of the above considerations, the stability constants of the binuclear complexes M_2GLA (containing metal ions in α- and γ-positions) can be given as the products of the corresponding formation constants of the mononuclear complexes. This statement implies the assumption that there is no significant difference between the inductive

effects of protonated and deprotonated α-amino groups. Thus:

$$\log \frac{[(Ca_2GLA)^+]}{[Ca^{2+}]^2[GLA^{3-}]} = 1.24$$

$$\log \frac{[(Mg_2GLA)^+]}{[Mg^{2+}]^2[GLA^{3-}]} = 2.34$$

Discussion

Hydrogen-bond formation between two basic donor atoms increases the protonation constants of one of the donor groups, and decreases that of the other by the same amount [36]. Since this effect was not observed in our system, the formation of hydrogen-bonds between geminal carboxylate groups could be neglected.

Several conclusions can be drawn from a comparison of the protonation microconstants. The difference between the inductive effects of a protonated and an acetylated α-amino group is reflected in the $\log k^{\gamma}$ values. This is the reason for the nearly equal $\log k^{\gamma}$ values for MA, GA, PNGLA and PGLA, which either do not contain an α-amino group or contain one in the acetylated form; these $\log k^{\gamma}$ values are higher than those for GLA, GLU and PCGLU, which contain an electrophilic protonated amino group in the α-position.

A similar tendency can be observed when the $\log k^{\alpha}$, $\log k_{\gamma}^{\alpha}$ and $\log k_{\alpha}^{\gamma}$ values are compared. The $\log k_{\alpha}^{\gamma}$ value for GLU was found to be equal to $\log k^{\gamma}$ for PCGLU (marked by an asterisk in Table II). Similarly, $\log k_{\alpha}^{\gamma}$ and $\log k_{\alpha\gamma}^{\gamma}$ for PNGLA are equal to $\log k^{\gamma}$ and $\log k_{\gamma}^{\gamma}$ for PGLA within experimental error. This suggests that the protonated and amidated carboxylate groups are equivalent with respect to their inductive effects. This is in accordance with previous observations [37], which have recently been verified by NMR spectroscopy [38].

When this equivalence is taken into consideration, the protonation macroconstant of PCGLU, $\log K_1 = 7.87$, can be regarded as the protonation microconstant of GLU and GLA $\log k_{\alpha}^{\alpha\text{-amino}}$, in spite of the fact that this species is not formed in a measurable quantity.

On the basis of the equivalence of the protonated and amidated carboxylate groups, the stability constants of the MH_{α}PNGLA complexes can be regarded as equal to the corresponding stability constants of the MPGLA complexes. These values are 0.1–0.2 log units lower than those of the fully deprotonated species, because of the higher electron-withdrawing effect of the protonated carboxylate group. Consequently, the same decrease can be expected for MH_{α}GLA in comparison with MGLA. These estimated values are designated by an asterisk in Table III.

If our results are extrapolated to biological systems, PGLA (which functionally models the GLA moiety in polypeptide chains) is able to bind calcium ions more strongly than free GLA at the pH of blood (pH = 7.3–7.5) as a result of two opposite effects: the electron-withdrawing effect of the acetylated amino group is smaller than that of the protonated one, and the amidated carboxylate group has a greater inductive effect than the deprotonated one. The stability constants, however, are much smaller than the calcium binding constants of vitamin K-dependent blood coagulation factors presented in the literature (for example: prothrombin, $\log K = 3.5$ [9]; Factor IX, $\log K = 4.0$ [6]). Consequently, additional stabilizing effects are responsible for the formation of high-affinity binding sites in the long-chain proteins.

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