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PH DEPENDENCE OF MAGNESIUM ION BINDING TO PROTHROMBIN FRAGMENT 1 AND γ-CARBOXYGLUTAMIC ACID-CONTAINING PEPTIDES VIA ^{25}MG NMR

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SUMMARY

The binding of magnesium ions to two tripeptides, L-Arg-D-Gla-D-Gla-OMe and Z-L-Arg(NO_2)-D-Gla-D-Gla-OMe, and to bovine prothrombin fragment 1 as a function of pH has been monitored by ^{25}Mg NMR spectroscopy. Binding to the tripeptide was dependent on peptide ionizations occurring at pH 4.6-4.8. The pH dependence of magnesium ion binding to fragment 1 reveals two inflection points at pH values of 4.2 and ca 7.5. The inflections at pH 4.6-4.8 and pH 4.2 may be attributed to the deprotonation of the third side chain carboxylic acid group of the double γ-carboxyglutamic acid sequence. The origin of the increased binding of magnesium ions to fragment 1 at pH values above 7 is unknown.

The importance of γ-carboxyglutamic acid side chain residues to the function of the vitamin K-dependent blood coagulation proteins has been the subject of intense recent investigation (1-6). The presence of Gla[2] residues in these proteins is now considered to be an essential requirement for the calcium ion-mediated phospholipid binding essential for catalytic processes involved in blood coagulation. In contrast to the behavior of calcium ions in this system, magnesium ions alone will not support fragment 1 or prothrombin phospholipid binding. As assessed by metal ion-induced quenching of fragment 1 fluorescence, magnesium and calcium ions bind to fragment 1 with approximately equal affinity. Furthermore, the detailed shape of the fragment 1 calcium ion binding isotherm is sensitive to the presence of magnesium ion on fragment 1. Such considerations have led Prendergast and Mann (6) to suggest that both

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[2]Abbreviations: Gla, γ-carboxyglutamic acid; I, L-Arg-D-Gla-D-Gla-OMe; II, Z-L-Arg(NO_2)-D-Gla-D-Gla-OMe.

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calcium and magnesium ion binding sites exist on fragment 1 and that some of these sites interact in some way.

MATERIALS AND METHODS

^{25}Mg was obtained from Oak Ridge National Laboratories in the form of magnesium oxide with 98.25 atom % of the desired isotope. Peptides studied were synthesized in this laboratory by previously published procedures. Bovine prothrombin fragment 1 was prepared in this laboratory. The protein was single-banded in SDS disc gel electrophoresis and had the appropriate amino acid composition. Deionized distilled water was employed throughout. All other chemicals used were reagent grade or better.

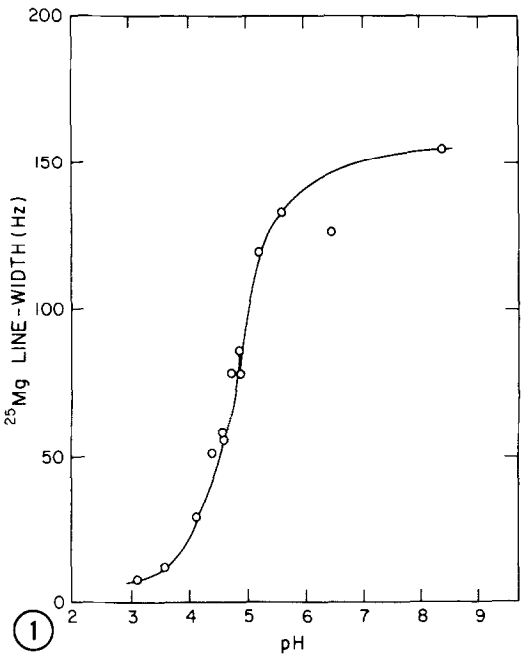
Peptide/Mg^{2+} samples were prepared as previously described (7). The ratio of the concentrations of peptide and magnesium ions was maintained at greater than one in order to minimize the incidence of non-chelated binding. The pH was adjusted as necessary by addition of neat triethylamine and/or 6 N HCl with good mixing.

The sample of fragment 1 was prepared by dialysis against 0.010 M Tris, 0.100 M NaCl, pH 8.3. $^{25}MgCl_2$ was added as a concentrated solution to this sample, the total volume being brought to 4.0 ml. The pH of individual samples was adjusted by <μl additions of 6 N HCl and/or 4 N NaOH.

NMR spectra were recorded on a Varian XL 100 FT spectrometer with a 23.5 kG field in spinning 18 mm sample tubes. The instrument had been modified for multinuclear operation in the manner described by Marshall et al. (8) The 18 mm probe was made by Nicolet Technology Inc. Field frequency lock was an external lock onto a ^{19}F sample built into the NMR instrument. A pulse width of 550 microseconds followed by an acquisition time of 1 sec (with no pulse delay) was employed for the spectra of samples with the lowest pH's. As the line-widths increased, the acquisition time was decreased, eventually down to 0.125 sec. For spectra with S/N > 10, ca 1000 transients were required at the lowest pH's, increasing to 5000 transients for the widest of the signals.

RESULTS AND DISCUSSION

The 1-156 sequence of prothrombin (Fragment 1) contains the ten γ-carboxyglutamic acid residues that are introduced into the protein in a post-ribosomal carboxylation step mediated by vitamin K. The γ-carboxyglutamic acid residues occur in pairs (or nearly so) and in at least three groupings the nearest neighbors are the basic amino acids, arginine or lysine. The formation of ion-pairs or simple polyelectrolyte effects due to the proximity of these positively charged side chains and the Gla carboxyl groups could affect the calcium and magnesium ion binding which is associated with this region of the protein. In order to establish whether nearest neighbor interactions could



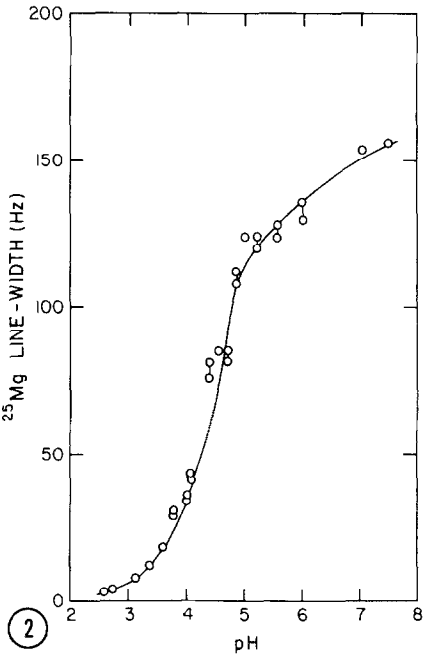


Figure 1. The dependence of $^{25}Mg^{2+}$ binding to L-Arg-D-Gla-D-Gla-OMe on pH. The concentrations of Mg and peptide are 18.1 mM and 28.9 mM, respectively. pH was varied from 3.12 to 8.37.

Figure 2. The dependence of $^{25}Mg^{2+}$ binding to Z-L-Arg(NO_2)-D-Gla-D-Gla-OMe on pH. The concentrations of magnesium ion and peptide are 18.9 mM and 33.7 mM, respectively. pH was varied from 2.59 to 7.47.

influence metal ion binding, the model system L-Arg-D-Gla-D-Gla-OMe (I) and the arginyl-blocked precursor Z-L-Arg(NO_2)-D-Gla-D-Gla-OMe (II) were studied using $^{25}Mg^{+2}$ NMR as previously described (7).

Both I and II bind Mg^{2+} with virtually the same binding constant as that already reported for the Z-D-Gla-D-Gla-OMe·Mg^{2+} complex (0.6 mM at pH 6.5) (7,9). The dissociation constants of the I·Mg^{2+} and II·Mg^{2+} complexes are 0.6 mM (pH 6.5) and 0.54 mM (pH 6.8), respectively. The pH dependence of the line-width of the $^{25}Mg^{2+}$ ions in the presence of I or II over the pH range 3 to 8 is illustrated in Figures 1 and 2. The line-width of $^{25}MgCl_2$ alone in solution is insensitive to pH over the range 2.0 to 9.7. At low pH values, the $^{25}Mg^{2+}$ line-width in the presence of peptide is effectively that of free magnesium ions in solution. The line-width increases markedly, indicating

binding, as the pH is increased. The resulting titration curves show inflections in the pH range 4.6-4.8, and there appear to be no appreciable differences between the curves for I and II. The inflection points are virtually identical, and the general curve shapes are very similar, although the slower leveling at high pH of the curve for the blocked peptide II may reveal an effect of the N-nitroguanidine group on the titration of the least acidic of the carboxyl groups of the double Gla sequence or may be an artifact peculiar to that experiment. The major feature of both plots is, however, the inflection at pH 4.6-4.8.

Märki et al. (9) have determined that the fourth ionization constant of H-L-Gla-L-Gla-OH is 4.7. Thus the deprotonation of the third side chain carboxyl of the Gla-Gla peptide appears to be necessary for the ligand system to be established and for significant metal binding to occur. Clearly the presence of the guanidino side chain has no effect on the ligand system of the peptide models.

A comparison of $^{25}Mg^{+2}$ binding to peptides with $^{25}Mg^{+2}$ binding to bovine prothrombin fragment 1 requires that the metal nuclei NMR method be extended to a much larger and more complex molecule than the models discussed above. The relaxation enhancement induced by the binding of a quadrupolar ion to a molecule is sensitive to the size of the complexing species. The line-width of $^{25}Mg^{2+}$ bound to the small peptides discussed above is ca 160 Hz. The apparent line-width of $^{25}Mg^{2+}$ bound to fragment 1 is ca 30 KHz. However, this value is actually a composite resulting from the binding of several magnesium ions to a single fragment 1 molecule.

The relative concentrations of magnesium and fragment 1 were adjusted such that all sites on the protein accessible to magnesium ions should be occupied. Sodium chloride was included in the solution in order to minimize non-specific association of magnesium ions with the protein. The variation of the $^{25}Mg^{2+}$ line-width as a function of the pH of a $^{25}Mg^{2+}$/fragment 1 solution is pictured in Figure 3. Virtually no magnesium ion binding is

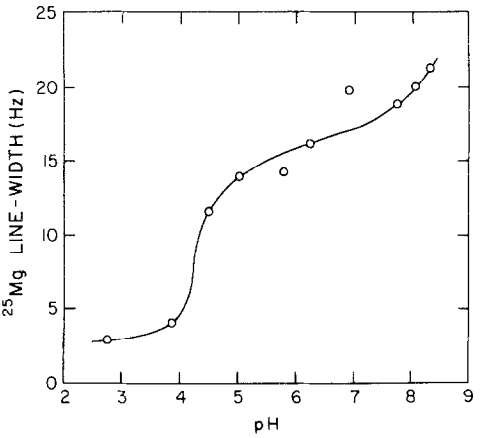


Figure 3. The dependence of $^{25}Mg^{2+}$ binding to prothrombin fragment 1 on pH. The concentrations of magnesium ions and protein (in 0.010 M Tris·HCl, 0.100 M potassium chloride) were 18.4 mM and 19.1 μM, respectively. pH was varied from 2.77 to 8.28.

apparent at low pH values. However, there is a sharp increase in binding as the pH is raised above 3.8. A major inflection point occurs in this titration curve at pH 4.2. Thus the basic shape of the titration curve obtained from either peptide models or the protein is similar up to pH 7. However at ca pH 7 a second substantial increase in magnesium ion binding to the protein begins. Hysteresis in the titration appears not to occur in this system since the line-width measured for the solution at pH 8.3 (the first spectrum to be recorded) falls on the line described by the other points of the titration.

Scott et al. (10) have studied the metal ion-induced quenching of the intrinsic fluorescence of bovine fragment 1. A pronounced pH dependence was noted where the affinity of protein for metal ion increased with increasing pH. Calcium binding as assessed by fluorescence quenching, increased twenty-fold from pH 6 to 8 while magnesium binding by fragment 1 increased only two-fold within the same range of pH. The apparent pK_a of protein side chains associated with metal binding was about 7.5.

The present data obtained by observation of the line-broadening associated with protein binding of $^{25}Mg^{+2}$ ions also indicate dependence on a protein pK_a of at least 7.5. The fact that the Gla-containing peptides (I,II) and bovine

fragment 1 exhibit similar metal ion binding behavior below pH 7 but quite different behavior above pH 7 suggest that functional side chains other than the carboxyl groups of Gla are involved in calcium and magnesium binding. Alternatively, Gla side chain carboxyl groups if involved must be located in microenvironments which significantly alter their prototropic behavior.

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