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Relative Affinity of Ca(II) and Mg(II) Ions for Human and Bovine Prothrombin and Fragment 1

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Summary: Equilibrium dialysis results are presented for Ca(II) and Mg(II) ion binding to human and bovine prothrombin and fragment 1. Ca(II) ions bind cooperatively, Mg(II) does not.

Prothrombin is an essential vitamin K-dependent protein in the coagulation cascade (1). Ca(II), but not Mg(II), ions are necessary for the proper phospholipid binding function of prothrombin and subsequent activation of the prothrombinase complex (2). Mg(II) ion, however, does exhibit a significant synergistic effect on the activation of prothrombin in the presence of low levels of Ca(II) ions (3). The physiological concentrations of both metals are in the mM range (4). It has been reasonably assumed that the metal binding properties of prothrombin are largely determined by the presence of 10 γ-carboxyglutamyl (Gla) residues near the amino terminus; for

ABBREVIATIONS: Gla, γ-carboxyglutamic acid, fragment 1; first 156 amino acid residues from N-terminal of prothrombin; reduced and alkylated fragment 1, reduction of the disulfide bonds of bovine fragment 1 with dithioerythritol followed by alkylation of the resultant thiols with iodoacetamide; 10 γ-MGlu fragment 1 in which all 10 Gla residues in bovine fragment 1 have been chemically modified to result in γ-methyleneglutamyl residues (15).

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this reason it has also been assumed that the first 156 residues of the N-terminus (fragment 1) of the intact protein (582 residues) constitutes a prothrombin model system. A previous review (5) summarizes the disparate metal ion binding results for prothrombin and fragment 1 (bovine and human). We have undertaken the current study to:

a) provide a direct comparison of Ca(II) and Mg(II) ion binding affinities in the human and prothrombin prothrombin system; and,

b) compare the Ca(II) and Mg(II) ion binding properties of human and bovine fragment 1 with the corresponding intact proteins.

MATERIALS AND METHODS

The procedure for the metal ion equilibrium dialysis experiment has been previously published (6, 7, 8, 9), as has the isolation and purification of human prothrombin (10) and human fragment 1 (10), bovine prothroobin (6), bovine fragment 1 (6), and reduced and alkylated fragment 1 (11). In all cases the data were analyzed using a Scatchard analysis (S vs $[\text{Metal free}]$; $S=\bar{v}/[\text{Metal free}]$, $\bar{v}=[\text{Metal bound}]/[\text{Protein total}]$ assuming a sequential loading of the ligands. The NLIN/DUD option of the SAS (Statistical Analysis System, Cary, NC) was used in conjunction with grid searches followed by nonlinear least squares fitting (6,7).

RESULTS

Scatchard plots derived from Ca(II) and Mg(II) ion equilibrium dialysis experiments are shown for human prothrombin (Fig. 1), and human fragment 1 (Fig. 2), bovine prothrombin (Fig. 3), bovine fragment 1 (7) (Fig. 4), and reduced and alkylated bovine prothrombin fragment 1 (Fig. 5).

The Scatchard plot for the binding of Mg(II) ions by all proteins was approximately a straight line; this suggests that all proteins bind Mg(II) ions to essentially equivalent, non-interacting sites. The experimental data for both human and bovine prothrombin extrapolated to seven Mg(II) ion binding sites for the proteins; the metal ion binding data for both human and

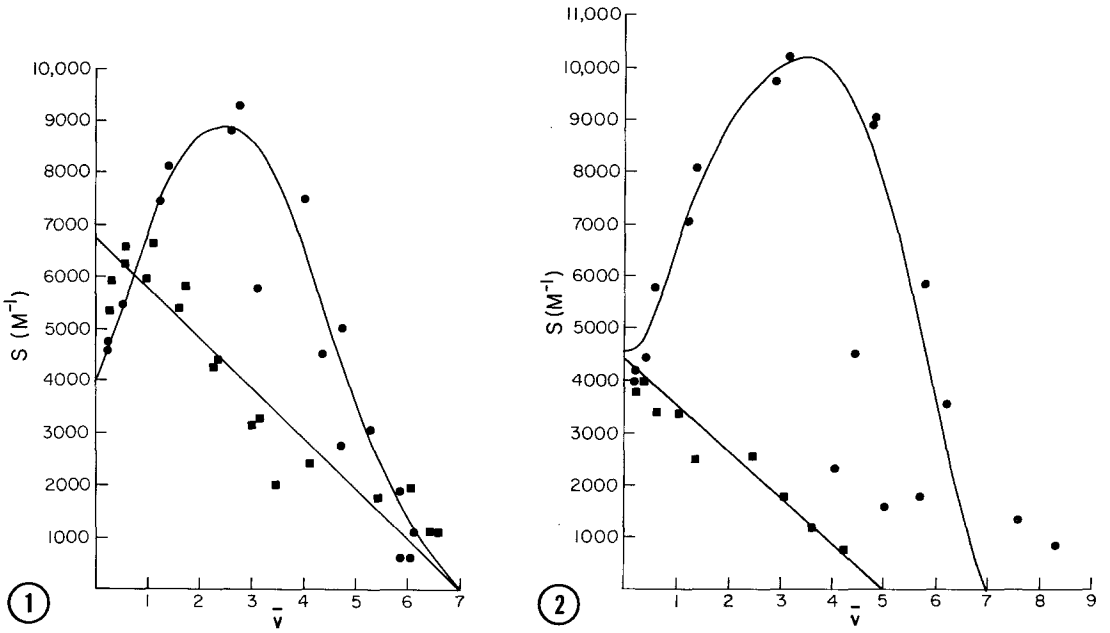


Fig. 1. Scatchard plot from the equilibrium dialysis data of human prothrombin with Ca(II) (● , protein concentration = 10 μM) and Mg(II) (■ , protein concentration = 20 μM). For Figs. 1 through 5, $\bar{v} = [\text{Metal}]_{\text{bound}}/[\text{Protein}]$, $S=\bar{v}/[\text{Metal}]_{\text{free}}$. The curves represent the least squares simulations (constants given in Table 1).

Fig 2. Scatchard plot from the equilibrium dialysis data of human prothrombin fragment 1 with Ca(II) (● , protein concentration = 20 μM) and Mg(II) (■ , protein concentration = 30 μM).

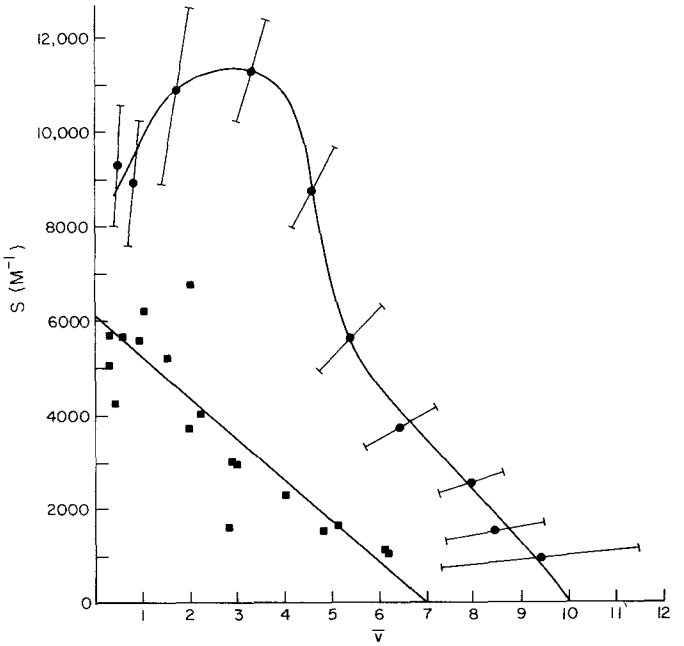
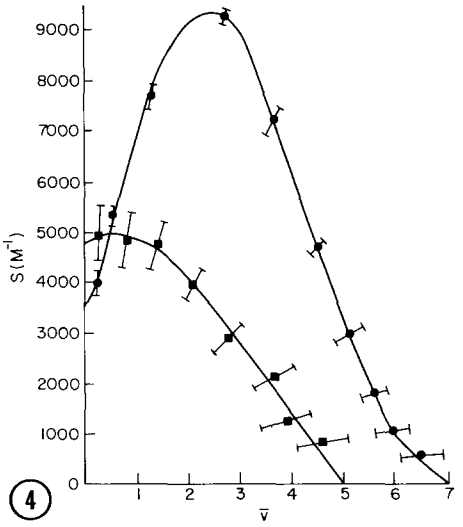


Fig 3. Scatchard plot from the equilibrium dialysis data of bovine prothrombin with Ca(II) (● , protein concentration = 10 μM) and Mg(II) (■ , protein concentration = 20 μM). Each Ca(II) ion data point represents the average of at least ten determinations and the error bars represent the experimental standard deviation for each point.



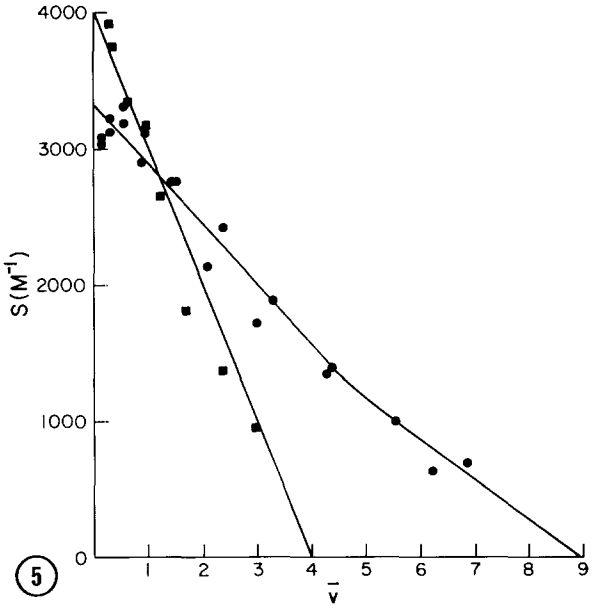


Fig. 4. Scatchard plot (taken from ref 7) from the equilibrium dialysis data of bovine prothrombin fragment 1 with Ca(II) (●, protein concentration = 20 μM) and Mg(II) (■, protein concentration = 30 μM). Each data point represents the average of at least four determinations and the error bars represent the experimental standard deviation for each point.

Fig. 5. Scatchard plot from the equilibrium dialysis data of reduced and alkylated bovine prothrombin fragment 1 with Ca(II) (●, protein concentration = 24 μM) and Mg(II) (■, protein concentration = 35 μM).

bovine fragment 1 extrapolated to five metal ions bound per protein molecule. The reduced and alkylated bovine fragment 1 was found to have four Mg(II) ion binding sites. In contrast to the Mg(II) ion binding data, human and bovine prothrombin and fragment 1 show enhanced cooperativity in the Ca(II) ion binding data. Human prothrombin and fragment 1 and bovine fragment 1 exhibit seven Ca(II) ion binding sites; bovine prothrombin extrapolates to approximately ten sites. The reduced and alkylated bovine fragment 1 was found to have approximately nine total Ca(II) ion sites.

A Scatchard analysis (Table 1) was conducted using the Adair equation (12) to simulate the Ca(II) ion binding by both human

Table I

Comparison of the Metal Ion Binding Ability
(All constants are given as M^{-1})

	Mg(II)		Ca(II)										
	Sites	k_{site}	Sites	K_1	K_2	K_3	K_4	K_5	K_6	K_7	K_8	K_9	K_{10}
Human Fragment 1	5	808	7	3780	1840	6790	4140	3100	1290	53			
Human Prothrombin	7	960	7	3770	2950	5630	5070	1110	354	44			
Bovine Fragment 1	5	1118	7	2720	4800	4200	5790	581	552	84			
Bovine Prothrombin	7	868	10	8160	3490	5970	4590	2510	1460	885	533	296	127
Reduced and Alkylated Bovine Fragment 1	4	1000	9	3324	1436	807	494	307	185	101	44	16	

and bovine prothrombin and fragment 1, while the Mg(II) ion binding data for all of the proteins was simulated using a single class Langmuir loading equation (13). The Ca(II) ion binding data for the reduced and alkylated bovine fragment 1 was simulated using a two class model (7) with one class containing seven total binding sites (k_{site}=463 M^{-1}) and a lower affinity class containing two sites (k_{site}=40 M^{-1}). The Ca(II) ion and Mg(II) ion equilibrium dialysis experiments were conducted on protein from the same reduced and alkylated bovine fragment 1 preparation; this preparation is also represented in the data for bovine fragment 1 presented in Fig 4.

The reproducibility between the duplicate Ca(II) ion binding experiments was good in all cases; for bovine prothrombin (Fig 3, the average of ten data sets from six preparations), however, the reproducibility of the Ca(II) ion binding affinity from preparation-to-preparation was only qualitative. This contrasted with the Ca(II) ion binding by bovine fragment 1 (Fig 4) for which preparation-to-preparation replication was good (14). The error bars represent the standard deviation for each point and include both experimental and preparation-to-preparation

reproducibility. Furthermore, no differences were observed at either 10 or 20 μM protein concentration (6). The Mg(II) ion binding data for bovine fragment 1 (Fig. 4) derives from two different preparations. The error bars for this case are larger than those found in the Ca(II) ion binding data due to the lower net binding of Mg(II) ions by the protein, some preparation-to-preparation differences between the two proteins, and to inherent difficulties associated with a short half-life probe (9). In the protein equilibrium dialysis experiments, the protein used in a duplicate equilibrium dialysis experiment derived from a single preparation.

One goal of this project was to provide a direct comparison of the intrinsic binding affinity of Gla residues in an intact protein for Mg(II) and Ca(II) ions. Previously, a modified prothrombin fragment 1 was reported (15) that contained seven equivalent, non-interacting Ca(II) ion binding sites (k_{site}=365 M^{-1}). This value for an intrinsic site constant for the binding of Ca(II) ions by the modified protein, averaged with the site constant found for the first class of the reduced and alkylated bovine fragment 1, leads to an estimated site binding constant for the binding of Ca(II) ions of k_{site}=414 M^{-1} ($\pm$ 69 M^{-1}). The average site binding constant for the binding of Mg(II) ions by the proteins reported here is 951 M^{-1} ($\pm$ 107 M^{-1}). We believe that these average site binding constants for the proteins examined for Mg(II) ion (951$\pm$107 M^{-1}) and Ca(II) ions (414$\pm$69 M^{-1}) are the site constants for Gla residues in the intact protein (the 10 γ-MGlu fragment 1 does not bind Ca(II) ions in this concentration range (6)). With the assumption that ethylmalonate provides a reasonable model of Gla, the site binding constants for Gla for these metal ions compares favorably

to the reported metal ion binding constants for ethyl malonate (K_{Mg}=422 M^{-1}, K_{Ca}=296 M^{-1}) (16).

CONCLUSIONS

In summary, we find:

1. Ca(II) and Mg(II) ion binding are very different in the intact prothrombin systems; modification of the protein normalizes the behavior of the two ions,
2. human and bovine prothrombin and fragment 1 exhibit enhanced cooperativity in the binding to Ca(II) ions whereas Mg(II) ions binds to essentially equivalent, non-interacting sites.
3. human and bovine fragment 1 are reasonable metal ion binding models for their larger precursors.

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REFERENCES

1. Suttie, J.W., and Jackson, C.M. (1977) Physiol. Rev. 57, 1-70.
2. Nesheim, M.E., Tracy, R.P., and Mann, K.G. (1984) J. Biol. Chem. Soc. 259, 1447-1453.
3. Prendergast, F.G., and Mann, K.G. (1977) J. Biol. Chem. 252, 840-850.
4. Iyengen, G.V., Kollmer, W.E., and Bowen, H.J. "The Elemental Composition of Human Tissues and Body Fluids", Verlag Chemie, 1978, New York, NY.
5. C. M. Jackson (1980) "Vitamin K Metabolism and Vitamin K-Dependent Proteins", J. W. Suttie (ed), Univ. Park Press, Baltimore, p.16.
6. Deerfield, D.W., II, Berkowitz, P., Olson, D.L., Wells, S., Hoke, R.A., Koehler, K.A., Pedersen, L.G., and Hiskey, R.G. (1986) J. Biol. Chem. 261, 4833-4839.
7. Deerfield, D.W., II, Olson, D.L., Berkowitz, P., Byrd, P.A., Koehler, K.A., Pedersen, L.G., and Hiskey, R.G. J. Biol. Chem. in press.

8. Olson, D.L., Deerfield, D.W., II, Berkowitz, P., Hiskey, R.G., and Pedersen, L.G. (1987) Anal. Biochem. 160, 468-470.
9. It was necessary to develop a Mg(II) ion equilibrium dialysis procedure using Mg-28, a short lived (21 h) isotope. The accurate binding results for Mg(II) ion herein are, along with ref 7 and 8, the first to be reported for any macromolecular system.
10. Kisiel, W., Hanahan, D.J. (1973) Biochim. Biophy. Acta 304, 103-113.
11. Henricksen, R.A., and Jackson, C.A. (1975) Arch. Biochem. Biophys. 170, 149-159.
12. Perlmutler-Hayman, B. (1986) Acc. Chem. Res. 19, 90-96.
13. Langmuir, I. (1916) J. Am. Chem. Soc. 38, 2221-2295.
14. Occasionally (≤15 % of the preparations), bovine fragment 1 was isolated that demonstrated different Ca(II) ion binding than that shown in Fig. 3 (T_m^{fq}=0.22 mM instead of T_m^{fq}=0.43, where T_m^{fq}=half maximal response for fluorescence quench).
15. Wright, S.F., Berkowitz, P., Deerfield, D.W., II, Byrd, P.A., Olson, D.L., Larson, R.S., Hinn, G.C., Koehler, K.A., Pedersen, L.G., and Hiskey, R.G. (1986) J. Biol. Chem. 261, 10598-10605.
16. Klæning, U. K.; Østerby, O. (1976) J. Chem. Soc. Faraday Trans 1 72, 513-525.