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Possible insights into metal ion recognition in calcium-binding proteins provided by complexing properties of ligands containing amide oxygen donors

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The formation constants of the ligand *N*,*N*,*N*′,*N*′-tetrakis(carbamoylmethyl)-ethylenediamine suggest that the amide oxygen is a stronger Lewis base in water than the alcoholic oxygen, or water, and that part of the selectivity for Ca^{2+} over Mg^{2+} shown by calcium-binding proteins such as calmodulin or annexin may be due to the higher affinity of Ca^{2+} for the O-donor of the Ca-binding amide groups present in such proteins.

Calcium as a second messenger in biology binds[1] to sites in proteins such as calmodulin, annexin, and troponin-C.[2–4] These proteins are switches triggered by Ca^{2+} when it enters the cytoplasm of the cell. Mg^{2+}, present in higher concentration in the cytoplasm, does not bind sufficiently strongly to these sites to interfere with triggering by Ca^{2+}. Falke *et al*.[5–7] studied binding of Ca^{2+} to bacterial proteins with sites resembling those of calmodulin, showing[5] selectivity for Ca^{2+} over Mg^{2+} of about 10^4. Possible origin[5–7] of such selectivity in a rigid cavity that might distinguish the large Ca^{2+} ion (ionic radius (r^+)[8] of 1.00 Å) from the small Mg^{2+} ion (r^+ = 0.74 Å) has been investigated. Usually proteins distort easily,[5,6,9] typically taking about 0.15 kcal mol^{-1} to expand the radius of a cavity from 0.9 to 1.1 Å. Site-directed mutagenesis studies of such proteins have suggested[7] that Ca/Mg selectivity is not here mainly governed by rigidity of the binding cavity.

In a number of proteins in the PDB (Protein Database[10]) with Ca^{2+} binding sites, two recurring themes are observable. First, there is at least one chelating carboxylate group,[11] as in the binding site of Ca^{2+} in annexin (Fig. 1). As has been discussed extensively,[12] small chelate rings bind with less steric strain to larger metal ions, so that these small four-membered chelate rings might promote selectivity for the large Ca^{2+} over the small Mg^{2+} cation. Second, which is the topic of interest here, there are one to three amide O-donor atoms coordinated to the Ca^{2+} (Fig. 1), from peptide linkages of the protein backbone, or from amide groups on asparagine and glutamine residues. Dudev and Lim[11] have carried out *ab initio* calculations that suggest that amide oxygens are stronger donors than the oxygen donor of water in situations of low dielectric constant, which might explain the embedding of such sites in a shell of hydrophobic residues.

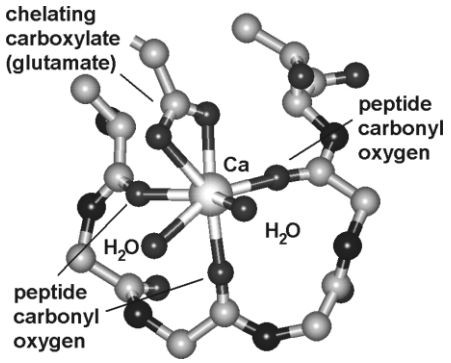

Fig. 1 Binding site of Ca^{2+} in annexin, drawn with coordinates from ref. 3. The Ca^{2+} is seven coordinate, held in the binding site by a chelating carboxylate from a glutamate residue, plus three amide oxygens derived from peptide linkages of the protein backbone. Two coordinated water molecules make up the rest of the coordination sphere.

To investigate the metal binding properties of the amide donor in water, the complexes of L1 (Fig. 2) have been studied. L1 has been reported by other workers[13–15] but not its formation constants (log K_1) with metal ions. The usual coordination of amides through the carbonyl oxygens to a metal ion, in this case for the L1 complex of Pb(II), except at higher pH, has been shown crystallographically,[14] as well as by us for the Cd(II) complex of L1 seen in the contents list for this article. Several ligands with one or two amide groups have been reported,[16] but there are several types of donor atom present in each of these ligands, so that it is not easy to distinguish the role of the amide oxygen donors. L1 has four pendant amide donors attached to an en (ethylenediamine) ligand. Mg^{2+} and Ca^{2+} have a low and approximately equal log $K_1$with en,[16] so that differences in log K_1 with L1 with these ions can be reasonably attributed to differences in affinity for the amide donors. L1 was synthesized as reported.[13] Log K_1 values were determined by glass electrode potentiometry.[17] The pK_a and log K_1 for L1 with Mg^{2+} and Ca^{2+}, as well as several other metal ions, are shown in the Table, together with log K_1 values[16] for L2 and en for comparison. In response to a comment by a referee, it is noted that although the structure of Cd(II) with L1 in the contents list has a coordinated nitrate, and the present formation constant study was carried out in 0.1M nitrate, nitrate is[16] a rather weak ligand, and would be coordinated to metal ions or their complexes with EDTAM in solution only very weakly.

The Table shows that the amide O-donors on L1 produce selectivity for Ca^{2+} over Mg^{2+} of almost 10^3. This, combined with the effects of the four-membered chelate rings formed by acetates, may account for part of the selectivity for Ca^{2+} over Mg^{2+} of about 10^4 found for Ca-binding sites.[5] Log K_1 values for L1 are larger than for L2, which has hydroxyalkyl O-donors[18] in place of amide O-donors in L1. Neutral O-donors vary widely[19] in their strength as Lewis bases. Amide donors (Table) are stronger Lewis bases towards larger metal ions such as Ca^{2+} than are alcoholic or water-derived O-donors. Log K_1 values for L1 and L2 give some insight into how alcoholic *versus* amide donors might affect Ca^{2+} binding strength and Ca^{2+}/Mg^{2+} selectivity. The log K_1 values for L1 and L2 suggest that the alcoholic oxygen from a serine would lower the Ca^{2+} binding strength of the serine-containing site in calpain.[20] A question is why the amide groups of L1 promote selectivity for Ca^{2+} over Mg^{2+}. There may be two main factors. One is that transfer of positive charge to the solvent is more important for small metal ions like Mg^{2+}, and amide groups are less efficient at this. A second factor may be sensitivity to distortion of M–O–C bond angles,[21] which will be more serious for Mg^{2+}, which will have more strongly directional bonding than Ca^{2+}. Note that the Ca–O–C angles involving the peptide oxygens in annexin in Fig. 1 range[3] from 142–166°, instead of the approximately 130° expected for such angles, as will be discussed in a future paper. It is the

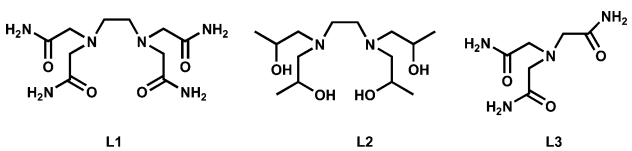

Fig. 2 Ligands discussed in this paper.

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Table 1 Formation constant for L1, L2, and en[a]

Lewis acid:	Ca^{2+}	Mg^{2+}	Sr^{2+}	Ba^{2+}	La^{3+}	Co^{2+}	H^+	References
Ionic radius (Å):	1.00	0.74	1.18	1.36	1.03	0.72	—	8
Log K_1 L1[b]:	3.29	≤0.6	2.30	2.15	5.19	5.94	4.36	this work
Log K_1 L2:	1.63	≤0.3	0.8	~0	2.90	6.1	8.67	16
Log K_1 en:	0.11	0.37	—	—	(1.4)[c]	5.5	9.92	14

[a] 25 °C and ionic strength 0.1 ($NaNO_3$). [b] Standard deviations in the determination of the log K_1 values is about 0.05. [c] Estimated in reference 18

directionality of M–O–C bonds that leads[21] to the greater stability of complexes of Ca^{2+} complexes than of Mg^{2+} with EDTA.[16]

Amide O-donors are the sole K^+ complexing groups[22] in K^+ ion channels, and are likely to occur in Ca^{2+} and Na^+ ion channels.[23] Studies of ligands containing amide donor groups could provide further insight into the metal-binding properties of proteins utilizing amide donors. The saturated N-donor, as found in L1, reduces[16,24] the affinity of ligands for Na^+ and K^+, and L1 does not appear to bind to Na^+ or K^+. L3 has a weak contribution to binding from its N-donors. The amide groups appear to be very electron-withdrawing, and L3 has a pK_a of only 2.6, which might improve binding to Na^+ and K^+. In order to remove the N-donor altogether, the aim is to study the metal binding properties of multidentate ligands containing amide O-donors only. What the present study has shown is that amide groups, as are present on L1, can bind Ca^{2+} quite strongly, and can lead to significant selectivity over Mg^{2+}.

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