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A neutral polypeptide–calcium ion complex

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SUMMARY

Proton magnetic resonance studies have been utilized to demonstrate calcium binding to a neutral polypeptide containing only peptide functional groups, *i.e. cyclo*-(–Gly–Gly–Val–Pro–)$_3$. This provides an explicit basis for the previously proposed neutral site theory for the binding of Ca^{2+} to polypeptides and protein wherein the peptide oxygens directly coordinate the Ca^{2+}.

In 1971 it was proposed that Ca^{2+} bind protein (most specifically elastin) at neutral sites–the peptide oxygens[1,2]. Previous proposals concerned with calcium binding to elastin had considered sulfhydryl groups[3], carboxylate anions in combination with threonine hydroxyls[4] and carboxyl groups in combination with amine groups[5]. This interest in calcium binding to elastin arises from its identification as the protein in the arterial wall which is responsible for the major deposition of calcium salts[6,7].

The proposal of acyl oxygens of peptides as the binding site was based on studies involving the complexation of cations by ion-transporting antibiotics and on the presence in elastin of a large amount of glycine and a limiting number of amino acids with functional side chains[8,9]. The glycine residues make probable the formation of β-turns[10], which have an end peptide oxygen oriented in a position to coordinate cations. The new binding site was substantiated in an implicit manner by the binding of Ca^{2+} to solubilized elastin under conditions where all sites were expected to be either protonated or neutral[2]. The proposal, which implicated glycines in inserting β-turns and in allowing other conformations with ion-binding capacity, suggested specific amino acid sequences which were expected to exhibit Ca^{2+} affinity. The first listed sequence was in analogy to valinomycin, a cyclic dodecadepsipeptide which has an optical isomeric sequence of $\overline{\text{(D–D–L–L)}_3}$. Replacing the D-amino

acid residues with glycine residues, it was suggested that a repeating sequence of Gly–Gly–L-amino acid–L-amino acid would favor calcium binding. Subsequently amino acid sequences have been determined by Gray and Sandberg (personal communication) for tropoelastin, a non-cross-linked elastin, from the aorta of copper-deficient pigs. The first eighteen residues are reported to be Gly–Gly–Val–Pro–Gly–Ala–Val–Pro–Gly–Gly–Val–Pro–Gly–Gly–Val–Phe–Phe–Pro–. With the exception of one residue, this contains the anticipated sequence (Gly–Gly–L-amino acid–L-amino acid) repeated four times.

Considering the basic repeat unit to be (Gly–Gly–Val–Pro), we have synthesized the linear and cyclic dodeca- and hexadecapeptides. The purpose of cyclization was to utilize, as an aid in solving the conformation of the molecule in solution, any symmetry that may result. Cyclization also serves to remove the carboxyl and amino end groups. The cyclic dodecapeptide $\overline{\text{L(Gly–Gly–Val–Pro)}_3\text{J}}$ is analogous to valinomycin. It may also be mentioned that other glycine-containing sequences proposed as having cation affinity[1] have been found as repeating units in the tropoelastin sequence (Gray, W.R. and Sandberg, L.B., personal communication). These sequences will be the subject of future communications.

We now report proton magnetic resonance studies on $\overline{\text{L(Gly–Gly–Val–Pro)}_3\text{J}}$ which demonstrate the complexation of Ca^{2+} to a polypeptide containing only peptide functional groups. Details of the secondary structure of the complex are discussed and two possible complete conformations, that differ by the direction in which the polypeptide chain wraps around the Ca^{2+}, are noted in analogy to the valinomycin–K^+ complex[11–15].

Fig. 1 shows the chemical shift of the peptide protons during Ca^{2+} titration. This demonstrates that (1) a complex is formed, (2) the complex does not involve displacement of peptide protons as occurs when Cu^{2+} binds polypeptides, and (3) complex formation results in a downfield shift of six of the nine peptide protons (three glycine and three valine) as commonly accompanies hydrogen bond formation.

Fig. 2 contains the temperature dependence of peptide proton chemical shift for the free and complexed polypeptide. The low temperature dependence for three glycine and three valine peptide protons in the complex indicates six solvent-shielded protons as occurs when peptide protons are intramolecularly hydrogen bonded[13]. The temperature dependence of the free polypeptide indicates a more involved situation where 3-fold symmetry is less apparent. Here, however, approximately three glycine peptide protons are weakly shielded from the solvent.

Hydrogen–deuterium exchange studies on the complex in methanol were inconclusive perhaps due to the presence of salts, as were methanol–trifluoroethanol solvent mixtures[16], due to the broadening of the spectra. Six peptide protons, however, exhibit large downfield shifts on Ca^{2+} addition; and the same six peptide protons exhibit decreased temperature dependence of peptide proton chemical shift. A reasonable interpretation of these results is the presence of six intramolecular hydrogen bonds in the calcium complex. This is a situation directly analogous to that of the valinomycin–K^+ complex[11–15] and suggests a series of β-turns in which the end peptides of the β-turns contribute their acyl oxygens to the direct coordination of the bare Ca^{2+}.

The above comparison of secondary structures is sufficient to warrant brief

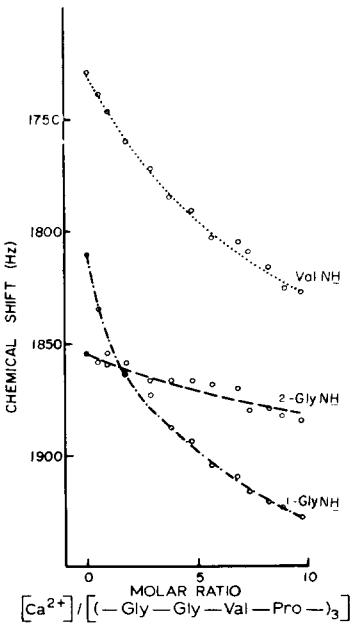

Fig. 1. Change in chemical shift of peptide protons on addition of $CaCl_2$ to a methanol solution of cyclo-(–Gly–Gly–Val–Pro–)$_3$. The spectra were obtained on a Varian HR 220 proton magnetic resonance spectrometer at 42 °C. Concentration of polypeptide was 29 mg/ml (31 mM). The complex exhibits only three resonances which, by inspection, (confirmed by decoupling experiments) are a valine doublet at high field followed by two glycine triplets at lower field. The valine NH and the 1-glycine NH resonances shift markedly to lower field on addition of $CaCl_2$. These shifts indicate complexation. The assignments of the glycine resonances were arrived at by noting chemical shifts of the tetrapeptides Pro–Gly–Gly–Val, Gly–Val–Pro–Gly, *etc.* The presence of only three resonances reflects the 3-fold symmetry in the complex.

discussion of the cyclododecapeptide–Ca^{2+} complex in terms of what is well established for the valinomycin–K^+ complex[12,15]. With a secondary structure of a series of β-turns and the restrictions due to the proline residue two complete conformations are possible which differ in the direction that the chain wraps around the ion. When the chain direction is the same as with the valinomycin–K^+ complex there is steric crowding of the valyl side chain and the δCH_2 of proline and there is a rather loose central core where a general preference for larger ions is apparent. When the chain direction is reversed a tighter structure is obtained which has a flat lipid face, a somewhat non-planar Val–Pro peptide ($\omega \simeq 25°$) and a tight central core wherein a high ion selectivity could occur.

Preliminary experiments, using Fig. 1 for the Ca^{2+} titration and a mole ratio of Me^{2+} to the cyclododecapeptide of 10, show Mg^{2+} to effect only about a 5 % shift and Sr^{2+} to effect about an 85 % shift, indicating a conformational energy minimum favoring Ca^{2+}. Utilizing circular dichroism studies on the cyclododecapeptide, a $\Delta[\theta]^{205}$ of $-1.6 \cdot 10^4 \pm 0.4 \cdot 10^4$ is observed on addition of Ca^{2+} at a mole ratio of 10, whereas an equivalent concentration of Na^+, within experimental, showed no effect. With the linear

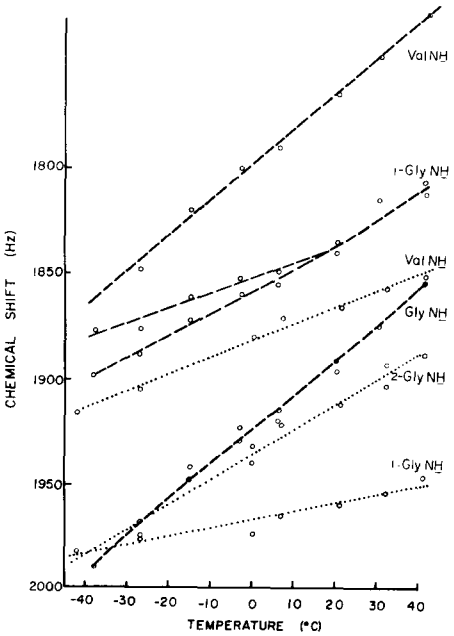

Fig. 2. Temperature dependence of peptide proton chemical shift; - - -, the free cyclododecapeptide $\overline{\text{(Gly–Gly–Val–Pro)}_3}$; . . . ., the Ca^{2+} complex. In the complex, the three valine NH and the three 1-glycine NH peptide protons (seen as two separate resonance peaks due to the 3-fold symmetry) exhibit a greatly decreased temperature dependence whereas the three 2-glycine NH, also seen as a single peak, have steeper dependences. This indicates that the six peptide protons are solvent shielded as occurs when intramolecularly hydrogen bonded[13].

dodecapeptide, *N*-formyl-(–Pro–Gly–Gly–Val–)$_3$ methyl ester, in trifluoroethanol at 29 °C and mole ratios of 1:1, $Ca(SCN)_2$ caused an average shift of 42 Hz in the valine NH protons whereas NaSCN caused an average shift of 35 Hz. A recent report by Wieland *et al.*[17] on antamanide first demonstrated that Ca^{2+} could complex with a polypeptide containing only peptide functional groups. In all solvents reported, including methanol, the affinity was greater for Na^+ than for Ca^{2+}. In the present case the affinity for the cations is less. Half complexation with Ca^{2+} in methanol occurs at a mole ratio of 3:1 for antamanide[17] and at 4:1 for the present cyclododecapeptide. But significantly $\overline{\text{(Gly–Gly–Val–Pro)}_3}$ is, to our knowledge, the first polypeptide, containing only peptide functional groups, to exhibit a selectivity favoring Ca^{2+} over Na^+. One explanation is that the conformational energy of the complex is slightly greater, such that the divalent cation is more effective in holding the polypeptide in the slightly distorted conformation.

Demonstration of the affinity of Ca^{2+} for neutral sites has marked implications as an organizing force in macromolecular assemblage, as for example, in membrane structure, in clot and atheroma formation, and in immunochemical processes as well as a driving force for the initiation of calcification. As an organizing force Ca^{2+} held in neutral sites could pair in a highly stereospecific manner with negatively charged groups of associating proteins or lipids. Relative to atheroma formation, one has the surprising effect of creating a

lipophilic region on binding such that there would be a synergism between calcium and lipid deposition. For the initiation of calcification, the affinity for neutral sites provides a driving force for positively charging the protein matrix until the affinity is matched by charge repulsion. The Ca^{2+}-charged protein then becomes a scavanger for negative ions which, when bound, relax the charge repulsion between bound Ca^{2+} and thereby allow further Ca^{2+} uptake and so on.

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