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Structural and functional importance of the *β*-turn in proteins. Studies on proline-containing peptides

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Abstract. We report here two sets of results on proline-containing linear peptides, one of which brings out the role of the *β*-turn conformation in the structure of nascent collagen while the other points to the functional importance of the *β*-turn in calcium-binding proteins. Based on the data on peptides containing the -Pro-Gly- sequence, we had proposed and experimentally verified that the *β*-turn conformation in these peptides is a structural requirement for the enzymic hydroxylation of the proline residues in the nascent (un-hydroxylated) procollagen molecule. Our recent data, presented here, on the conformation of peptides containing both the -Pro-Gly- and -Gly-Pro- sequences reveal that while the *β*-turn in the substrate molecule is required at the catalytic site of prolyl hydroxylase, the polyproline-II structure is necessary for effective binding at the active site of the enzyme. Thus, peptides containing either the *β*-turn or the polyproline-II structure alone are found to act only as inhibitors while those with the polyproline-II followed by *β*-turn serve as substrates of the enzyme. In another study, we have synthesized the two linear peptides: Boc-Pro-D-Ala-Ala-$NHCH_3$ and Boc-Pro-Gly-Ala-$NHCH_3$ each of which adopts, in solution, a structure with two consecutive *β*-turns, as judged from circular dichroism, infrared and nuclear magnetic resonance data. Drastic spectral changes are seen in these peptides on binding to Ca^{2+}. Both the peptides show a distinct specificity to Ca^{2+} over Mg^{2+}, Na^+ and Li^+. A conformational change in the peptides occurs on Ca^{2+} binding which brings together the carbonyl groups to coordinate with the metal ion. These results imply a functional role for the *β*-turn in Ca^{2+}-binding proteins.

Keywords. *β*-Turn; proline peptides; collagen; prolyl hydroxylase; calcium-binding proteins; protein structure.

Introduction

The complex three-dimensional structure of a protein molecule has often been sought to be understood in terms of the simpler structural elements like the α-helix, the *β*-structure, the *β*-turn and, in the case of collagen, the polyproline-II (PP-II) helix. These secondary structures are put together in well-defined super-secondary and higher-order arrangements that eventually impart the ultimate biological function to the protein. Except in the case of fibrous proteins and a few globular proteins, the functional importance of the individual secondary structures is not well understood. We have been involved in the past several years in the characterization of the various secondary structures, particularly the *β*-turn. Ever since its description by Venkatachalam (1968), a large number of studies have been devoted to the *β*-turn, mainly because of its

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Abbreviations used: PP-II, Polyproline-II; P_t, *β*-turn potential; CD, circular dichroism.

important structural role in globular proteins, peptides, hormones and ionophores (Smith and Pease, 1980). Our own interest in the *β*-turn originally arose from our studies on the conformational aspects of proline hydroxylation in collagen (Ananthanarayanan, 1983a,b) and on the positional preferences of amino acid residues in the *β*-turn (Ananthanarayanan *et al*., 1984). In the course of these studies, we have discovered not only the structural usefulness of the *β*-turn but, more recently, its functional role as well. In this paper, we present some of our results on proline-containing linear peptides which bring out the structural importance of *β*-turn in the nascent procollagen molecule and its functional importance in some of the calcium-binding proteins. Our data will be presented in the form of a review; details of the experimental methods and a more comprehensive discussion of the results have been, or will be, presented elsewhere.

Proline hydroxylation in collagen

Amongst the several post-translational modifications undergone by the nascent polypeptide chains of procollagen, the enzymic hydroxylation of selected proline residues is of singular importance since incomplete hydroxylation results in the structural instability and impaired secretion of the collagen molecule (Bornstein and Traub, 1979). Considerable attention has therefore been paid to the understanding of the conformational and mechanistic details of proline hydroxylation. The enzyme, prolyl hydroxylase, is known to display a high degree of specificity in that only the 3rd position proline in the Gly_1-X_2-Pro_3- sequence is hydroxylated and not all the 3rd position proline residues are equally hydroxylated (Prockop *et al*., 1976). In our laboratory, we have sought to understand the nature of the specific conformation in the substrate that is recognized by prolyl hydroxylase and the conformational consequences of proline hydroxylation in collagen. On the basis of several experimental and theoretical considerations, we proposed a few years ago the so-called *β*-turn hypothesis in answer to the substrate specificity of prolyl hydroxylase (Brahmachari and Ananthanarayanan, 1979). According to this postulate, the -Pro-Gly-segments in the nascent procollagen polypeptide chains would, as found in several globular proteins and synthetic peptides (Ananthanarayanan *et al*., 1984; Brahmachari *et al*., 1982), show preference for *β*-turn conformation which then would act as the recognition site for the enzymic hydroxylation. As a corrolary to this postulate, we also proposed that, after hydroxylation, the resulting -Hyp-Gly- segments would take up the PP-II helical structure, so that a conformational change from a previously folded (*β*-turn-containing) structure to a rigid helix would be the consequence of proline hydroxylation in collagen.

In a more recent study (Chopra and Ananthanarayanan, 1982), we have provided experimental support for both our postulates by (a) synthesising simple *β*-turn peptides and studying their interactions with chick embryo prolyl hydroxylase and (b) studying the conformational change in polypeptide models of collagen during the enzymic hydroxylation process. In particular, the *β*-turn tripeptides: Boc-Pro-Gly-Ala-OH, Boc-Pro-Gly-Val-OH and Boc-Pro-D-Ala-Ala-OH were found to act as inhibitors of the enzyme, while the pentapeptide Boc-Gly-Val-Pro-Gly-Val not only inhibited the

hydroxylation of the synthetic substrate but was itself a substrate for the enzyme. In contrast, none of the tripeptides was found to be hydroxylated. While these results indicated that the β-turn conformation was indeed recognized by prolyl hydroxylase, the tripeptide data pointed out that the minimal peptide sequence for a β-turn is necessary but not sufficient for proline hydroxylation. In addition, the observed inhibitory effect of native collagen and polyproline (in the PP-II form), neither of which can sustain the β-turn, was not explained. There was also no data available for our assumption (Brahmachari and Ananthanarayanan, 1979) that the synthetic substrate $(Pro\text{-}Pro\text{-}Gly)_n$ in the 'denatured' form in which it is hydroxylated, contained β-turn segments at the Pro-Gly junctions. There was thus a need for a modification of the β-turn hypothesis.

With this objective, we have recently carried out additional experiments on the interaction of several synthetic peptides with prolyl hydroxylase. The data obtained on di- and tripeptides are presented in table 1. It is seen that, contrary to our initial expectation, several dipeptides, in addition to the tripeptides, significantly inhibited the hydroxylation of the synthetic substrate without themselves serving as substrates. Since the β-turn is not feasible in the dipeptides, these results pointed to the recognition of a non-β-turn (in addition to the β-turn) conformation by the enzyme. Extending these studies further, we synthesized a series of peptides which had additional residues besides those minimally needed to form the β-turn. The data obtained with several tetra- and pentapeptides are shown in table 1. All the peptides are found to be hydroxylated although the extent of hydroxylation varies.

Table 1. Interaction of synthetic peptides with prolyl hydroxylase.

Peptide[a]	Inhibition(%)[b,c]	Hydroxylation[b,c]	Conformation[d,e]
Boc-Pro-Gly-Ala-OH	90		β-turn
Boc-Pro-Gly-Val-OH	80		β-turn
Boc-Pro-D-Ala-Ala-OH	70		β-turn
Boc-Pro-Gly-OH	40		'bent structure'
Boc-Pro-D-Ala-OH	50		'bent structure'
Boc-Gly-Pro-OH	35		'rigid' structure
Boc-Pro-Pro-Gly-$NHCH_3$		3·8	PP-II + β-turn
Boc-Pro-Pro-Gly-Pro-OH		6·6	PP-II + β-turn
Boc-Pro-Pro-Gly-Pro-$NHCH_3$		7·8	n.a.
Boc-Val-Pro-Gly-Val-OH		14·5	PP-II + β-turn
Boc-Gly-Val-Pro-Gly-Val-OH		23·8	n.a.

[a] Peptide concentration used was 10 mM for inhibition and 20 mM for hydroxylation experiments.
[b] Expressed with respect to $(Pro\text{-}Pro\text{-}Gly)_5 \cdot 4H_2O$ as substrate.
[c] Average of 3 to 6 trials.
[d] For references, see text.
[e] n.a.: not available.

An explanation for the observations was sought by an examination of the conformation of the peptides used in these studies. The tripeptides shown as inhibitors in table 1 had earlier been shown by us to favour the β-turn conformation

(Ananthanarayanan, 1983a). The structures of the dipeptides were deduced from available crystallographic data, which showed that Boc-Pro-Gly-OH (Benedetti, 1977) and Boc-Pro-D-Ala-OH (Cameron, T. S. and Ananthanarayanan, V. S., unpublished results) have a 'bent' conformation in which the ϕ, ψ values for the Pro residue are close to those observed for the second residue in type I or type II β-turn ($\phi \cong -60°$ and $\psi \cong 150–175°$) while those for the Gly and D-Ala residues are such that they would cause a bend similar to that observed for the third residue in type I or type II β-turn, respectively. These dipeptides have thus a 'partial β-turn' structure. The crystal structure of Boc-Gly-Pro-OH (Tanaka *et al*., 1977), on the other hand, is that of an extended conformation where the ϕ, ψ values for the Gly residue are high ($\cong 180°$). Theoretical calculations on the conformation of X-Pro and Pro-X sequences support the above experimental data (Zimmerman and Scheraga, 1977). Extending these observations to longer peptides which contain adjacent X-Pro and Pro-X sequences, one would anticipate that these will exhibit an extended structure followed by a bent structure. Such indeed is found to be the case for Boc- Pro- Pro-Gly-$NHCH_3$ (Tanaka *et al*., 1979) and Boc-Val-Pro-Gly-Val-OH (Yagi *et al*., 1983) whose crystal structures are available. In fact, these peptides are seen to adopt what may be called a "PP-II + β-turn" conformation where the N-terminal X-Pro part is in the PP-II conformation while the -Pro-Gly-X part assumes the β-turn. A recent study of the minimum energy conformation of N-acetyl-Pro-Pro-Gly-OH and $(Pro\text{-}Pro\text{-}Gly)_n$ in the non-helical ("denatured") form shows that the -Pro-Pro-Gly-Pro- segment would indeed favour the PP-II + β-turn conformation (Lee *et al*., 1984). We may therefore reasonably expect this rather unique conformation to be prevalent in the other tetra- and pentapeptides shown in table 1. Noting that all of them are substrates of prolyl hydroxylase (table 1), we conclude that the conformational criterion for the enzymic proline hydroxylation is the presence of the PP-II + β-turn in the substrate molecule. The inhibition data on the di- and tripeptides obtained by us (table 1) and by others on native collagen and polyproline referred to earlier, may readily be explained if the PP-II structure is considered to be the requirement at the binding site of the enzyme, while the β-turn is necessary at the catalytic site. A sketch of our new model for the conformation of prolyl hydroxylase substrates is shown in figure 1. This model is found to account for all the available experimental data on the substrate specificity of prolyl hydroxylase. A detailed interpretation of these data, together with a study of the effect of neighbouring residues on the extent of hydroxylation will be presented elsewhere.

Calcium binding by linear synthetic peptides

In addition to its role in proline hydroxylation described in the preceding section, the β-türn has also been implicated in several other functions such as glycosylation, phosphorylation, antigen-antibody interaction, etc. (Smith and Pease, 1980). An interesting role for the β-turn in calcium-binding proteins was pointed out a few years ago by Vogt *et al.* (1979). These authors examined the conformation of the calcium-binding regions of several homologous and non-homologous protiens by computing the β-turn potential (P_t) of all possible tetrapeptide sequences in these regions. This led them to suggest a correlation between the position and linear density of β-turn forming

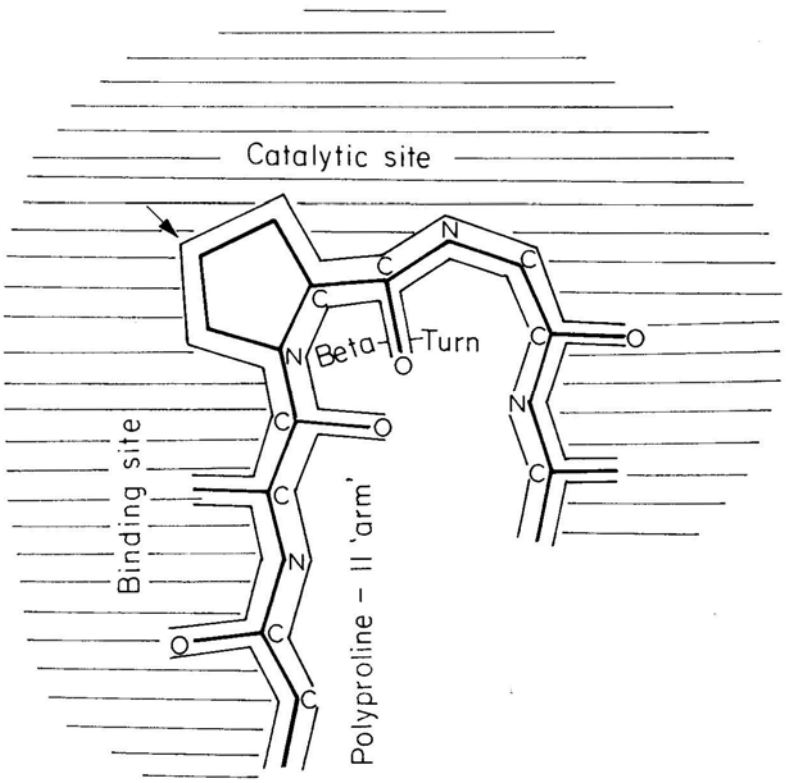

Figure 1. Schematic representation of the proposed model of the substrate of prolyl hydroxylase. The arrow indicates the position of hydroxylation.

residues and their ability to bind calcium. They also observed that a 'doublet' containing two consecutive tetrapeptide segments with high P_t values was involved in the calcium-binding portion of the 'loop' regions of homologous muscle proteins such as parvalbumin, troponin-C, etc. In other proteins like *staphylococcus* nuclease and thermolysin, they found a predominance of *β*-turns, though not necessarily occurring as doublets, in the calcium-binding regions. It is, in fact, interesting that several of these *β*-turn tetrapeptide segments have the -Pro-X- sequence in the middle (Vogt *et al*., 1979). In view of our special interest in proline -containing *β*-turn peptides referred to earlier, we decided to test whether these would bind calcium with any degree of specificity.

Our initial attempts were made with the tripeptide Boc-Pro-D-Ala-Ala-OH which has been shown to take up the type-II *β*-turn in the solid state by X-ray crystallography (Cameron, T. S. and Ananthanarayanan, V. S., unpublished results) and in solution (Attah-Poku, S. K. and Ananthanarayanan, V. S., unpublished results). The addition of increasing amounts of Ca^{2+} to a solution of this tripeptide in water caused a change in its circular dichroism (CD) spectrum indicative of the collapse of the *β*-turn conformation similar to what was observed when the peptide solution was treated with

6 M guanidine hydrochloride (GuHCl) or 8 M urea. In other words, there was no specific binding of Ca^{2+} by the tripeptide. We therefore synthesized an extension of this peptide containing a N-methyl amide group at the carboxyl end, *viz.* Boc-Pro-D-Ala-Ala-$NHCH_3$. This tetrapeptide (which we will refer to as the D-Ala peptide) is potentially capable of forming a set of consecutive β-turns, similar to the 'doublet' pattern observed by Vogt *et al.* (1979) in many calcium-binding proteins. The actual conformation of this tetra-peptide was deduced from IR and NMR data in organic solvent media which confirmed the presence of two consecutive 4 $\rightarrow$ 1 hydrogen bonds in the molecule involving the carbonyls of the Boc group and the Pro residue on the one hand and the NH's of the Ala residue and the terminal amide group, respectively, on the other. The CD spectral data in aqueous and organic solvent media were compatible

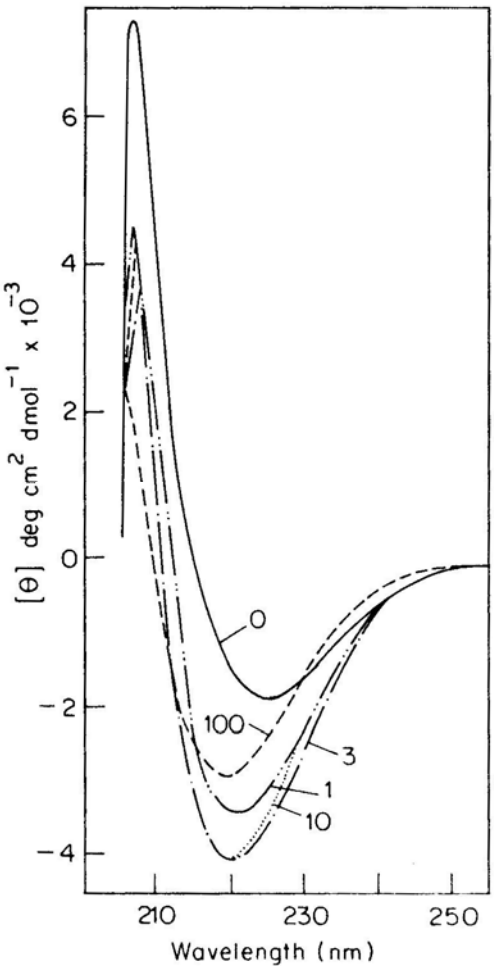

Figure 2. CD spectra of Boc-Pro-D-Ala-Ala-$NHCH_3$ in acetonitrile at the indicated $[Ca^{2+}]$/[Peptide] (ratio of molar concentrations).

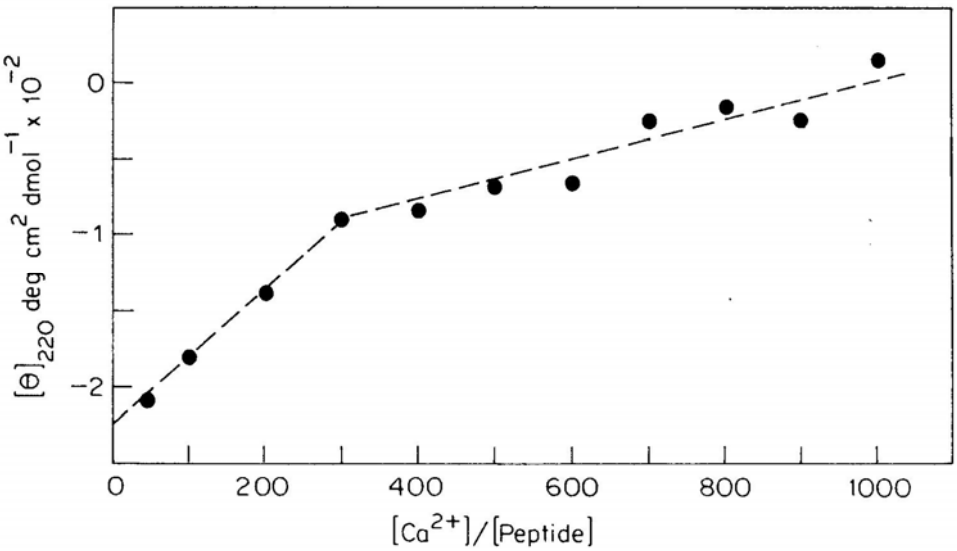

Figure 3. Plot of molar ellipticity of Boc-Pro-D-Ala-Ala-$NHCH_3$ at 220 nm against the [Ca^{2+}]/[Peptide] molar ratio in water.

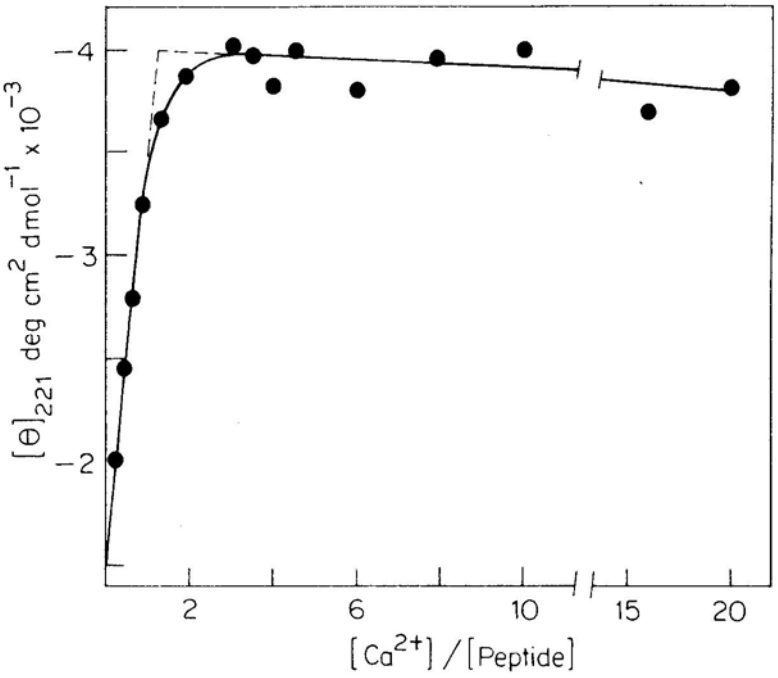

Figure 4. Plot of molar ellipticity of Boc-Pro-D-Ala-Ala-$NHCH_3$ at 221 nm against [Ca^{2+}]/[Peptide] molar ratio in acetonitrile.

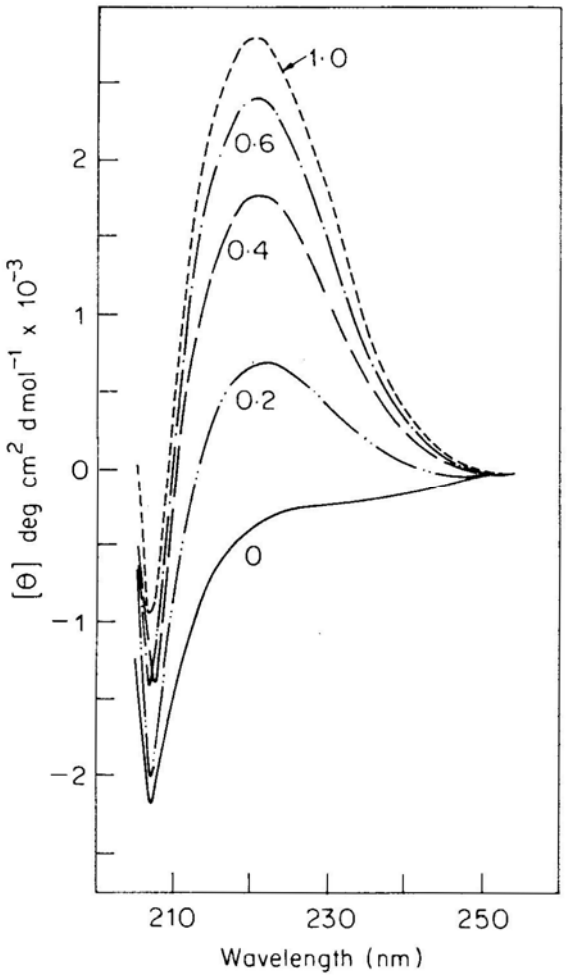

Figure 5. CD spectra of Boc-Pro-Gly-Ala-$NHCH_3$ in acetonitrile at the indicated [Ca^{2+}]/[Peptide] ratios.

with the presence of the β-turn conformation. The details of the spectral data and their interpretation will be given elsewhere (Rehse, P. H., Attah-Poku, S. and Ananthanarayanan, V. S., unpublished results). (A schematic representation of the consecutive β-turn conformation of the D-Ala is presented in a later part of this paper; see figure 9a).

Titration of the D-Ala peptide with Ca^{2+} caused a remarkable change in the CD spectra both in aqueous and acetonitrile (figure 2) solutions. In water, the CD spectral change caused by Ca^{2+} addition was found to be opposite to that observed on GuHCl or urea treatment (data not shown). This indicates a definitive binding of the Ca^{2+} ion by the tetrapeptide. However, a large molar excess of Ca^{2+} was found to be necessary to achieve saturation of the binding in water while a nearly 1:1 molar ratio of Ca^{2+} to the peptide was sufficient to effect saturation in acetonitrile (figures 3 and 4). This may be

explained in terms of the interaction between the peptide and water which is less favourable for the intramolecular hydrogen bonded conformation of the peptide than the relatively non-polar acetonitrile.

That the consecutive *β*-turn conformation is minimally necessary for calcium binding was further confirmed by the effect of Ca^{2+} addition on the CD spectral data (not shown) of Boc-Pro-D-Ala-Ala-OCH_3, Boc-Pro-D-Ala-OH and Boc-Pro-D-Ala-$NHCH_3$, none of which indicated any significant binding to Ca^{2+}. We then proceeded to examine whether the D-Ala residue can be replaced by the more naturally occurring Gly residue, particularly since the Pro-Gly sequence is also quite conducive to *β*-turn formation as seen in the earlier section. We therefore synthesized Boc-Pro-Gly-Ala-$NHCH_3$ (which we will refer to as the Gly peptide) and characterized its solution conformation by CD, NMR and IR techniques as was done for the D-Ala peptide. Like the latter, the Gly peptide was also found to have a two consecutive 4→1 hydrogen-bonded *β*-turn conformation. The CD spectral changes that take place on titrating this peptide with Ca^{2+} in acetonitrile are shown in figure 5. These data are plotted in

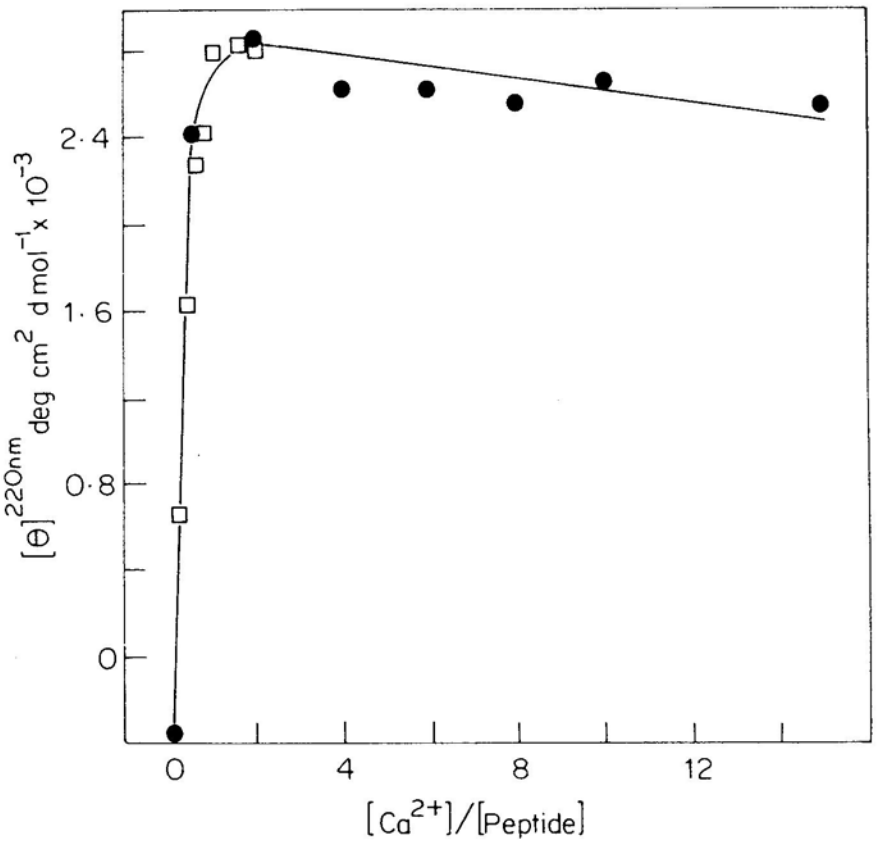

Figure 6. Plot of molar ellipticity of Boc-Pro-Gly-Ala-$NHCH_3$ at 220 nm against the $[Ca^{2+}]$/[Peptide] molar ratio in acetonitrile.

figure 6 to show the approximate stoichiometry of Ca^{2+} binding, which is seen to be 1:1 (ion : peptide), similar to the case of the D-Ala peptide (figure 4).

Both the D-Ala peptide and Gly peptide showed a considerable degree of specificity towards binding the Ca^{2+} ion when compared with other ions like Mg^{2+}, Na^{+} or Li^{+}. This is seen from the extent of the CD spectral changes on ion binding; the data for the D-Ala peptide are shown in figure 7.

The binding of Ca^{2+} by both the D-Ala- and Gly peptides was further monitored by ^{1}H- and ^{13}C-NMR spectroscopy. The data obtained with the D-Ala peptide in acetone-d_6 and their interpretation are given below; the data on the Gly peptide were quite similar and are therefore not described. The chemical shifts of the NH protons of the D-Ala ($N^{2}H$) and Ala ($N^{3}H$) residues and $NHCH_3$ ($N^{4}H$) group in the peptide were monitored during the addition of increasing amounts of Ca^{2+} (in the form of calcium Perchlorate). The data are shown in figure 8. The largest change in the chemical shift is seen to occur for the $N^{3}H$ followed by the $N^{4}H$ protons, both of which are involved in intramolecular hydrogen bonds in the free peptide (figure 9a), while

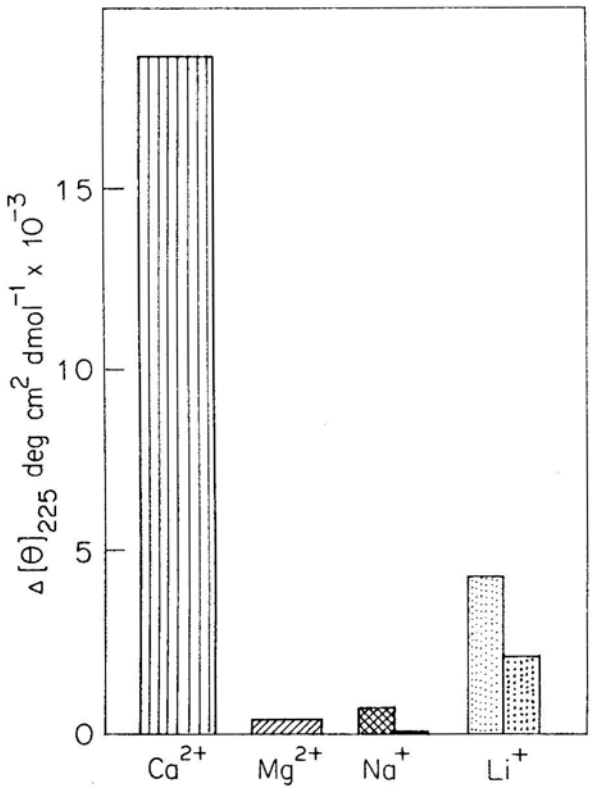

Figure 7. Histograms showing the extent of ion binding, as monitored by change in molar ellipticity at 225 nm, by Boc-Pro-D-Ala-Ala-$NHCH_3$ in acetonitrile. The [ion]/[Peptide] ratio is 4 for Ca^{2+} and Mg^{2+}. The data for Na^{+} and Li^{+} are shown in two ways: in terms of molar concentrations (left side boxes) and ionic strength (right side boxes) of $Ca(ClO_4)_2$.

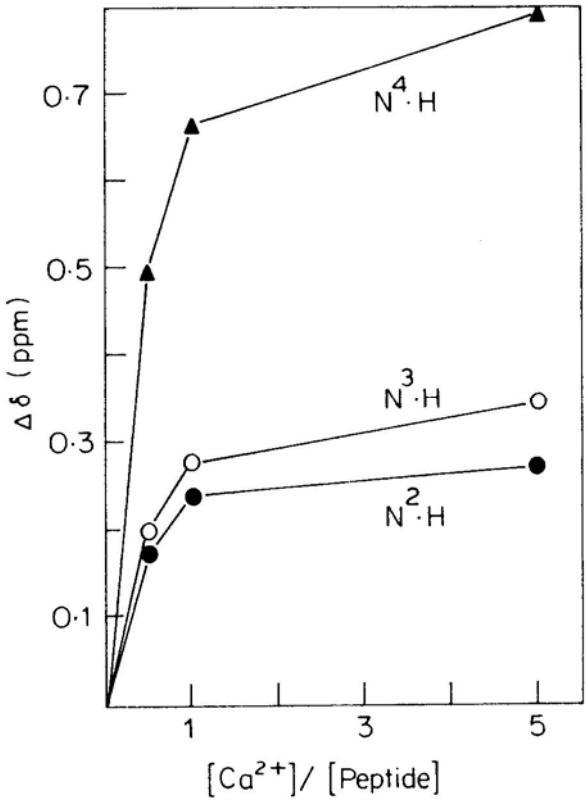

Figure 8. Change in the chemical shifts of the indicated amide protons in Boc-Pro-D-Ala-Ala-$NHCH_3$ on titration with Ca^{2+} in acetone-d_6.

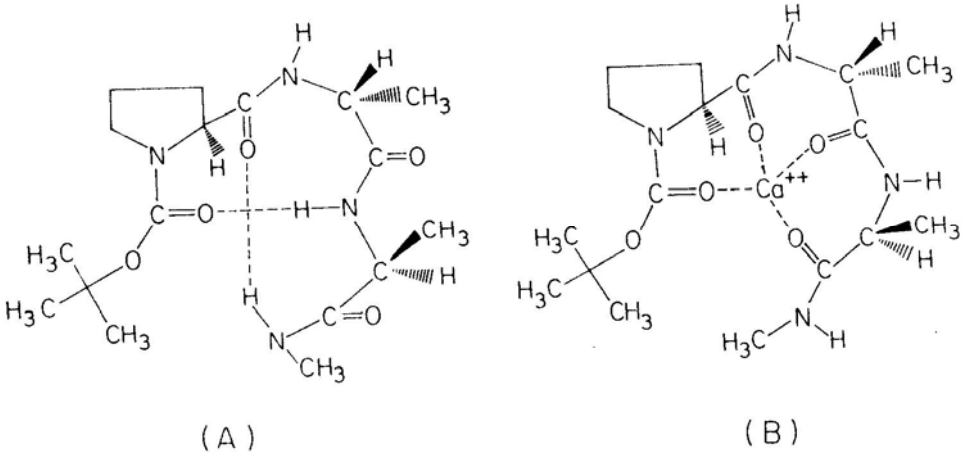

Figure 9. Schematic diagram of the conformation of the Boc-Pro-D-Ala-Ala-$NHCH_3$, (a) before and (b) after complexing with Ca^{2+}.

relatively much less change is seen for the $N^2\underline{H}$ proton which is not hydrogen-bonded. The ^{13}C-NMR data show parallel changes in the chemical shifts of the carbonyl carbons on Ca^{2+} addition. The data, shown in figure 10, reveal a relatively large change in the chemical shift of the $\underline{C}^4 = O$ of the Ala residue. An intermediate but significant change occurs for the $\underline{C}^3 = O$ of the D-Ala residue while the $\underline{C}^1 = O$ and $\underline{C}^2 = O$ chemical shifts are least affected. Recalling that the $N^3\underline{H}$ and $N^4\underline{H}$ are hydrogen bonded, respectively, to the $\underline{C}^1 = O$ and $\underline{C}^2 = O$ groups while the $\underline{C}^3 = O$ and $\underline{C}^4 = O$ are not involved in intramolecular hydrogen-bonding in the free peptide, the observed changes in the N$\underline{H}$ and $\underline{C} = O$ chemical shifts (figures 8 and 10) indicate that an interesting conformational change takes place in the tetrapeptide on calcium binding. This change involves the breaking of the intramolecular hydrogen-bonding in the free peptide (which would account for the significant changes in the $N^3\underline{H}$ and $N^4\underline{H}$ chemical shifts) and the coordination of all the four carbonyl groups to the metal ion in the complex (which involves considerable change in the environment of the $\underline{C}^3 = O$ and $\underline{C}^4 = O$ groups with much less change in $\underline{C}^1 = O$ and $\underline{C}^2 = O$ groups). The proposed conformation of the calcium-binding form of the D-Ala peptide is shown in figure 9b. The remaining two coordination positions of the Ca^{2+} ion in the complex may reasonably be expected to be fitted by the solvent molecules. Our study appears to be the first example of conformation-dependent binding of calcium by small (*i.e.* only four residue-long) linear peptides.

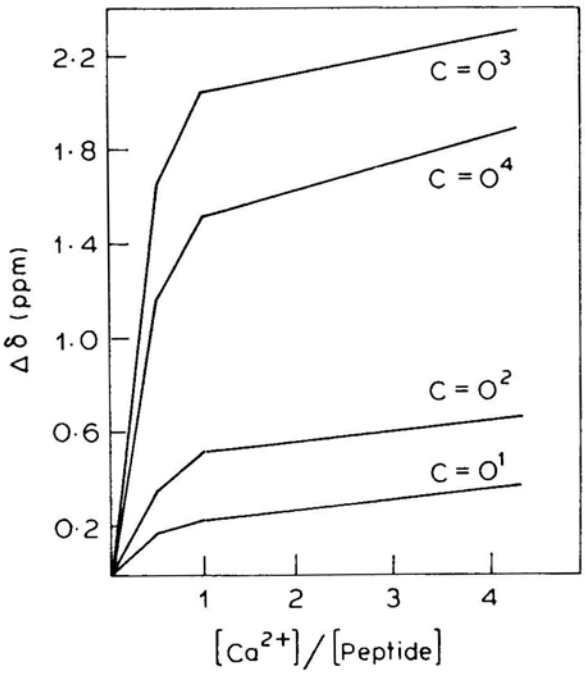

Figure 10. Change in ^{13}C chemical shifts of the indicated carbonyl carbons in Boc-Pro-D-Ala-Ala-$NHCH_3$ on titration with Ca^{2+} in acetone-d_6.

Concluding remarks

Using proline-containing peptides we have, in the two types of studies described above, examined the active site geometry of collagen prolyl hydroxylase and the conformation of calcium-binding regions in protein, respectively. In each case, the *β*-turn is found to play an important role, both structurally and functionally. In the case of proline hydroxylation in collagen, the *β*-turn serves as a structural requirement (in conjunction with the PP-II conformation) in the substrate molecule thereby enabling the function of the hydroxylating enzyme. In calcium-binding proteins, the *β*-turn is seen to be the necessary initial conformation which, on binding calcium, undergoes a transition to a different conformation. It may be noted that conformational changes have been observed in proteins as a result of calcium-binding although these have not been directly correlated with the change taking place in the *β*-turn part of the loop region.

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