Conformational study of a collagen peptide by ^{1}H NMR spectroscopy: observation of the ^{14}N-^{1}H spin-spin coupling of the Arg guanidinium moiety in the triple-helix structure

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Abstract CB2, a CNBr peptide of 36 residues from type I collagen α1(I) chain has been studied by NMR spectroscopy as a function of temperature. At low temperature, the guanidinium protons of Arg_9 showed sharp 1:1:1 NMR triplets around 6.95 ppm, characteristic of ^{14}N coupled protons ($^{1}J_{NH}$=52 Hz) when the quadrupolar relaxation rate is drastically reduced. These spectral characteristics and the low temperature coefficient of the 1:1:1 triplets ($\Delta\delta/\Delta T$ of −3.6 ppb/°C) suggest that the H atoms of the protonated guanidinium moiety of Arg_9 in the triple helix are slowly exchanging with bulk water, most likely involved in hydrogen bonds. On the basis of conformational energy computations on a model segment of type I collagen (Vitagliano, L., Némethy, G., Zagari, A. and Scheraga, H.A. (1993) Biochemistry 32, 7354–7359), similar to CB2, our data could indicate that the guanidinium group of Arg_9 form hydrogen bonds with a backbone carbonyl of an adjacent chain probably by using the N_ε hydrogen, leaving the four N_η hydrogens bound to water molecules that must be in slow exchange with bulk water and that could therefore be considered structural elements of the trimeric α1(I) CB2 triple helix. The behaviour of Arg_9 has been investigated also in terms of equilibrium between random monomer and helical trimer conformations controlled by temperature. The thermal unfolding process was found to be reversible and the melting point resulted to be 17°C.

Key words: Collagen; Collagen peptide; Nuclear magnetic resonance; ^{14}N quadrupolar effect; Triple helix stability

1. Introduction

Collagen triple helix is composed of three extended polypeptide chains having polyproline II-like conformation and being folded around each other in a supercoiled rod-like structure [1–3]. Every third residue is a Gly, small enough to accommodate the close packing of the three staggered chains. Collagen is constituted by $(GlyXY)_n$ repeats in wich X and Y positions are very often occupied by the imino acid proline (Pro) and hydroxyproline (Hyp), respectively. It has been demonstrated that the sequence GlyProHyp is the most stabilising triplet for the triple-helix conformation. By using host-guest peptides such as acetyl-$(GlyProHyp)_3$-$GlyXY(GlyProHyp)_4GlyGly$-amide, it has been very recently demonstrated that the incorporation of a GlyProArg guest triplet confers stability as great as that of the GlyProHyp peptide unit [4]. Upon heating, $(GlyProHyp)_8$ and its derivative containing the GlyProArg triplet showed a sharp transition with a common melting temperature value, T_m, of 45.5°C. The preference of Arg and Hyp for the Y position [5] suggested to the authors a similarity in their stabilising effect: they proposed a mechanism of stabilisation consisting of a possible interaction of the guanidinium group of Arg with the peptide backbone and/or with the water network in the triple helix. According to these authors, the guanidinium moiety might participate in intrachain hydrogen bonding to the backbone carbonyl groups or to the Arg carbonyl group of a neighbouring chain, in agreement with a previous computer calculation reported by Vitagliano et al. [6].

In the present study, the behaviour is reported of an Arg residue in Y position of a real segment of a type I collagen, the fragment CB2 from the α1(I) chain (see Scheme 1), as studied by NMR spectroscopy as a function of temperature.

2. Materials and methods

Peptides CB2 (residues 4–39 of the α1(I) chain) has been prepared and purified from acid-soluble type I collagen of calf skin by a combination of gel filtration and reverse-phase chromatography, as will be described elsewhere. The sequence of the 6 N-terminal residues for the peptide gave a single sequence and an estimated purity of ≥95%.

^{1}H-NMR spectra have been acquired on a Bruker DMX-500 spectrometer, by using a 6 mM sample of CB2 in 50 mM tetradeuteroacetic acid, pH 2.9. Chemical shifts were referred to TSP. The curve fitting of the area variations as a function of temperature was performed with a sigmoid logistic function: the inflection point represents the midpoint temperature of the monomer/trimer conformational transition.

3. Results

CB2 is a CNBr peptide from the α1(I) chain of type I collagen. It contains 36 residues, 33% being imino acids. Circular dichroism and NMR spectra showed that CB2 molecules fold into a triple helical conformation at low temperature as other collagen peptides do and in agreement with previous reports on CB2 [9–11]. Arg_9 is in position Y and represents a suitable probe to experimentally verify the hypothesis set forth by Yang et al. [4] concerning the possible stabilising effect of the Arg residue on the collagen structure.

^{1}H-NMR experiments performed on a sample of 6 mM CB2 in 50 mM deuteroacetic acid, at pH 2.9, are reported in Fig. 1 as a function of temperature. At temperatures below 23°C an interesting 1:1:1 pattern (separation of 52 Hz) of sharp resonances can be observed around 6.95 ppm almost overlapping a minor, slightly broader, 1:1:1 triplet, while the rest of the spectrum becomes broader and broader upon de-

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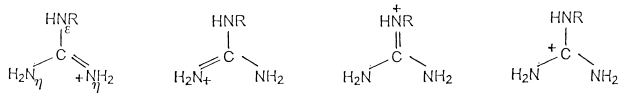
res. n.	4	9	11	39
CB2	GPS GPR GLO GPO GAO GPQ GFQ GPO GEO GEO GAS GPM*			

Scheme 1. Amino acid sequence of CNBr peptides from the α1(I) chain of type I collagen calf skin. Human and bovine sequences of both peptides are identical [7,8]. M* stands for homoserine residue deriving from the cleavage of methionyl residues with CNBr.

creasing temperature, due to the increasing content of the triple helix. In a previous paper [12] some of us have observed the same spectral pattern in a small peptide dissolved in dimethylsulfoxide and ascribed it to the ^{14}N coupled guanidinium protons of the arginyl residue, which can be represented by the following mesomeric forms:



The broader triplet was tentatively assigned to the NH_ε proton of the guanidinium group on the basis of its area compared to that of the major triplet (area ratio about 1:4). A deeper analysis of the minor triplet revealed the presence of a 1:1:1 triplet of 1:1:1 triplets (Fig. 2), most likely deriving from a $^1J_{N\varepsilon H\varepsilon} = 52$ Hz and a $^3J_{N\eta H\varepsilon} = 1.7$ Hz (with one of the two η nitrogens), respectively.

^{14}N-^1H spin-spin couplings are seldom observed due to quadrupolar relaxation which induces severe resonance broadening [13]. The influence of the quadrupole moment on the nuclear relaxation is given by:

$$1/T_Q = 3/8 \times (1 + \varepsilon^2/8) \times (e^2qQ/h)^2 \times \tau_c$$

where $1/T_Q$ is the quadrupolar relaxation time; e^2qQ is the quadrupole coupling constant dependent upon the electric field gradient at the nucleus, eq, and the nuclear quadrupole moment, eQ; the factor ε describes the deviation of the electric field gradient from axial symmetry; τ_c is the molecular correlation time. If relaxation is fast, the guanidinium protons see only a time average of the possible ^{14}N spin states ($m = 1$, 0 and -1) and the expected splitting into a 1:1:1 triplet vanishes. For the arginyl residue in water, in fact, the result is usually an extensive broadening for the proton signals. Indeed, the ^{14}N-^1H are detectable only in the presence of a positive charge that lowers the field gradient at the nitrogen [14]. In the extreme narrowing limit, the efficiency of quadrupolar relaxation is directly proportional to the correlation time, which is, in turn, directly proportional to the solution viscosity, in agreement with the Stokes equation ($\tau_c = V\eta/kT$, where V is the volume of a spherically symmetric molecule, η is the viscosity of the medium, k is the Boltzmann's constant and T is the temperature). It follows that ^{14}N line width increases by decreasing the temperature and increasing the viscosity of the medium. In fact, the use of supercritical fluids allowed the experimental measure of some spin-spin couplings in cases where one or two of the nuclei involved were quadrupolar. A nice example was reported by Robert and Evila [15] for ammonia in water at 28°C in supercritical ethylene. In that case, they obtained a strong signal enhancement probably due to a specific interaction of NH_3 with water that increases the charge symmetry around the nitrogen, resulting in a less efficient net relaxation.

In the case of CB2, at low temperatures, where the peptide has a triple helix conformation, the quadrupolar relaxation of the guanidinium nitrogens seems to be suppressed, even if the viscosity of the medium is expected to increase (due to both the effect of temperature and the presence of trimer) and the correlation time to become longer.

Moreover, the line shape of the guanidinium protons can further be broadened by the exchange process with water. Indeed, the labile protons may become decoupled from ^{14}N if the exchange rate constant (k) is such that $2\pi k \gg J_{NH}$. For CB2 the 1:1:1 triplet separation of 52 Hz indicates that k is not much greater than 0.1 min^{-1}, a value that can be taken as characteristic of relatively slowly exchanging guanidinium protons.

In CB2, the analysis of the temperature coefficient of the 1:1:1 triplets indicated that the guanidinium moiety was solvent shielded in the triple helix, being characterised by a $\Delta\delta/\Delta T$ of -3.6 ppb/°C, which suggests a possible involvement of guanidinium protons in hydrogen bonds. Upon increasing temperature, the unfolding process was accompanied by the area decrease of the 1:1:1 guanidinium triplets in favour of the broad signal at about 6.5 ppm (see Fig. 1), typical of the arginyl group in the monomeric form of collagen.

The thermal transition from trimer to monomer was monitored following the area variation of the highest field component of the 1:1:1 guanidinium triplets with respect to the methyl resonances of Leu_{11} at 0.8 ppm. These signals belonged to the δ protons of both assembled and unassembled forms of the leucyl residue and kept a constant area during the whole thermal transition, the chemical shifts of the two forms being very close. The melting point resulted to be 17°C (Fig. 3) and the unfolding process was found to be fully reversible, since unfolding and refolding cycles were performed repeatedly during NMR experiments, even if the equilibrium was reached slowly.

4. Conclusions

We propose here that our data could have a structural valence. Vitagliano et al. [6] proposed a model on the N-terminal segment of type I collagen triple helix. This trimeric segment incorporates two copies of CB2. On the basis of conformational energy computations, the authors showed that the triple helix is stabilised by side-chain interactions and that the guanidinium group of the arginyl residues contributes to stability by means of hydrogen bonds with backbone carbonyl groups.

According to this model, we think that in CB2 the $N_\varepsilon H$ group of the guanidinium moiety acts as hydrogen donor to a backbone carbonyl of an adjacent chain. This configuration introduces additional constraints due to the planarity and geometry of the protonated guanidinium group. It follows that the two $N_\eta H_2$ groups point out from the triple helix (see Fig. 1 in [6]). The side chains of spatially adjacent residues (in particular Leu_{11}) are not able to interact with the NH_2 groups through side-chain side-chain hydrogen bonds or salt bridges. It follows therefore that these NH_2 groups are interacting with water molecules. However, as mentioned

above, one of the characteristics of the NMR signal of the guanidinium protons is their slow exchange with their surroundings ($\Delta\delta/\Delta T = -3.6$ ppb/°C). The conclusion is therefore that the water molecules interacting with the guanidinium NH_2 groups are, in their turn, slowly exchanging with bulk water. They are therefore to be considered as structural elements of the triple helix formed by peptide CB2. In other words, the arginyl guanidinium group can act as a coordination centre for water molecules, as the hydroxyl group of hydroxyproline does. Brodsky and coworkers [4] made a similar hypothesis in their studies on the determinants of stability for the triple helix.

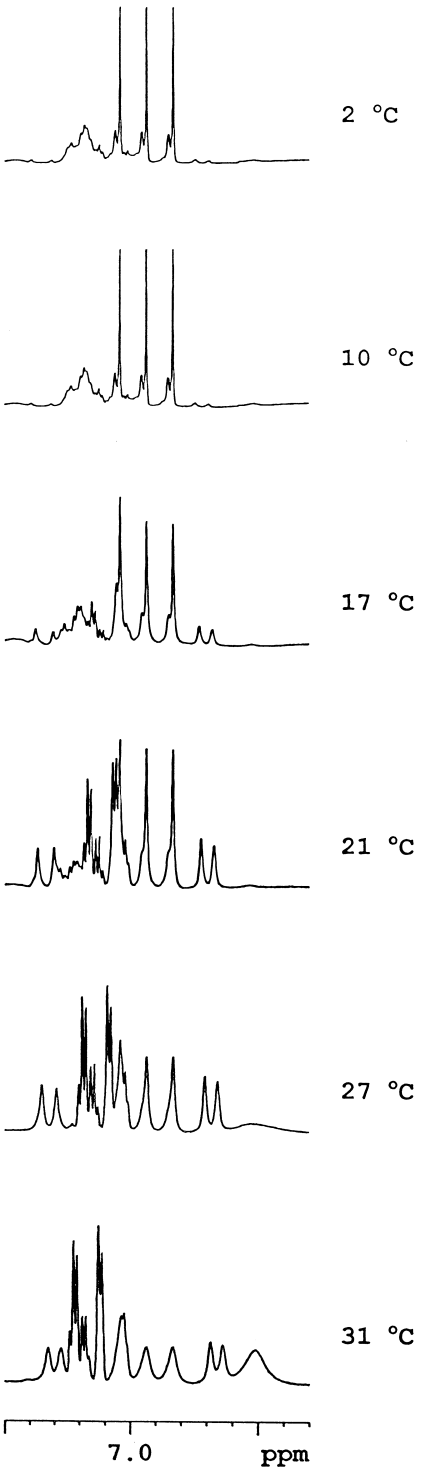


Fig. 1. ^{1}H-NMR spectra of CB2 in 50 mM deuteroacetic acid, pH 2.9, at various temperatures from 2 to 31°C: region containing the guanidinium resonances of Arg_9.

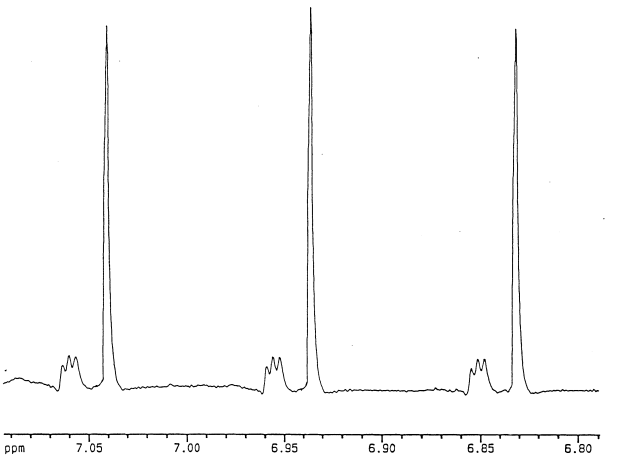


Fig. 2. ^{1}H-NMR spectra of CB2 in 50 mM deuteroacetic acid, pH 2.9, 2°C: expanded region containing the guanidinium resonances of Arg_9. Resolution enhancement was achieved by multiplying the free induction decay by a Gaussian function characterized by a line broadening factor (LB) of −2 and a maximum (GB) of 0.4.

CB2 contains only one arginyl residue. We do not know whether all arginines in position Y have the same behaviour or whether the sequence influences it. We are currently analysing peptide α1(I) CB4 which contains four arginyl residues. Our preliminary results show that a low NMR signal for the guanidinium group is present. We do not know yet whether this low signal intensity is due only to a percentage of trimers for CB4 lower than for CB2.

The hydration structure for a collagen peptide was clearly demonstrated in the ordered packing of the peptide crystal

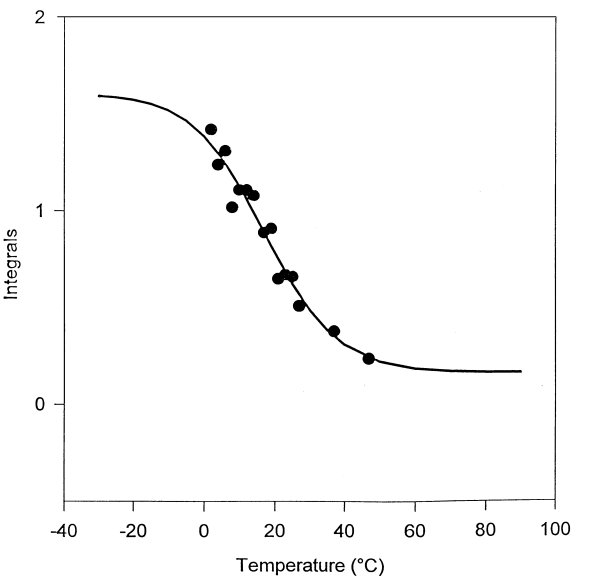


Fig. 3. ^{1}H-NMR thermal transition profile determined by monitoring the area variation of the high field component of the 1:1:1 triplets of the guanidinium protons of Arg_9 with respect to that of the methyl resonance of Leu_{11} at 0.8 ppm.

structure [16]. In the present paper, the experimental NMR evidence we have obtained for free collagen molecules in solution was interpreted by us with the presence of water as a structural element of the collagen triple helical conformation.

We await independent confirmation of our interpretation. If it is wrong, our data maintain the characteristics of a set of constraints for any collagen model.

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