Int. J. Peptide Protein Res. 16, 1980, 241–244

REACTION OF BOVINE PANCREATIC RIBONUCLEASE A WITH 6-CHLOROPURINE RIBOSIDE 5′-MONOPHOSPHATE

Nuclear Magnetic Resonance Studies of the Corresponding S-Peptide

XAVIER PARÉS,* PERE PUIGDOMÈNECH† and CLAUDI M. CUCHILLO

Department of Biochemistry, Faculty of Sciences and Institute of Fundamental Biology, Autonomous University of Barcelona, Barcelona, Spain

Received 26 October 1979, accepted for publication 23 April 1980

The n.m.r. spectra of native S-peptide and of S-peptide II, a derivative obtained after reaction of bovine pancreatic ribonuclease A with 6-chloropurine riboside 5′-monophosphate, both in D_2O and in urea-d_4, were obtained with a 270 MHz Fourier transform spectrometer. From these spectra it was possible to assign most of the proton resonances of the peptide and the position of the labelling group, the α-NH_2 of Lys-1, was also deduced.

Key words: 6-chloropurineriboside 5′-monophosphate; n.m.r.; S-peptide; ribonuclease A.

Parés *et al.* (1978, 1980) showed that cl^6-RMP first interacts and then reacts with ribonuclease A giving a major nucleotydylribonuclease derivative, derivative II, the reaction site being an amino group of Lys-1. In the present work the S-peptide II was studied by means of n.m.r. spectrometry to confirm the reaction site and to elucidate which of the two NH_2 groups (α or ε) of Lys-1 had been substituted. To this end the spectra of the S-peptide II and native S-peptide are compared, both in urea-d_4 and in D_2O.

Although native S-peptide has been extensively studied by means of n.m.r. spectrometry no complete assignment of most of the resonance signals has been reported. In the present work a detailed assignment of most of the resonance signals was obtained by using a 270 MHz spectrometer with Fourier transform.

Present addresses: *Biophysics Research Laboratory, Peter Bent Brigham Hospital, Harvard Medical School, Boston, MA 02115, U.S.A.
†Max Planck Institut für Molekulare Genetik, Ihnestrasse 63–73, 1 Berlin 33, W. Germany.

Abbreviations: cl^6-RMP, 6-chloropurine-9-β-D-ribofuranosyl 5′-monophosphate. DSS, sodium 2,2-dimethyl-2-silapentane sulphonate.
Definitions: Ribonuclease S, a derivative of ribonuclease A obtained by specific cleavage with subtilisin of the peptide bond between amino acid residues 20–21. S-peptide, the ribonuclease S fragment containing amino acid residues 1–20. S-protein, the ribonuclease S fragment containing amino acid residues 21–124. S-peptide II, the S-peptide corresponding to derivative II.

MATERIAL AND METHODS

Nuclear magnetic resonance spectra of proton resonances were recorded in a Bruker WH-270 Fourier transform spectrometer at 270 MHz. Samples were prepared in H_2O and freeze-dried, and were dissolved directly in ^2H_2O

0367–8377/80/040241–04 $02.00/0

at a concentration of 2.5 mg·ml^{-1}. The pH was measured directly in the n.m.r. tube using a microelectrode. No correction of pH was done for heavy water solutions. If necessary, the pH was adjusted by adding 0.1 M-NaO^{2}H or 0.1 M-^{2}HCl. Urea-d_4 (MSD isotopes) was added as a solid and the final concentration was calculated from the final volume. Samples of 0.4 ml were introduced in 5-mm standard tubes and the spectra were obtained at 20° by transforming the sum of 10000 free induction decays accumulated over periods of about 2.5 h. Shifts were measured relative to sodium 2,2-dimethyl-2-silapentane sulphonate and integration of peak areas was carried out by weighing. All spectra were systematically treated with an exponential multiplication for resolution enhancement.

Derivative II, and the corresponding S-peptide, were prepared according to Parés *et al.* (1980).

RESULTS AND DISCUSSION

Assignment of S-peptide resonances

The S-peptide holds 144 protons, of which 41 are linked to either nitrogen or oxygen atoms and are exchangeable with the deuterium of the solvent, D_2O; the remaining 103 protons give the spectra shown in Fig. 1 (high field) and Fig. 2 (low field). The assignment of the S-peptide proton resonances was first made on the spectra in 6 M-urea-d_4 solution (Figs. 1D and 2D). In these conditions only the S-peptide primary structure determines the spectrum signals, thus allowing the use of the proton chemical shifts of the random coil polypeptides reported by McDonald & Phillips (1969). Besides these values the previous assignments of Finn *et al.* (1972) and Ihnat (1972) were also considered in the spectrum interpretation. The acetate ion resonance at 1.98 p.p.m./DSS was used as an internal reference.

Some characteristics in the spectrum which cannot be attributed to the S-peptide must be noticed: The broad small signal near 1 p.p.m. is originated by methyl resonances of S-protein traces. The acetate resonance is the sharp peak detected at 1.98 p.p.m. The 4.5–6 p.p.m. spectrum region is not indicated since the HDO signal and the urea-d_4 residual protons appear in this region. This represents the loss of some HC_α signals, which are therefore not included in the interpretation of the present spectrum.

Study of the nucleotide resonances in the S-peptide II spectra

Figs. 1A, 1B, 2A and 2B show the resonance spectra of the S-peptide II in urea and D_2O solutions. The resonances of the nucleotide that labels the peptide are clearly distinguished in the aromatic region of the spectra (Fig. 2). The assignment of the proton resonances was done from the data of Ts'O (1969, 1974), and from the spectrum of 6-methylaminopurine riboside (not shown), which would be a good model of the labelling group assuming that the nucleotide is linked to an NH_2 group of the peptide (Parés *et al.*, 1980).

Some features can be noticed when comparing the spectrum of the S-peptide II with that of the native S-peptide. The H-2′ resonance was overlapped under the HDO signal. The low intensity of the H-2 signal in the S-peptide II may be due to a low relaxation time of this proton that may produce an apparent loss of intensity when using Fourier transform techniques. The 6-methylaminopurine spectrum in a 60 MHz instrument (Perkin-Elmer R12A) shows the same intensity for the H-2 and H-8 resonances, supporting this interpretation. The H-2 resonance is a double peak in D_2O and a single peak in the spectrum of the S-peptide II in urea, but the downfield shoulder shows it is not homogeneous. Therefore it is possible to conclude that there are two different forms of the label in the S-peptide II. The chemical shift difference between the two H-1′ doublets is 0.07 p.p.m. and that between the H-2 singlets −0.04 p.p.m. These values fit very well with the chemical shift differences between purine nucleoside and purine nucleotide resonances (Ts'O, 1974). The homogeneity of the H-8 resonance may be explained by a distant situation of this proton from the 5′-phosphate, due to the peptide steric hindrance, since changes in the H-8 resonance between nucleoside and nucleotide would occur mainly be-

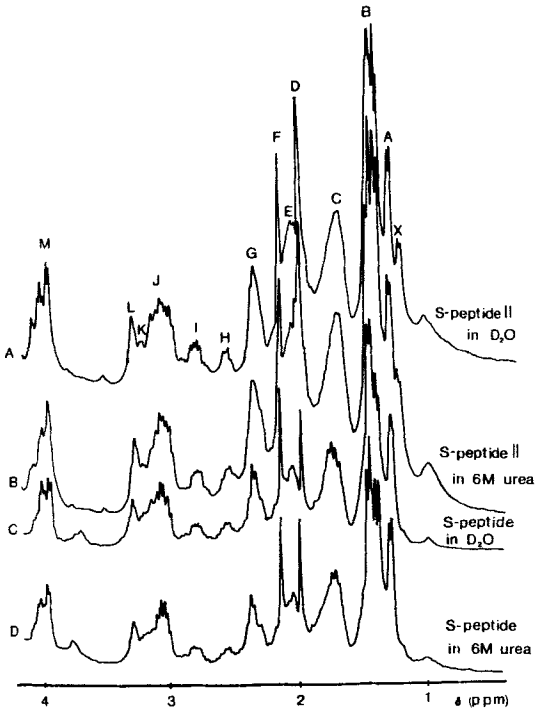

FIGURE 1

High field n.m.r. spectra of S-peptide II and native S-peptide. (A) S-peptide II in D_2O. (B) S-peptide II in 6 M-urea. (C) Native S-peptide in D_2O. (D) Native S-peptide in 6 M-urea. The assignments are as follows: A (δ 1.2–1.3) are 2 CH_3 from Thr-3 and 17. B (δ 1.3–1.55) are 5 CH_3 from Ala-4, 5, 6, 19 and 20 and 2 γCH_2 from Lys-1 and 7. C (δ 1.6–1.9) are 1 γCH_2 from Arg-10, 2 βCH_2 from Lys-1 and 7 and 2 δCH_2 from Lys-1 and 7. D + E (δ 1.9–2.15) are 5 βCH_2 from Glu-2 and 9, Arg-10, Gln-11 and Met-13. F (δ 2.15) is 1 CH_3 from Met-13. G (δ 2.25–2.45) are 3 γCH_2 from Glu-2 and 9 and Gln-11. H (δ 2.5–2.65) is 1 γCH_2 from Met-13. I (δ 2.7–2.85) is 1 βCH_2 from Asp-14. J (δ 2.95–3.15) is one proton of 1 βCH_2 from Phe-8 and 2 ϵCH_2 from Lys-1 and 7. K (δ 3.15–3.25) is 1 βCH_2 from His-12. L (δ 3.25–3.35) is one proton of 1 βCH_2 from Phe-8 and 1 δCH_2 from Arg-10. M (δ 3.9–4.05 and 4.1–6) are 3 βCH_2 from Ser-15, 16 and 18, 2 βCH from Thr-3 and 17 and 20 αCH.

cause of the direct interaction of this proton with the phosphate group. It can be concluded that the two forms of the label in the S-peptide II are nucleotide and nucleoside in an approximate ratio of 4:1 (calculated from the peak area). The loss of the phosphate group in a fraction of the S-peptide II was also demonstrated by high-voltage electrophoresis of the tryptic digestion peptides of the S-peptide II (Parés *et al.*, 1980).

By comparing the area of the H-8 resonance with one proton area of the S-peptide (i.e. $\frac{1}{5}$ of the area of the Phe resonance) it can be concluded that approximately 60% of the S-peptide II preparation studied is labelled with the nucleotide, the remaining 40% being native S-peptide.

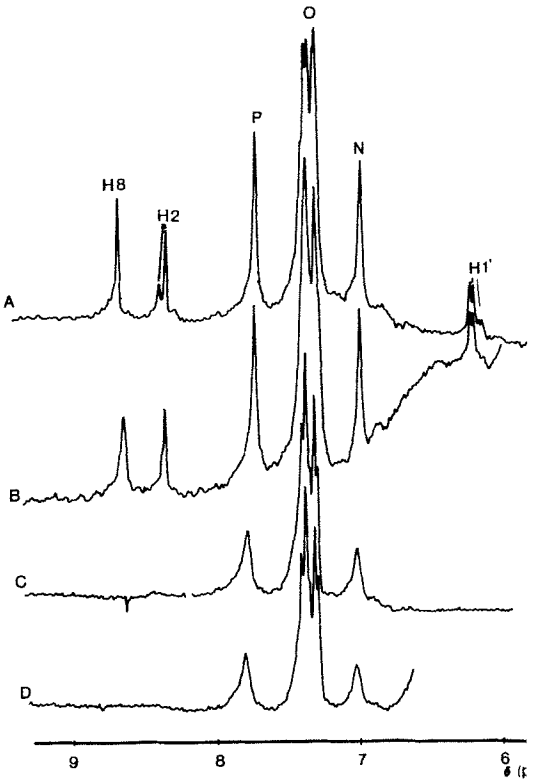

FIGURE 2

Low field n.m.r. spectra of S-peptide II and native S-peptide. (A) S-peptide II in D_2O. (B) S-peptide II in 6 M-urea. (C) Native S-peptide in D_2O. (D) Native S-peptide in 6 M-urea. The assignments are as follows: N (δ 6.98) is 1 CH-4 from His-12. O (δ 7.25–7.5) are 5 CH(aromatic) from Phe-8. P (δ 7.75) is 1 CH-2 from His-12. The peaks labelled H1', H2 and H8 correspond to the protons of the labelling nucleotide in positions 1', 2 and 8, respectively.

Localization of the labelling site

If the spectra of S-peptide II and native S-peptide in 6 M-urea (Figs. 1B, 1D, 2B and 2D) are compared, the spectral changes may be interpreted as produced by the covalent linkage of the nucleotide in the S-peptide II.

In comparison with the native S-peptide spectrum the S-peptide II spectrum presents the following main differences: 1) a new doublet (X) at 1.21 p.p.m., 2) a relative decrease of signal A intensity; 3) variation of signal B multiplicity and 4) differences in multiplicity of signals I and J. Other variations cannot be attributed to the presence of the label, i.e. peak D changes, because of the greater quantity of acetate ion in the S-peptide II preparation. Differences in multiplicity of C, F and O can be due to a lower resolution of the S-peptide II spectrum.

The appearance of the new doublet (X) at 1.21 p.p.m. must be considered in relation to the decrease in the area of the doublet A at 1.26 p.p.m. that corresponds to threonine-CH_3. It is evident that part of this methyl group is shifted 0.05 p.p.m. upfield in the S-peptide II.

Variation in resonances B (lysine γ-CH_2) and J (lysine ϵ-CH_2) may be produced by the presence of the label linked to an amino group of a lysine as already proposed from other experiments (Parés *et al.*, 1980). The different shielding of all these resonances could be originated from electronic density change due to the covalent linkage, change in local magnetic field due to the proximity of the aromatic ring of the label, or both.

To elucidate which group (α or ϵ) the label is linked to, the n.m.r. spectrum of the model 6-methylaminopurine riboside can be useful. The CH_3 signal of this nucleoside should appear some tenths of p.p.m. downfield from the ϵ-CH_2 resonance, if it is assumed that the label is linked to the ϵ-NH_2 of Lys-1. The CH_3 of the model appears in the spectrum at 3.4 p.p.m., but no new resonance is detected near this region in the S-peptide spectrum. Therefore the purine base is not linked to the ϵ-NH_2. It is then concluded, by exclusion, that the reaction site is the α-NH_2 of Lys-1.

The best protons to diagnose a substitution in the α-NH_2 would be the β-CH_2 of Lys-1, but their resonance frequency is in a region overlapping with many other protons (band C) and it is not possible to draw any direct conclusion from the spectrum. The resonance X of the threonine can only be explained by a close proximity between Thr-3(CH_3) and the purine ring linked to Lys-1 which would allow the methyl to fall in the induced magnetic field, even in the S-peptide random coil conformation (Fig. 1B). This supports the α-NH_2 of Lys-1 as the substitution site as it is much closer to the methyl group of Thr-3 than the ϵ-NH_2 of Lys-1. Model building shows the feasibility of this explanation.

There are some differences between the spectra of the peptides in D_2O and in 6 M-urea and studies are in progress to see whether they are due to changes in conformation or to solvent effects.

ACKNOWLEDGEMENTS

The authors are grateful to Drs. E.M. Bradbury and C. Crane-Robinson of the Biophysics Lab. (Portsmouth Polytechnic, U.K.) for the use of the 270 MHz spectrometer and to the Departament de Química Orgànica, Facultat de Ciències, Universitat Autònoma de Barcelona, for the use of the 60 MHz spectrometer. X.P. received a fellowship from the Ministerio de Educación y Ciencia.

REFERENCES

Finn, F.M., Dadok, J. & Bothner-By, A.A. (1972) *Biochemistry* **11**, 455–461

Ihnat, M. (1972) *Biochemistry* **11**, 3483–3492

McDonald, C.C. & Phillips, W.D. (1969) *J. Am. Chem. Soc.* **91**, 1513–1521

Parés, X., Arús, C., Llorens, R. & Cuchillo, C.M. (1978) *Biochem. J.* **175**, 21–27

Parés, X., Llorens, R., Arús, C. & Cuchillo, C.M. (1980) *European J. Biochem.* **105**, 571–579

Ts'O, P.O.P. (1969) in *Fine Structure of Biological Macromolecules* (Fasman, G.D. & Timasheff, S.N., eds.), p. 49, M. Dekker, New York

Ts'O, P.O.P. (1974) in *Basic Principles in Nucleic Acid Chemistry* (Ts'O, P.O.P., ed.), vol. I, pp. 453–584, Academic Press, New York

Address:
Prof. *C.M. Cuchillo*
P.O. Box 8
Universitat Autònoma de Barcelona
Bellaterra
Spain