CHARACTERIZATION OF AN ACETYL GROUP AT THE *N*-TERMINAL POSITION OF α-AMYLASE BY NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

P. COZZONE and G. MARCHIS-MOUREN

Institut de Chimie Biologique, Faculté des Sciences, Place V. Hugo, 13-Marseille (3°), France

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1. Introduction

Porcine pancreatic α-amylase is a monomeric enzyme consisting of a single polypeptide chain, about 470 residues long [1]. Previous chemical studies [2] have shown that the *N*-terminal end of this chain is blocked by an acetyl group. However, the characterization of an acetyl as DNP-acetyl hydrazide is not very good, because of the poorly specific absorption spectrum of the compound. A more accurate identification is therefore necessary. Nuclear magnetic resonance (NMR), widely used in structural studies of biological molecules, [3] is specially precise in the determination of acetyl groups since the 3 corresponding protons give a singlet signal due to uncoupling. However this technique was unsuccessful for total amylase due to limited sensitivity (see Discussion); it was thus necessary to cleave the amylase chain by cyanogen bromide; 9 fragments were obtained [4]. End group determinations were carried out on these peptides; only one possessed no free *N*-terminal group [5]. This peptide numbered VIII was analysed by NMR.

2. Experimental procedure

N-Acetylated samples were dissolved in a 1/1 mixture of formic acid and deuterium oxide and evaporated under vacuum. This process was repeated 3 times. The standard sample tube filled with 500 μl was used routinely; when necessary a spherical microcell (capacity: 35 μl) was employed. All spectra were recorded at 30° on a Varian Associates model HA 100 NMR Spectrometer operating at a frequency of 100 MHz. Tetramethylsilane (TMS) was used as an internal reference for all chemical shift measurements, using the scale. The sweep width of all spectra was 500 Hz from the TMS signal (sweep time 250 sec).

N-Acetyl amino acids and *N*-acetylglucosamine were obtained from Fluka and Sigma, formic acid was purchased from Fluka A.G. and D_2O from Merck. The GAR Histone *N*-acetyl tripeptide was a gift from Dr. Sautière.

3. Results and discussion

Various *N*-acetylated compounds were used as models. The NMR spectra of 4 *N*-acetylaminoacids and *N*-acetylglucosamine are given in fig. 1. As shown a singlet signal is obtained at about 7.90 ppm in each case.

For a better comparison with the *N*-blocked amylase peptide, NMR analysis of the *N*-acetyl tripeptide obtained from tryptic digest of the GAR histone was carried out. The sequence of this peptide: *N*-acetyl-Ser-Gly-Arg, is known from the work of Dr. P. Sautière [6]. The various amino acids can be identified in the spectrum: Ser (H_α and H_β giving a second order multiplet at τ = 5.82 ppm), Gly (the two uncoupled H_α giving a singlet at τ = 5.94 ppm) Arg (multiplet of H_β and H_γ from τ = 7.70 to τ = 8.35 ppm; triplet of H_δ at τ = 6.33 ppm; triplet of H_α at τ = 5.74 ppm), while the acetyl signal comes out as a single band at the expected value (fig. 3a). The profile obtained with peptide VIII (fig. 3b) is not so simple due to the 26 amino acid residues contained in this peptide and to higher magnification. However,

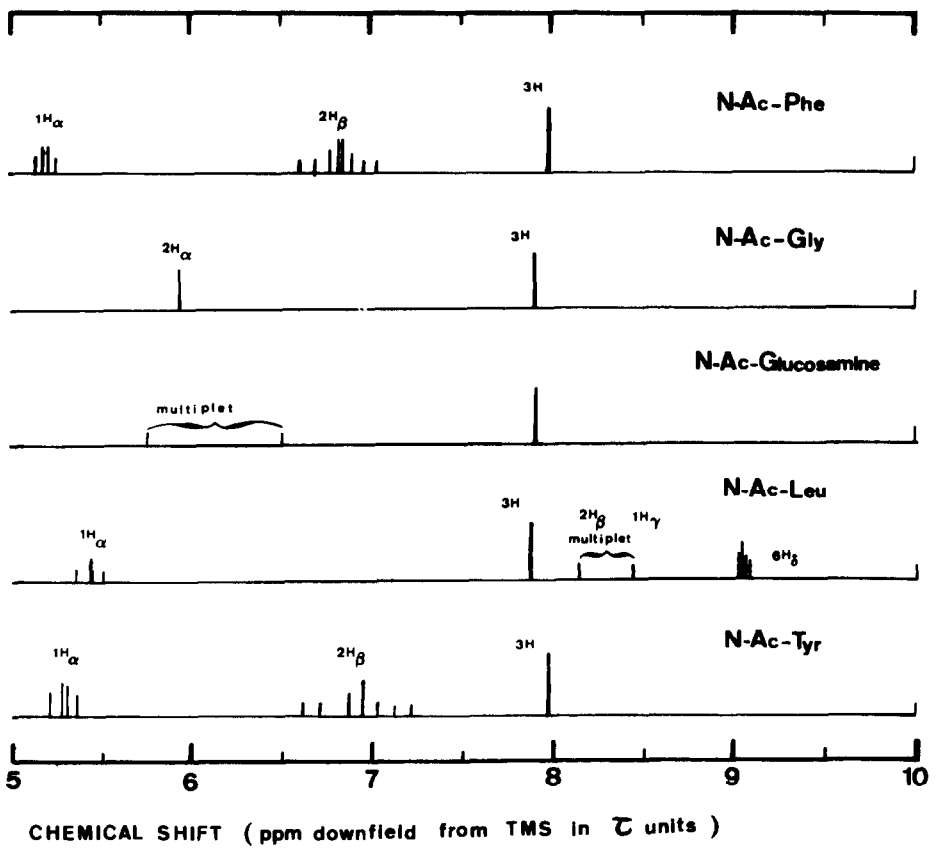

Fig. 1. NMR spectra of some *N*-acetylated biological compounds. The spectra were obtained at 100 MHz; solutions 0.1 M HCOOH – D_2O (50:50, v/v) were used. A partial attribution of signals is given. The acetyl protons appear from $\tau = 7.90$ to $\tau = 7.97$ ppm.

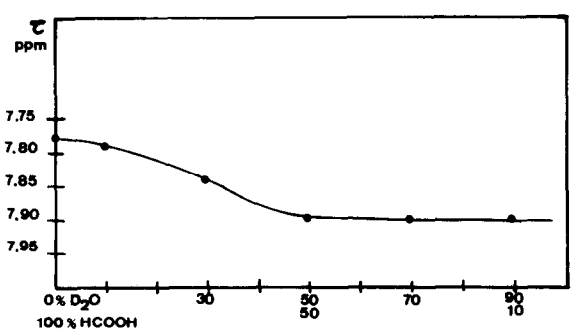

Fig. 2. Chemical shift (in τ units downfield from TMS) of acetyl protons of *N*-acetyl-leucine as a function of D_2O: HCOOH ratio.

despite this background, a high singlet signal is obtained at the acetyl frequence. All other peptides from amylase give a negative result.

Acetyl group determination by NMR is very convenient since the samples can be recovered after analysis. However, two facts should be kept in mind: 1) the specificity of the signal; 2) the sensitivity. The acetyl chemical shift may slightly vary depending on the solvent and on the amino acid next to the acetyl group. Possible effect due to the amino acid side chain has already been examined (fig. 1). In order to check the extent of variation due to the solvent, the *N*-acetyl leucine spectrum was recorded at different formic acid concentrations in deuterium oxide (fig. 2). No change was found between 0-50% formic acid although a small shift of the signal position occurred between 50-100%. The acetyl singlet is then very spe-

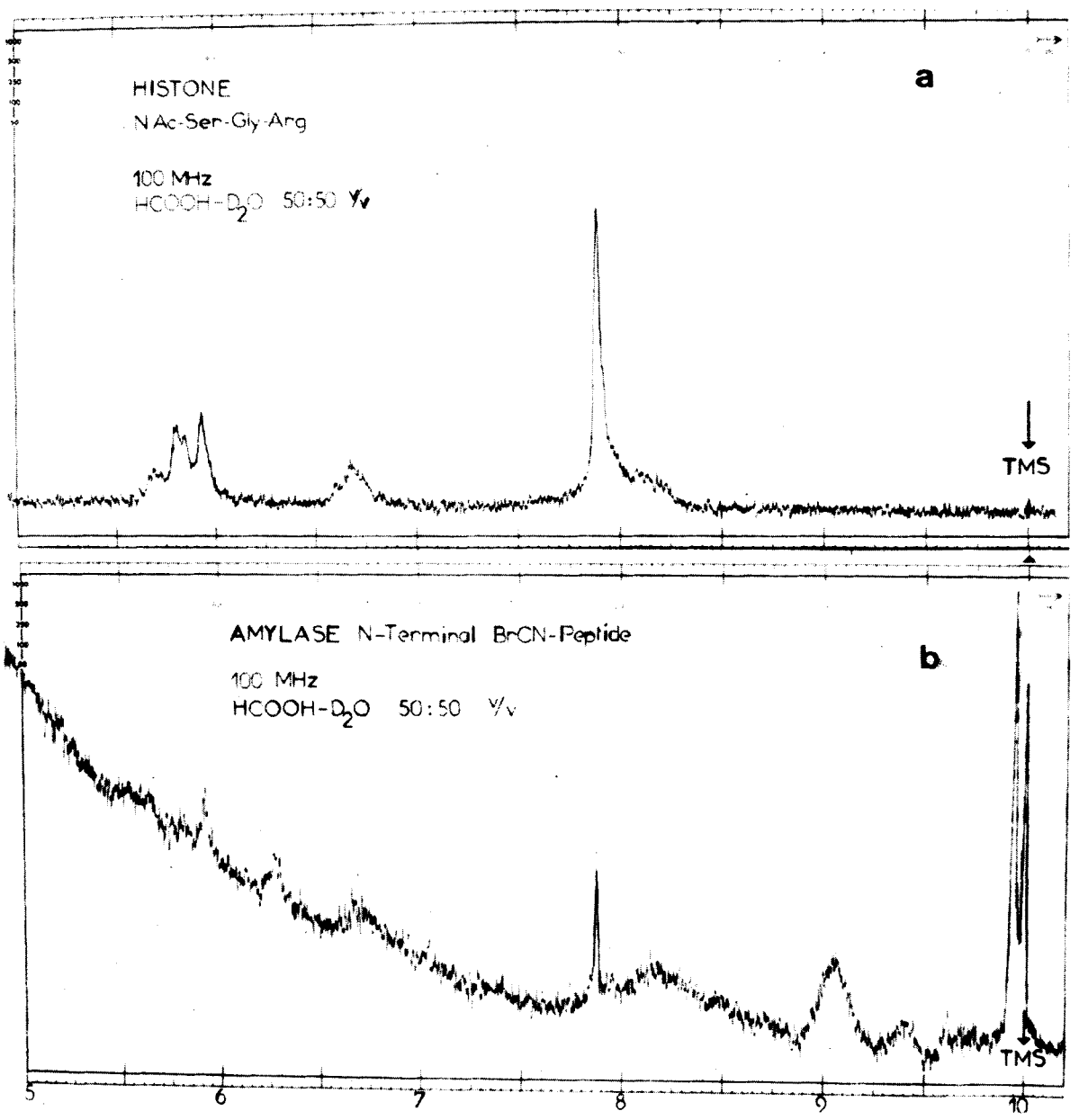

Fig. 3. NMR spectra of *N*-terminal peptides of porcine thymus GAR histone (3a) and porcine pancreatic α-amylase (3b). The microcell was used instead of the standard sample tube. The absorption near the TMS signal on the spectrum 3b is due to an impurity in the TMS solution.

cific and in peptide analysis cannot be mistaken for the only singulet signal given by methionine in the same range.

About 1-2 μmoles of acetyl peptides are needed when using the microcell, but the sensitivity can be increased more than ten times by using the computing system CAT C1024. However, the sensitivity decreases with increasing molecular weight of the peptides, because of the multiplicity and width of the signals due to various side chain protons.

In conclusion, although the method is highly specific and sensitive, the characterization of *N*-acetyl groups by NMR spectroscopy does not yet apply to large peptides and proteins. The identification of an acetyl group in the *N*-blocked terminal peptide of amylase definitely proves that this enzyme is an acetylprotein.

Acknowledgements

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