CONFORMATIONAL DYNAMICS OF PORCINE PANCREATIC COLIPASE: A 360 MHz PROTON NUCLEAR MAGNETIC RESONANCE STUDY

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Received 6 August 1976

1. Introduction

The present study is devoted to colipase II, one of the two main molecular forms of porcine pancreatic colipase [1,2]. This small protein has a molecular weight of 8700 and consists of a single polypeptide chain of 84 amino acid residues [3,4]. Its amino acid sequence has been determined [3] and corresponds to a rather compact structure maintained by 5 disulfide bridges [4]. The biological role of colipase is of great importance since it acts in vivo as a cofactor preventing the inhibitory effect of physiological concentrations of bile salts on the intraduodenal lipolysis of dietary triglycerides [1,5,6]. In an attempt to explain the physiological effect of colipase, binary and ternary associations of colipase, lipase and bile salts have been recently investigated under a variety of experimental conditions [7–13]. Typically, colipase has been shown to bind stoichiometrical amounts of bile salt micelles and to form a binary complex that pancreatic lipase can in turn recognize. It is likely that these binding and recognition processes involve specific conformational changes creating and/or unveiling appropriate sites on the colipase molecule [11].

The potential of high resolution n.m.r. in describing the conformational rearrangements of proteins under various perturbing factors (pH, temperature, ionic strength, ligand binding etc...) is now widely recognized. In this paper, I report and describe the first n.m.r. spectra of porcine colipase, obtained with the help of the most recent advances in the instrumentation for high field proton n.m.r. spectroscopy. Several resonances can be ascribed to specific residues or classes of residues and provide useful natural probes to study the conformational dynamics of colipase II in solution.

Abbreviations: n.m.r., Nuclear Magnetic Resonance. DSS, Sodium 2,2-dimethyl-2-silapentane-5-sulfonate. HMDS, Hexamethyldisiloxane. ppm, parts per million.

2. Material and methods

Porcine pancreatic colipase II was a generous gift of Mrs. M. Astier and Dr. M. Charles [1]. Fourier Transform n.m.r. spectra were obtained on the Bruker HXS-360 MHz spectrometer located at the Stanford Magnetic Resonance Laboratory (Stanford University California, USA). This spectrometer is interfaced with a Nicolet NIC-80 16K computer equipped with a Nicolet NIC-294 disc system. Protein samples were dissolved in pure D_2O (Diaprep) at a concentration of 1.5 mM. and pH was adjusted with dilute NaOD and DCl. The pH values were measured on a Radiometer pH Meter Model 26 employing a thin combination glass electrode and are given without correction for deuterium isotopic effect. Chemical shifts were measured with respect to HDO and subsequently converted to HMDS by measuring the position of HDO resonance with respect to an external capillary of HMDS. No correction for bulk magnetic susceptibility was applied. For the sake of a better comparison with other n.m.r. studies of proteins, chemical shifts were finally expressed in ppm from DSS. All spectra were recorded at 30°C using 5 mm precision n.m.r. cells (Wilmad). Sensitivity enhancement was achieved using negative exponential multiplication of the free

induction decays collected after 90° R_F pulses for an acquisition time of 1.63 s. Digital resolution was 0.6 Hz. In some cases, a 180° - τ - 90° pulse sequence was used to reduce the residual HDO resonance.

3. Results and discussion

The 360 MHz proton n.m.r. spectrum of colipase II is shown in fig.1. From high field to low field, it displays a series of methyl resonances down to around 1 ppm, the remaining resonances of the amino acid side chains between 1.5 and 4 ppm, the α-H resonances from 4 to 5 ppm, the residual HDO peak at 4.30 ppm and the resonances of the aromatic protons between 6 and 8 ppm.

3.1. *High field region of the spectrum*

The methyl resonance region contains several signals at unusual shielded positions with one well-resolved resonance having a negative chemical shift (−0.31 ppm). This effect is a direct manifestation of the three-dimensional arrangement of colipase II since the exceptional high field position of these resonances can be readily attributed to high field ring current shifts that are induced mainly in the shielded methyl groups of leucine, isoleucine and valine whose side chains are proximal (above or below) to the faces of an aromatic ring. These shielded resonances are not found in the spectra of aged and/or denatured colipase and then reflect in a very sensitive fashion aspects of the folded conformation of the protein. The −0.31 ppm resonance is not affected by an increase of pH from 5 to 12 and might correspond to a methyl-aromatic ring interaction involving rather uncharged aromatic residues, i.e. Phe 45 and/or Phe 77.

Another feature of this high field region is the presence of a large and intense resonance centered at 0.52 ppm. This signal is obviously exogeneous and arises from impurities presumably firmly bound to the protein, since repeated lyophilisations and prolonged dialyses can reduce the intensity of the peak but fail to eliminate it. These methyl- (and eventually methylene-) rich contaminants could correspond to the large quantities of butanol used during colipase purification, the degradation products of diisopropyl-fluorophosphate used to prevent proteolytic cleavage of colipase and/or some remaining lipids which might be recognized by the high affinity binding site that colipase has been shown to possess [9,10].

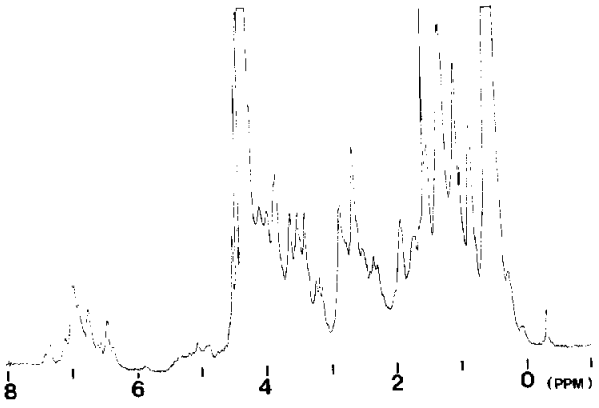

Fig.1. Proton n.m.r. spectrum at 360 MHz of a 1.5 mM solution of colipase II in D_2O at pH 8.75. 500 scans.

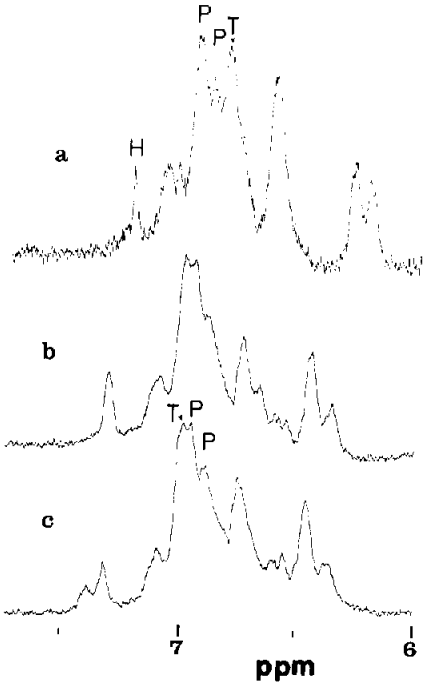

Fig.2. Aromatic region of the 360 MHz spectrum of 1.5 mM colipase II at different pH values. (a) pH 10.2; 250 scans (b) pH 9.65; 500 scans (c) pH 8.75; 500 scans. H: Histidine resonance, P: Phenylalanine resonance, T: Tyrosine resonance

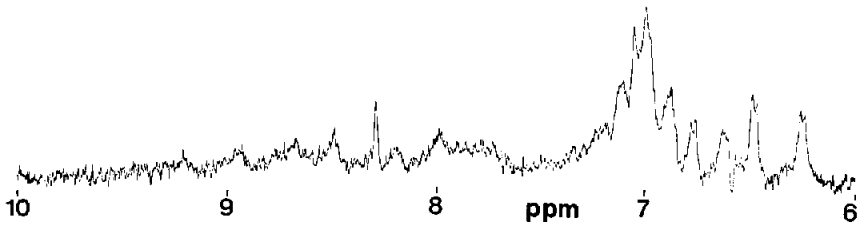

Fig.3. Low field region (6 to 10 ppm) of the 360 MHz spectrum of 1.5 mM colipase II at pH 6.0 in D_2O. 500 scans. Age of the solution: 12 h.

3.2. *Low field region of the spectrum*

Colipase II contains 2 phenylalanine, 2 histidine and 3 tyrosine residues, and is devoid of tryptophan. The region of the n.m.r. spectrum downfield from 6 ppm bears the resonances of the corresponding 26 protons (figs.1,2 and 3). Assignments of all resonance lines to particular residues is not yet possible and will require further work, presently in progress. However, several assignments of resonances to a class of specific aromatic protons can already be made. The low field doublet at 7.43 and 7.35 ppm (figs.1 and 2c) corresponds to the two H-2 protons of the imidazole ring of His 25 and His 81. This assignment is made on the basis of the expected chemical shift of this type of protons [14], the area of the signals and their pH sensitivity. The complex pattern observed upfield from 7.2 ppm reflects the absorption of the remaining 24 protons, i.e. the two H-4 imidazole protons of His 25 and His 81, the 10 benzene ring protons of Phe 45 and Phe 77, the 6 orthophenyl protons and the 6 meta-phenyl protons of Tyr 48, 51 and 52 (figs.1 and 2). The peaks at 6.90 and 6.97 ppm, whose chemical shift is pH insensitive (fig.2) are assigned to phenylalanyl protons together with most of the resonance at 7.10 ppm which does not shift significantly between pH 6 and 11. At least one H-2 proton of imidazole shifts upfield in the range of pH 8–10 (fig.2) thus indicating a His p*K*a value above 8 or a cooperative interaction with a tyrosine residue. All the other lines upfield from 6.90 ppm (fig.2b) are related to Tyr resonances and titrate in the 8 to 11 range of pH.

A better resolution of the aromatic region is achieved at lower pH (fig.3). Each of the 4 upfield resonances has an area corresponding to 2 protons, appears as a doublet with a characteristic coupling constant of 7.5–8 Hz [14] and titrates from pH 8 to 12. They are specifically ascribed to the Tyr ring protons and the most shielded 3 peaks at 6.24, 6.48 and 6.63 ppm can be tentatively assigned to the 3,5-orthophenyl protons of the 3 Tyr residues since ortho protons are generally found upfield to meta protons [14]. The doublet at 6.89 ppm would then correspond to the two 2,6-metaphenyl protons of one of the three Tyr residues. The region of the spectrum between 6.80 and 7.40 ppm (fig.3) contains a total of 16 protons, i.e. 10 Phe ring protons, 4 Tyr ring protons and the two H-4 protons of His 25 and His 81. The absolute integration of resonance area is based on the assumption that the doublet at 6.24 ppm corresponds to 2 protons. These complex overlapping lines are more difficult to resolve. However, the broad lowfield signal centered at 7.20 ppm is obviously related to the two H-4 imidazole protons of the 2 His residues and shifts upfield to overlap with Phe and Tyr resonances at higher pH (see fig.2c). In addition, the sharp peak at 7.05 ppm in fig.3 can be assigned to Tyr protons (presumably 2,6-metaphenyl protons) and shifts at basic pH (see peak T in fig.2). Finally, the pH insensitive complex lines at 6.96 and 6.88 ppm (fig.3) are assumed to arise from Phe ring protons (see peaks P in fig.2).

This distribution of aromatic resonances indicates that colipase II, although a small protein, is large enough to possess local magnetic environments inducing chemical shift differences for analogous protons of residues belonging to the same class. The separation of Tyr resonances (fig.3) is particularly striking in this respect and is directly related to a well-defined spatial arrangement of the colipase molecule. The 3 Tyr residues, although very close in the sequence (positions 48, 51 and 52) clearly

undergo very different three-dimensional environments.

The low field 7.5 to 10 ppm region of the spectrum in fig.3 displays together with the H-2 imidazole resonances of His, some broad resonances accounting for a total intensity of more than 10 protons. These lines are most intense in freshly prepared D_2O solutions of colipase II at neutral pH. They decay as a function of time and are still present after one day at ambient temperature. They disappear rapidly after colipase solution is altared whether heated above 70°C or brought to pH 12. On the basis of what is observed for other proteins [15–17], these resonances can be assigned to buried NH and/or OH protons whose exchange with deuterons is particularly slow. The increase of exchange rate with pH and temperature indicates that these buried hydrogens become increasingly exposed at higher pH values and temperature. The existence of such slow exchanging protons which are shielded from interaction with the solvent is another manifestation of the spatial structure of colipase II and can be used as a reliable monitor of its conformational dynamics.

3.3. *Interaction with bile salts*

A preliminary study of the association of colipase II and sodium taurodeoxycholate micelles in 10 mM NaCl shows a specific line broadening and loss of fine structure of the Tyr resonances confirming the involvment of these residues in the binding process as already demonstrated by ultraviolet spectrometry [10]. Other specific perturbations of the protein spectrum are observed (e.g. a 0.06 ppm down-field shift of the −0.31 ppm signal) and suggest rather discrete rearrangements of the colipase structure upon binding of bile salt micelle (e.g. disruption of intramolecular methyl–aromatic interaction). Further work on these molecular associations is in progress.

Acknowledgements

The author thanks Professor P. Desnuelle and Dr M. Charles for helpful discussions and is particularly indebted to Professor O. Jardetzky, Chairman of the Stanford Magnetic Resonance Laboratory (Stanford University, USA) for his support and interest, and for providing access to the 360 MHz facilities. This work is supported by a CNRS grant (Contrat ATP 2188).

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