PROTON NMR STUDY AT 360 MHz OF PORCINE PANCREATIC COLIPASE

Identification of aromatic resonances

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1. Introduction

Colipase is a small protein cofactor found in the pancreatic secretion of mammalians [1,2]. The biological role of colipase is of critical importance in vivo since it counteracts the inhibitory effect of physiological concentrations of bile salts on the intraduodenal lipolysis of insoluble dietary triglycerides [1–3]. In addition, colipase has a direct specific effect on interfacial lipolysis by protecting lipase from surface denaturation in the absence of detergents [4–6]. Considering that these binding and recognition processes are likely to involve specific structural changes creating and/or unveiling appropriate sites on the colipase molecule, we have undertaken a systematic NMR study of colipase conformational properties in solution. A general interpretation of the colipase II spectrum has already been reported [7]. Colipase II is one of the two major forms of the cofactor isolated in [8]; its covalent structure (84 residues) has been fully elucidated [9,10] and corresponds to a compact central core crosslinked by 5 disulfide bridges with two terminal tails. Two other forms of the cofactor have been obtained [11,12] and some characteristics of 3 aromatic residues of a 106 residue long form of colipase have been investigated by NMR spectroscopy [13].

A new direct purification of porcine colipase from pancreatic tissue homogenate in the presence of Triton X-100 has been achieved [14]. The major form (80%) thus obtained and designated as colipase A is highly stable and is similar to the one in [11,12].

Abbreviations: DSS, sodium 2,2-dimethylsilapentane 5-sulfonate; FID, free induction decay

In the present paper, we report the complete interpretation of the 6–8 ppm region of the proton spectrum of colipase A, at pH 10 and 40°C. The resonances of all aromatic side chains have been identified and some basic properties of the tyrosine ring dynamics are discussed.

2. Materials and methods

All proton Fourier Transform NMR spectra were obtained at 360 MHz on a Brüker HXS-360 spectrometer located at the Stanford Magnetic Resonance Laboratory, Stanford. Additional experiments were carried out in Karlsruhe (Brüker Physik AG) on a similar spectrometer. Colipase was prepared as in [14] and assayed for activity in the triolein–gum arabic system [15]. Colipase A samples were dissolved in D_2O (Diaprep) and heated for 1 h at 70°C then submitted to repeated lyophilisations from D_2O to achieve complete replacement by deuterons of exchangeable protons. In a typical experiment, 200 FID were collected in 0.3 ml 0.6 mM colipase A solution at 40°C (acquisition time, 1.8 s) then Fourier transformed with a digital resolution of 0.5 Hz. Quadrature detection and sensitivity enhancement through negative exponential multiplication of FID were routinely used. Chemical shifts are expressed in ppm from internal DSS. All pH values are not corrected for the deuterium isotope effect at the glass electrode.

3. Results and discussion

Colipase A contains 2 phenylalanine, 3 tyrosine

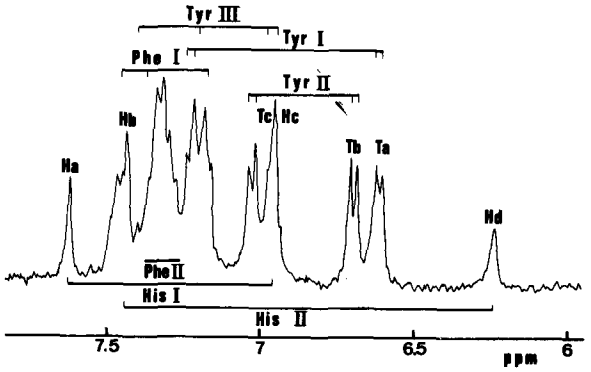

Fig.1. Identification of aromatic resonances on the 360 MHz spectrum of colipase A in D_2O at pH 10 and 40°C. The numbering of residues is arbitrary.

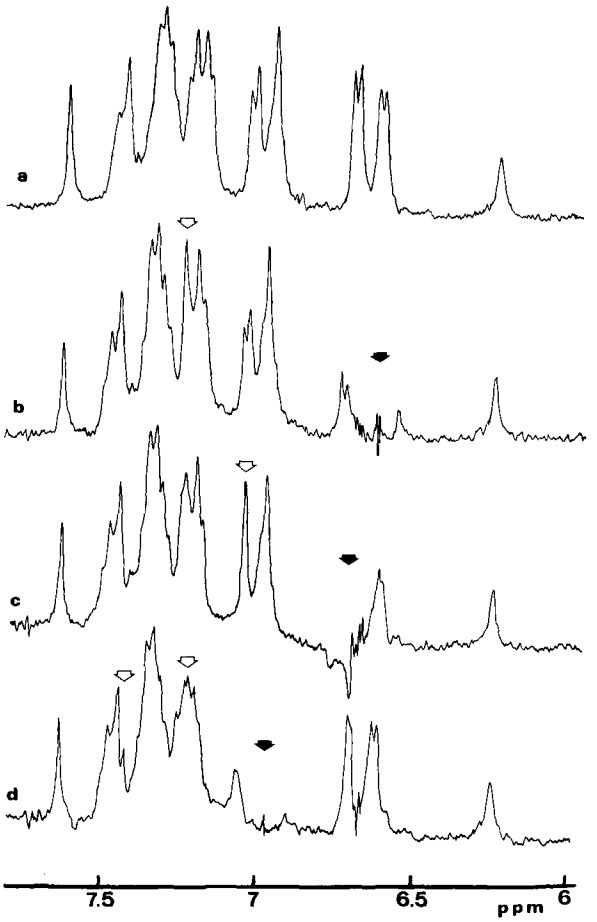

Fig.2. Selective spin decoupling experiments in the aromatic region of the colipase A spectrum: (a) colipase A spectrum at pH 10; (b) irradiation at 6.96 ppm; (c) irradiation at 6.62 ppm; (d) irradiation at 6.70 ppm.

and 2 histidine residues; it is devoid of tryptophan. The low field (6–8 ppm) region of the spectrum is presented in fig.1. If one assumes that the signal at 7.63 ppm corresponds to a 1 proton resonance, the total area measurement is consistent with the expected 26 aromatic protons with no indication of the presence of remaining resonances from exchangeable protons. The same total is obtained when choosing as a reference for integration the 1 proton singlet at 6.24 ppm or either one of the two well-resolved 2 proton doublets centered at 6.62 ppm and 6.70 ppm. The resonances of aromatic protons have been identified on the basis of:

(i) The chemical shifts [16];
(ii) The area of the signals;
(iii) Their pH sensitivity, i.e., pK_a values and magnitude of titration shifts;
(iv) The spin coupling patterns;
(v) The results of double resonance experiments.

3.1. *Tyrosine resonances*

A selection of the perturbed spectra obtained by systematic selective irradiation of all coupled signals is shown on fig.2. Homonuclear decoupling of the upfield doublet at 6.62 ppm (Ta) causes the collapse of the doublet structure centered at 7.23 ppm (fig.2b) and vice-versa. Both resonances are pH sensitive and titrate with pK_a 10.4 (table 1). They are considered to reflect the AA′XX′ spin system of the same tyrosine ring (Tyr I). Similarly, the two doublets of area two centered at 6.70 (Tb) and 7.05 ppm are mutually decoupled by selective double irradiation (fig.2c). Their pH dependence (pK_a 10.3) is also characteristic of a tyrosine residue and they are consequently ascribed to the AA′XX′ spin system of a second tyrosine (Tyr II). Since the 3,5-orthophenyl protons of tyrosine are usually found upfield from the 2,6-metaphenyl protons [16], the two doublets of area 2 at 6.62 ppm and 6.70 ppm are identified as the 3,5-orthophenyl protons of Tyr I and Tyr II, respectively. Moreover, the magnitude of the titration shifts of these two resonances is, as expected for orthophenyl protons, significantly larger than for the two other doublets centered at

Table 1
Chemical shifts, spin systems and titration parameters of the aromatic protons of pancreatic colipase A at 40°C

Residue	Spin system	Ring proton position	Number of protons	δ (ppm) at pH 10.0	δ_0	Δ	pK_a
Tyr I	AA'XX'	3,5 (Ta)	2	6.62	6.45	0.24	10.4
		2,6	2	7.23	7.12	0.13	10.4
Tyr II	AA'XY'	3,5 (Tb)	2	6.70	6.52	0.28	10.3
		2,6	2	7.05	6.90	0.17	10.3
Tyr III	AA'MX	3,5 (Tc)	2	6.96	6.57	0.39	11.3
		2 or 6	1	7.20	6.99	0.21	11.3
		2 or 6	1	7.40			n.d.
Phe I	AA'MXX'	3,5 or					
		2,6	2	7.17			
		4	1	7.36			
		3,5 or					
		2,6	2	7.46			
Phe II	AA'BB'C	all protons	5	7.32			
His I		2 (Ha)	1	7.63	7.63	1.03	8.0
		4 (Hc)	1	6.95	6.95	0.39	8.1
His II		2 (Hb)	1	7.44	7.44	1.17	6.9
		4 (Hd)	1	6.24	6.24	0.40	6.9

δ_0 is the chemical shift of the unprotonated form and Δ is the chemical shift change upon protonation. Positive values of Δ indicate downfield shifts

7.23 ppm and 7.05 ppm which then correspond to the 2,6-metaphenyl protons of Tyr I and Tyr II, respectively (table 1).

The signal of area 3 centered at 6.96 ppm consists of a 1 proton resonance assigned to the C (4) proton of a histidine residue (see below) and a 2 proton doublet (Tc) exhibiting a titration curve with an app. pK_a 11.3 characteristic of a tyrosine residue (Tyr III). Selective irradiation of this signal induces a perturbation at two other complex resonances centered at 7.20 ppm and 7.40 ppm (fig.2d). The resonance at 7.20 ppm is of area 1 while direct integration of the signal at 7.40 ppm is impossible. Nevertheless, it is clear that the aromatic ring of Tyr III is described by a AA'MX spin system. The AA' part of the system (6.96 ppm doublet) can be assigned on the basis of chemical shifts to the 3,5-orthophenyl protons of the ring (table 1).

3.2. *Phenylalanine resonances*

The coupled signals of area 2 centered at 7.17 ppm and 7.46 ppm and the 1 proton signal at 7.36 ppm are identified as the AA'MXX' spin system of one of the two phenylalanines (Phe I). These resonances are all mutually coupled and pH insensitive. However, above pH 11, a slight shift of the whole system is observed in relation with the pH-induced loosening of the colipase spatial arrangement. The resonance at 7.36 ppm corresponds to the C (4) paraphenyl proton of Phe I while the ortho- and metaphenyl protons are found under the doublet-like signals. The two 2,6-metaphenyl protons would more likely resonate at 7.17 ppm since they are generally more shielded than the protons in the ortho position [17].

The multiplet of area 7 centered at 7.32 ppm contains the five ring protons of Phe II together with two other protons belonging to Phe I (7.36 ppm) and Tyr III (7.40 ppm). The aromatic resonances of Phe II constitute then a AA'BB'C or ABCDE spectrum at pH 10; a pure AA'BB'C pattern is however observed above pH 11.

3.3. *Histidine resonances*

The imidazole ring protons are readily identified on the basis of chemical shifts and pH sensitivity. The 1 proton singlets at 7.63 ppm (Ha) and 6.95 ppm (Hc) (fig.1) titrate simultaneously with similar pK_a 8.0 and 8.1. They correspond to the C (2) and C (4) protons, respectively, of the same histidine ring (His I). In a similar manner, both 1 proton resonances at 7.44 ppm (Hb) and 6.24 ppm (Hd) titrate with pK_a 6.9 and are assigned to the C (2) and C (4) protons of the second histidine residue (His II). The analysis of the specific interactions of His I and II with other titratable groups will be described [18].

However, one can indicate that the high pK_a value of His I is expected to arise from the close proximity of a negatively-charged group contributed by a carboxyl group or from the indirect interaction with a carboxyl group through a hydrogen bond [19]. In addition the low pH region of the titration curve of the proton displays an additional inflexion corresponding to pK_a ~5.0. On the basis of these 2 observations, a tentative assignment of His I to the His 30 residue is proposed, taking into account the adjacency of Asp 31. The second histidine residue, His II, would then correspond to the solvent-exposed His 86.

3.4. *Dynamics of tyrosine and phenylalanine rings*

Although very close in the sequence, the 3 Tyr residues are subjected to different spatial environments as is demonstrated by the spread of their chemical shifts (table 1). They also differ with respect to their mobility on the NMR time scale.

The AA'MX spectrum observed for Tyr III indicates a magnetic non-equivalence of the protons at positions 2 and 6 while the two other symmetry-related 3,5-orthophenyl protons resonate as a doublet. This situation which is analogous to the one described for Tyr 23 in the basic pancreatic trypsin inhibitor [17] is a consequence of different environments experienced locally by the two metaphenyl protons and that restricted motion of the aromatic ring in the interior of the protein cannot average out. The life time with respect to 180° flips about the C_β–C_γ bond axis of Tyr III can thus be estimated to be > 0.02 s at 40°C. The apparent high pK_a value of Tyr III (which can be tentatively assigned by difference to Tyr 53) is also indicative of a buried residue which requires a preliminary high pH-induced loosening of colipase structure before coming accessible to the solvent and hence titratable.

The symmetry of the spin systems observed for Phe I, Phe II, Tyr I and Tyr II indicates that at 40°C and pH 10, their aromatic rings rotate freely about the C_β–C_γ bond axis. This observation suggests that these residues occupy in the 3-dimensional structure a relatively exposed position which allows a freedom of rotation large enough to account for the averaging out of the symmetry-related ring protons. The finding of pK_a values for Tyr I and II close to the one corresponding to 'free' tyrosine [16] confirms the location of these two residues at the surface of the protein globule. It seems then reasonable to assign Tyr I and II as a pair to the two adjacent Tyr 56 and 57 on the polypeptide chain. Their accessibility and mobility make them the best candidates to correspond to the two external tyrosines detected by differential ultraviolet spectrophotometry and found to be involved in the binding of colipase to polar or apolar micelles [20].

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