360 MHz LASER PHOTO-CIDNP OF PORCINE PANCREATIC COLIPASE A

Study of the aromatic surface residues

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1. Introduction

Colipase is a protein of low molecular weight (about 11 000) found in the pancreatic secretion of mammals [1]. Its physiological role is: (i) to allow lipase to adsorb at lipid–water interface in the presence of high concentrations of bile salts, (ii) to stabilize the enzyme in its fully active form under large variations of surface energy [2–5]. The adsorption of colipase to the substrate–water interface is now accepted as the first step of the lipolytic process and it can be related to the presence of a particular domain on the surface of the coprotein [6]. Proton NMR, fluorescence and spectrophotometric studies on porcine colipase have indicated that two tyrosine and one histidine residues are located in a hydrophobic region of the protein that might constitute at least a part of the lipid binding site on colipase [7–11].

Here we report a series of laser photo-CIDNP experiments performed on porcine colipase A [11]. The photo-CIDNP method is based on the generation of nuclear spin polarization in a reversible photoreaction of a dye with aromatic residues [12]. The side-chains of tyrosine, histidine and tryptophan residues can be polarized when they are accessible to the photo-excited dye [13,14]. Porcine colipase A contains 3 tyrosine, 2 histidine and 2 phenylalanine residues. It is devoid of tryptophan. The sequence of the polypeptide chain and the position of the 5 disulfide bridges have been fully elucidated [15,16]. The results obtained using the photo-CIDNP technique on colipase A clearly indicate that 2 out of the 3 tyrosines of the protein are surface residues. In contrast, the 2 histidine residues are buried at low pH. The accessibility of 1 histidine residue (His I) is pH dependent and requires a basic pH to be fully achieved.

Abbreviations: CIDNP, chemically induced dynamic nuclear polarization; NMR, nuclear magnetic resonance; ppm, parts per million, DSS, sodium 2,2-dimethylsilapentane 5-sulfonate; FID, free induction decay

2. Material and methods

Porcine colipase A was purified from pancreatic tissue homogenate by the detergent method [11]. Colipase activity was assayed at pH 9.0 and 25°C with the triolein gum arabic system in the presence of bile salts or sodium taurodeoxycholate (Sigma) [17].

^{1}H NMR spectra were obtained at 360 MHz using a Bruker HX-360 spectrometer operating in the pulse Fourier transform mode. For the photo-CIDNP spectra, a Spectra Physics model 171 argon ion laser was used as the light source. Difference spectra were obtained by taking alternating 'light' and 'dark' free induction decays and subtracting the spectra after Fourier transformation. For the light spectra, the sample containing the protein and a flavin dye was irradiated by 0.6 s laser pulse (7 W, multiline) prior to data acquisition. A full description of the method has been given in [12]. The dye used in this work, 3-carboxymethyl lumiflavin, was synthesized by Dr F. Müller (Wageningen).

3. Results and discussion

3.1. *Photo-CIDNP difference spectrum of colipase A*

The conformational dynamics of colipase A has been studied by proton NMR spectroscopy [8,18] and the resonances of all aromatic ring protons have been identified [19]. The pK_a values of the aromatic titratable residues have also been determined [8].

In fig.1, the dark spectrum (fig.1a) and the dark–light difference spectra (fig.1b) are shown. The most prominent features of the CIDNP difference spectrum are the two strong emission lines centered, in the aromatic region, at 6.68 ppm (Ta) and 6.75 ppm (Tb). On the basis [8], these lines can be assigned to the orthoprotons of Tyr I and Tyr II, respectively (Tyr 56 and Tyr 57). Two other emission lines are observed at 7.30 ppm (Ta′) and 7.04 ppm (Tb′) and correspond to the small negative polarization of the *meta* protons of Tyr I and Tyr II. This effect arises from a transfer of polarization from *ortho* protons by a crossrelaxation mechanism [20,21]. In small molecules, transfer by this mechanism yields polarization of opposite sign [20], but in proteins that have longer correlation times for molecular tumbling, the polarization is transferred with retention of sign [13,14,21]. It can be concluded that Tyr I and Tyr II are surface residues.

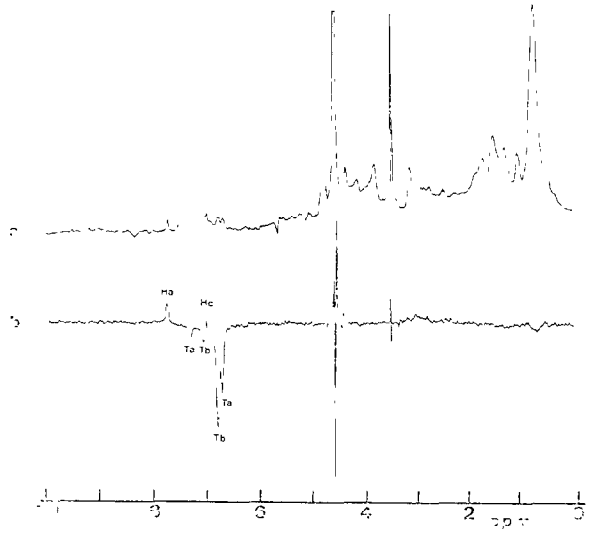

Fig.1. 360 MHz proton NMR spectra of porcine colipase A 1.5 mM in ^2H_2O and 0.4 mM flavin, at pH 8.8 and 35°C: (a) dark spectrum, (b) photo-CIDNP difference spectrum (light spectrum, obtained by 0.6 s irradiation of the sample, minus dark spectrum). Light and dark FID were taken alternately (20 scans).

This is in accordance with their 'normal' pK_a values (about 10.3) and with the symmetry of their NMR spectra (pairs of doublets), which indicates that the tyrosine rings are free to make rapid 180° flips about the C_β–C_γ bonds.

At pH 8.8, two absorption lines are also present in the CIDNP spectrum of colipase, at 7.72 ppm (Ha) and 7.00 ppm (Hc). These positive lines correspond to the C-2 and C-4 protons of His I which has been tentatively assigned to His 30 [8].

3.2. *pH dependence of the photo-CIDNP difference spectrum of colipase A*

The photo-CIDNP difference spectra of porcine colipase A at acidic, neutral and basic pH are given in fig.2. At pH 2.2, a very strong CIDNP effect is observed, in particular in the aromatic region of the spectrum. The two well-resolved doublets at 6.58 ppm and 6.82 ppm correspond to the two equivalent *ortho*

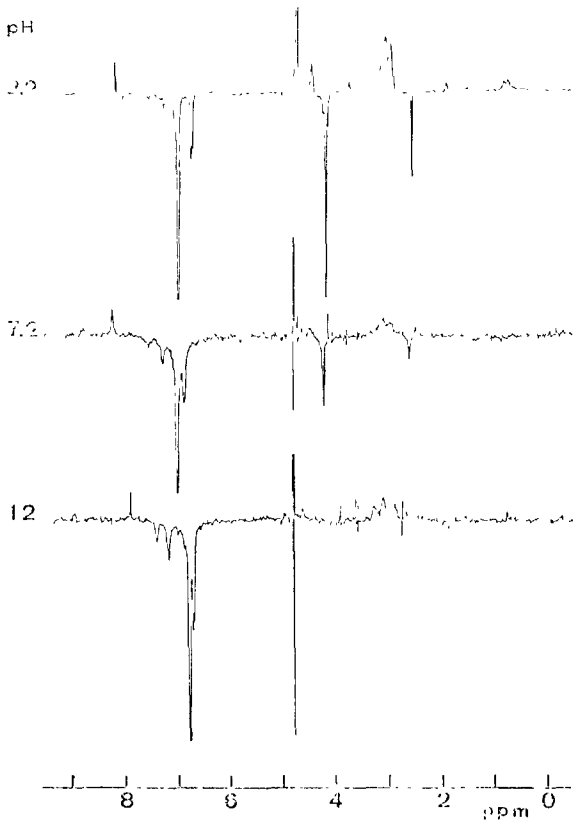

Fig.2. 360 MHz photo-CIDNP difference spectra of porcine colipase A: (a) pH 2.2; (b) pH 7.2; (c) pH 12. Concentrations used are: 1.5 mM colipase and 0.4 mM flavin. 30°C, 20 scans. The gain of the pH 2.2 spectrum is 50% that of the others.

protons of Tyr I and Tyr II, respectively, with the *meta* protons showing a weaker emission effect due to crossrelaxation, at 7.38 ppm and 7.11 ppm. Identification of the resonances at 3.00 ppm and 3.11 ppm (tyrosyl-CH_2 protons) and at 4.36 ppm (CH proton) is facilitated by the strong enhancements observed at low pH. The line at 4.36 ppm is probably a tyrosyl-CH proton polarized by transfer of polarization from the adjacent-CH_2 groups. Since the ϵ-proton of Tyr II is most strongly polarized, most of the polarization around 3.1 ppm and at 4.36 ppm probably also belongs to Tyr II being the most exposed tyrosine residue.

Other polarized lines are visible in the low pH CIDNP spectrum at 2.60 ppm, 4.15 ppm and 8.15 ppm. These 3 lines belong to the flavin dye and correspond to the methyl groups at positions 8 and 10, and to the aromatic proton at position 6, respectively [12]. No polarization is observed from the histidines C-2 protons in the region of 8–9 ppm which is indicative of a buried position for the 2 histidine residues of colipase at acidic pH.

Photo-CIDNP effects for 2 tyrosine residues can be observed in the spectra taken at higher pH as well, although they are not as strong as at low pH. The relatively larger enhancement of the *meta* protons of both Tyr I and Tyr II is likely to be related to some aggregation of the protein (dimerization) at high pH favouring crossrelaxation due to slower average tumbling rates.

The 3 lines belonging to the flavin disappear at basic pH. In contrast, an extra absorption line is visible at pH 7.2 and an increase in pH is accompanied by growing absorption intensity of this line, centered at 7.61 ppm at pH 12. This signal is pH dependent and corresponds to the C-2 proton of His I. The C-4 proton resonance overlaps with the line of Tyr II *meta* protons. It is likely that the accessibility of His I (His 30) at pH above 7 involves conformational changes in the spatial structure of the protein.

3.3. *Temperature dependence of the CIDNP difference spectrum*

Fig.3 shows the photo-CIDNP spectra of colipase taken at various temperatures. The spectrum at 67°C is not much different from that taken at 35°C, except the disappearance of the lines from the methyl 10 and from the aromatic proton at position 6 of the flavin. At higher temperatures, the resonance of the orthoprotons of Tyr II is shifted downfield and overlaps with the corresponding resonance of Tyr I. The

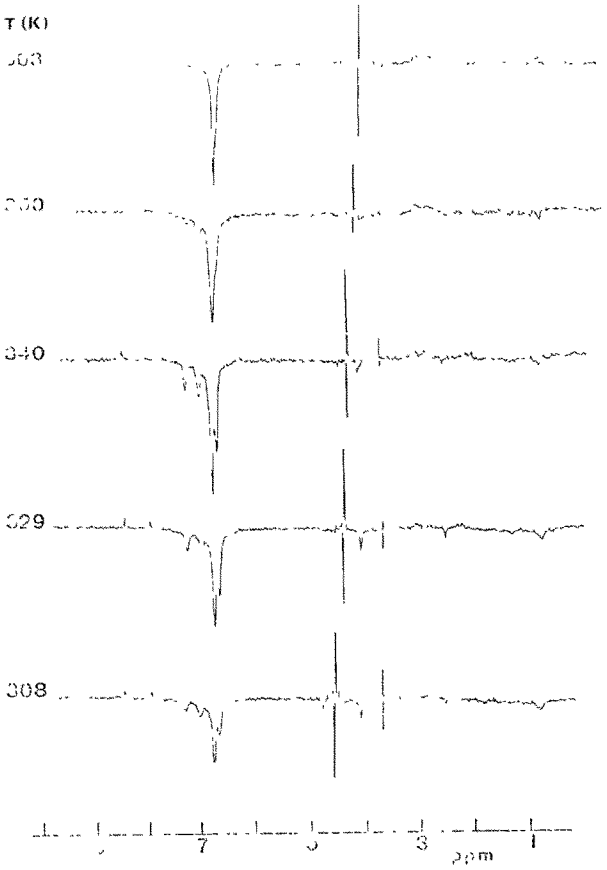

Fig.3. Temperature dependence of the photo-CIDNP difference spectrum of colipase A at pH 7.2 (2.0 mM colipase, 0.4 mM flavin, 20 scans).

broadening of the signal belonging to the C-2 proton of His I [8] is correlated with the disappearance of the absorption line centered at 8.47 ppm. Although the full spectrum of colipase at 90°C (not shown) indicates a partial unfolding of the protein, the CIDNP evidence suggests that Tyr III is situated in a tightly-folded region of colipase, this residue remaining buried at high pH and temperature values.

4. Conclusion

In the absence of any crystallographic data on colipase A, the laser photo-CIDNP experiments described here provide valuable information about the spatial location of the tyrosine and histidine residues of the protein. Two tyrosine residues are

identified at the surface of the globule while the third tyrosine remains unaccessible at any pH and temperature. The 2 histidine residues are similarly buried but a pH-induced conformational rearrangement of colipase allows the exposure of His I at basic pH. These results are particularly important with respect to the existence of a hydrophobic aromatic site (lipid binding site) which has been identified on pancreatic colipase A [8] and shown to contain 2 tyrosine residues (exposed) and one histidine residue in a cluster of apolar residues. The critical role of aromatic surface residues surrounded by hydrophobic side chains in the recognition of organized lipids, by proteins has also been emphasized recently in phospholipase A2 [22] and the phosphatidylcholine exchange protein [23].

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