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^{1}H-NMR AND PHOTOCHEMICALLY-INDUCED DYNAMIC NUCLEAR POLARIZATION STUDIES ON BOVINE PANCREATIC PHOSPHOLIPASE A_2

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Summary

Proton-NMR resonances of trytophan 3 and tyrosine 69 in bovine pancreatic phospholipase A_2, its pro-enzyme and in Ala^1-transaminated protein were assigned using photochemically-induced dynamic nuclear polarization (photo-CIDNP) as such or in combination with spin-echo measurements. In addition assignments were made by suppression of cross-relaxation effects using short (0.1 s) high-power laser pulses.

Phospholipase A_2 (EC 3.1.1.4) catalyzes specifically the hydrolysis of the fatty acid ester bond at the 2-position of 1,2-diacyl-*sn*-glycero-3-phosphocholines [1]. The enzyme is obtained from pancreatic tissues as its zymogen, pro-phospholipase A_2. The pro-enzyme is converted into phospholipase A_2 by tryptic cleavage of the N-terminal heptapeptide pGlu-Ala-Gly-Leu-Asn-Ser-Arg, for the bovine enzyme [2].

Both the zymogen and phospholipase A_2 catalyze the hydrolysis of monomeric short-chain phosphatidylcholines. However, only phospholipase A_2 is able to catalyze the hydrolysis of micellar aggregates of the phospholipid substrates as well. Hydrolysis of micellar substrates takes place at rates which are several orders of magnitude higher than those for monomeric substrates [3].

Porcine phopholipase A_2 has recently been shown to form specific complexes with micellar phospholipids [4]. Trp^3 and one or more Tyr residues

Abbreviations: pH*, uncorrected pH meter reading in ^2H_2O; photo-CIDNP, photochemically-induced dynamic nuclear polarization; pGlu: pyroglutamic acid residue; TSP, sodium 3-trimethylsilyl [2,2,3,3-$^2H_4$] propionate.

were found to be perturbed by lipid binding, as indicated by ^{13}C-NMR [5], fluorescence [6] and ultraviolet difference spectoscopy [6]. In contrast, the zymogen does not form these specific complexes.

In order to gather more insight into the interaction with micelles, it was considered important to elucidate further the ^{1}H-NMR spectrum of phospholipase A_2 and its zymogen. Previously assignments have been made to ^{1}H resonances of His residues in pancreatic phospholipase A_2 and pro-enzymes [7]. Furthermore, photo-CIDNP signals of Trp^3, Tyr^{69} and Tyr^{123} (the latter in the porcine pro-enzyme only) have been indentified [8,9]. Some Trp^3 photo-CIDNP signals were tentatively assigned [9]. It has to be noted that the His protons in phospholipase A_2 do not give rise to photo-CIDNP signals. In this paper all Trp^3 ^{1}H resonances will be unambiguously assigned using photo-CIDNP.

Assignments were aided by combining photo-CIDNP and spin-echo measurements. In addition, information was obtained via suppression of cross-relaxation effects by using short laser light pulses. Lyophilized pure protein samples [10] were dissolved in $^{2}H_2O$ (1.5—2 mM protein), containing 0.28 M NaCl at pH* 5. 360 MHz ^{1}H-NMR spectra were recorded at 40°C using quadrature detection. About 1000 free induction decays were recorded and accumulated at approx. 1 s repetition time. Resolution was enhanced by multiplying free induction decays with the exponential exp $[a\cdot(t/T)-b\cdot(t/T)^2]$ [11, 12] where t is time, T is acquisition time and a and b are empirical constants, generally being 25 and 1.5, respectively. Dioxan was added as internal standard. Chemical shifts are reported relative to sodium 3-trimethylsilyl [2,2,3,3-$^{2}H_4$]-propionate (TSP), using 3.747 ppm as the chemical shift difference between dioxan and TSP. (3-*N*-Carboxymethyl)lumoflavin was the dye used in the photo-CIDNP experiments. This dye was a generous gift from Dr F. Müller.

Usually 0.6 s (7 W, multiline) laser pulses were applied and 10 to 20 free induction decays accumulated. Short pulse experiments involved 0.1 s laser pulses at high power (15 W). A program was written to combine the photo-CIDNP pulse routine with e.g. the 90° - τ - 180° - τ radiofrequency pulse sequences for the spin-echo measurements. At a delay time of 60 ms the accumulation of 20 free induction decays was found to be sufficient.

Part of the aromatic region of the 360 MHz ^{1}H-NMR spectrum of bovine phopholipase A_2 is shown in Fig. 1A. This spectrum is complicated by the fact that the enzyme contains seven tyrosines, four phenylalanines, two histidines and one tryptophan. Not shown in this figure is the singlet H-2 resonance of His^{115} at 8.76 ppm. The H-2 resonance of the active site residue [13] His^{48} is strongly shifted upfield [7], probably due to the proximity of the Tyr^{52} ring [7,14].

Fig. 1B shows the corresponding part of the photo-CIDNP difference spectrum of the same enzyme preparation. Apart from the emission doublet of Tyr^{69} H-3,5 resonance at 6.90 ppm, the spectrum shows absorption peaks of Trp^3 protons: two doublets, one at 7.57 ppm, the other at 7.47 ppm, a singlet at 7.51 ppm and a complex resonance at 7.28 ppm. As only the H-2 (singlet), H-4 (doublet) and H-6 (triplet) resonances of Trp^3 are directly polarized by the photo-CIDNP effect [15], it appears that the spectrum in Fig. 1B also contains indirectly enhanced resonances.

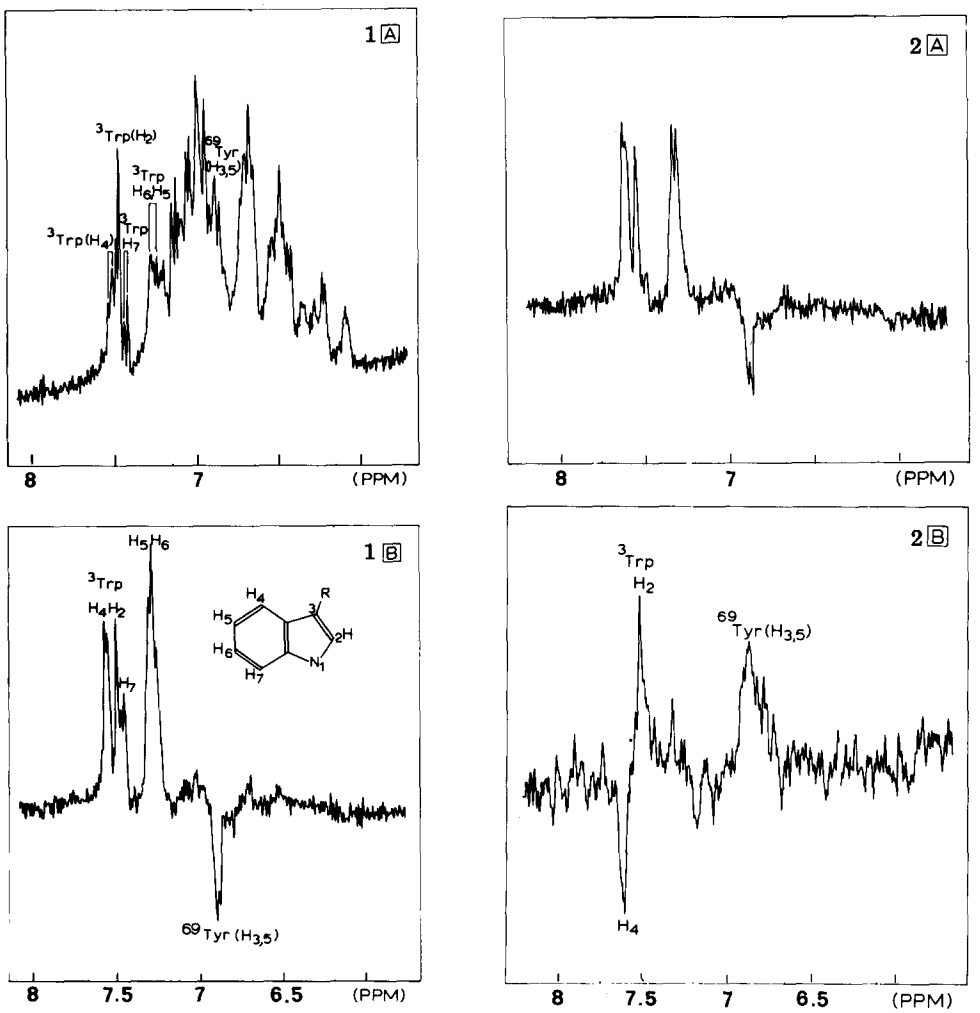

Fig. 1. A. Low-field region of the resolution enhanced in ^{1}H-NMR spectrum of bovine phopholipase A_2 at pH* 5.0, 40°C in the presence of 0.28 M NaCl. B. Resolution enhanced photo-CIDNP difference spectrum of the same enzyme sample in the presence of 0.2 mM flavin dye. Corresponding resonances are indicated in A and B; resonance positions are not affected by the dye. The inset in B shows the indole ring and numbering of the tryptophan residue.

Fig. 2. A. Low-field region of the resolution enhanced photo-CIDNP difference spectrum of bovine phospholipase A_2. Short (0.1 s) laser pulses were applied to avoid indirect polarization via cross-relaxation. B. Photo-CIDNP spin-echo spectrum of bovine phospholipase A_2; 20 transients were accumulated using a 60 ms delay between the 90° and 180° pulses. Conditions were as above.

Transfer of polarization can occur via dipolar cross-relaxation to protons close to the primary polarized protons [15]. Because the cross-relaxation-induced effect lags behind the directly generated polarization, the two effects can be separated. This was done by applying short (0.1 s) high-power laser pulses to the sample, thereby suppressing cross-relaxation effects. The resulting spectrum (Fig. 2A) is indeed simpler than that of Fig. 1B, showing the directly polarized resonances of H-4 at 7.57 ppm, H-2 at 7.51 ppm and H-6 at 7.29 ppm of Trp^3 and of H-3,5 resonances of Tyr^{69} at 6.90 ppm. For tryptophan the most

likely candidates for cross-relaxation effects are the non-directly polarizable protons in the indole ring, i.e. H-5 and H-7. In the case of tyrosine a similar transfer is often observed from the 3,5 protons to the 2,6 protons [15].

Comparison of Figs. 1B and 2A then allows assignments to be made to the H-7 doublet at 7.47 ppm and the H-5 triplet at approx. 7.27 ppm, both of Trp^3. The corresponding resonances in the ^{1}H-NMR spectrum (Fig. 1A) are assigned as shown in the figure.

The multiplicity in homonuclear coupled spin systems can be determined by a spin-echo experiment using the phase modulation of the spin-echo. Doublets are inverted at a delay $\tau = (2J)^{-1}$ (J is the spin-spin coupling constant) in the Carr-Purcell experiment, whereas singlets and the centre lines of triplets stay in phase.

In order to confirm the multiplicity of the observed photo-CIDNP signals, at 90° - τ - 180° - τ pulse sequence [16] was applied to the sample following the laser pulse. Fig. 2B shows the phase modulation in the spin-echo at a delay of 60 ms. This firgure clearly demonstrates the doublet nature of the H-4 resonance of Trp^3 and the H-3,5 resonance of Tyr^{69}, both being fully inverted at 60 ms delay time (J is approx. 8 Hz for both resonances).

The triplet H-5 and H-6 resonances of Trp^3 are no longer observed in the spin-echo, probably due to a short spin-spin relaxation time (T_2) for these protons.

Using the same techniques, Trp^3 and Tyr^{69} resonances were assigned for the bovine pro-enzyme as well as for Ala^1-transaminated bovine phospholipase A_2. In the latter protein the N-terminal amino group of Ala^1 was replaced by a keto group (Ref. 17, and Slotboom, A.J. unpublished observations). The results of these experiments are summarized in Table I. This table also gives the chemical shift values for Tyr^{69} H-2,6 resonances, taken from the photo-CIDNP difference spectra and confirmed by selective homonuclear decoupling.

TABLE I

RESONANCE POSITIONS RELATIVE TO TSP OF BOVINE ENZYME SPECIES DISSOLVED IN ^2H_2O AT pH* 5.0, 40°C IN 0.28 M NaCl

Species	Chemical shift (ppm)						
	Trp^3					Tyr^{69}	
	H-2	H-4	H-5	H-6	H-7	H-3,5	H-2,6
Bovine phospholipase A_2	7.51	7.57	7.27	7.29	7.47	6.90	7.18
Bovine prophospholipase A_2	7.54	7.69	7.26	7.30	7.41	6.71	6.98
Bovine transaminated phospholipase A_2	7.37	7.58	7.24		7.43	6.79	7.06
Random coil protein	7.32	7.73	7.28		7.54	6.90	7.19

The chemical shift values of the Tyr^{69} resonances and those of the H-5, H-6 and H-7 protons of Trp^3 in bovine phospholipase A_2 are very close (within 0.05 ppm) to the values for a random coil protein. The H-2 and H-4 of Trp^3 resonate at lower and higher field, respectively, than their random coil positions.

The Trp^3 and Tyr^{69} proton resonances are fairly insensitive to temperature changes. Only the H-2 resonance of Trp^3 gradually shifts upfield in the photo-CIDNP spectrum towards its random coil position at 7.3 ppm when the temperature is raised to 80°C.

Comparing the photo-CIDNP spectra of bovine phospholipase A_2 and the pro-enzyme, the main differences are the resonance positions of H-4 of Trp^3 and H-3,5 of Tyr^{69} (Table I). In phospholipase A_2 the Tyr^{69} residue shows proton resonances at random coil values, while these resonances are upfield shifted (by 0.2 ppm) in the bovine pro-enzyme. On the other hand, the H-4 proton of Trp^3 resonates at 7.69 ppm in the pro-enzyme, close to its random coil value (7.73 ppm), while its position has shifted upfield by 0.12 ppm, in bovine phospholipase itself. These data indicate that Tyr^{69} is an exposed surface residue in the bovine enzyme, while Trp^3 is probably more shielded from the solvent in the enzyme as compared to the pro-enzyme.

Fluorescence studies on porcine phospholipase A_2 and pro-enzyme have shown [6,18], that Trp^3 is present in a more hydrophobic environment in the enzyme than in the pro-enzyme. This agrees with the conclusion reached above that Trp^3 is more buried in the enzyme than in the pro-enzyme.

Previous photo-CIDNP ^{1}H-NMR studies on bovine phospholipase A_2 demonstrated that the H-2 and H-4 resonances of Trp^3 shift upfield and downfield, respectively, towards their random coil positions with increasing pH (note that the preliminary assigned H-2 and H-4 resonances should be interchanged in that article). Thus, at high pH values the Trp^3 residue becomes more exposed. Under these conditions phospholipase A_2 no longer binds to micellar substrates [9]. The Trp^3 proton resonances are now very close to those of the pro-enzyme, which is also unable to bind micelles.

It has been proposed [9] that the ability of the protein to bind micellar substrates is correlated with the formation of a H-bridge between the Ala^1 NH_3^+ group and a negatively charged residue in the protein. Indeed Ala^1-transaminated pancreatic phospholipases A_2 no longer bind to micelles. The chemical shift data for the bovine transaminated enzyme (Table I) generally give resonance positions in between those of the enzyme and pro-enzyme, respectively. The only exception is the H-2 resonance position of Trp^3 at 7.37 ppm, being close to its random coil value.

The present paper demonstrates that the photo-CIDNP method as such or combined with special pulse sequences can be conveniently used to provide data needed for peak assigments in the ^{1}H-NMR spectra of the protein species studied. The assigned resonances can be used to identify conformational differences between different related proteins, or to monitor changes in the protein upon binding of micelles. The latter possibility is currently investigated.

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