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Proton NMR resonance assignments and surface accessibility of tryptophan residues of a dimeric phospholipase A_2 from *Trimeresurus flavoviridis*

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Proton NMR spectra of a dimeric phospholipase A_2 from *Trimeresurus flavoviridis* have been recorded. N-1 proton resonances of the tryptophan indole rings have been detected and assigned to specific positions, Trp-3/Trp-30, Trp-68 and Trp-108, by comparing the spectra of the enzyme derivatives with tryptophans oxidized to differing extents. Photo-CIDNP experiments have revealed that Trp-68 and Trp-108 are exposed while Trp-3 and Trp-30 are buried in the molecule. This is consistent with the X-ray crystal structure of a homologous phospholipase A_2 from *Crotalus atrox* where residues 3 and 30 are located at a dimer interface, but inconsistent with the results of stepwise oxidation of tryptophan residues.

Phospholipase A_2; Tryptophan; ^{1}H-NMR; Photo-CIDNP; Surface accessibility; *N*-Bromosuccinimide oxidation

1. INTRODUCTION

Phospholipase A_2 (EC 3.1.1.4) hydrolyzes the second ester bond of 1,2-diacyl-*sn*-phosphoglycerides. Phospholipase A_2 from Japanese Habu snake, *Trimeresurus flavoviridis,* is composed of two non-covalently associated, identical subunits, each of which consists of 122 amino acid residues [1]. The sequence shares high homology with that of a dimeric phospholipase A_2 from *Crotalus atrox* [2], whose crystal structure has been determined [3,4]; 70% (80%) of the residues in the *T. flavoviridis* enzyme are preserved (kept in a type-conservative manner) in the *C. atrox* enzyme. The deduced crystal structure of *C. atrox* phospholipase A_2 shows unusual features in that most residues in the putative catalytic site are located at a dimer interface and are shielded from the bulk solvent [4]. How the substrate approaches the catalytic center in such dimeric phospholipase A_2 is a mystery; there may be a difference in dimer topology between the enzyme in solution and that in the crystal; calcium binding or substrate binding may induce a conformational change in the enzyme so that the catalytic site becomes open for the substrate.

Here, we have recorded ^{1}H-NMR spectra of *T. flavoviridis* phospholipase A_2 in H_2O solution and assigned N-1 proton resonances from 4 tryptophan residues, two of which are expected to be located at a dimer interface. The photochemically induced dynamic nuclear polarization (CIDNP) technique has been applied to probe the surface accessibility of tryptophan residues and the results are compared with those of stepwise oxidation of tryptophan residues [5].

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2. MATERIALS AND METHODS

Phospholipase A_2 was purified from the venom of *T. flavoviridis* [6], and oxidation of tryptophan residues was carried out with *N*-bromosuccinimide (NBS) at pH 4.0 according to [5].

270-MHz and 400-MHz ^{1}H-NMR spectra were recorded on Bruker WH-270 and AM-400 spectrometers. Photo-CIDNP experiments were performed at 360 MHz on a Bruker HX-360 spectrometer as in [7]. NMR spectra in H_2O solutions were measured with presaturation of a water signal (figs.2,3) or by a 1-1 pulse technique (fig.1) [8].

3. RESULTS AND DISCUSSION

N-1 proton resonances of tryptophan residues are expected to occur at around 10 ppm. The low-field region of a 400-MHz ^{1}H-NMR spectrum of *T. flavoviridis* phospholipase A_2 (in H_2O solution) is shown in fig.1A. A group of overlapping resonances at 6.4–9.2 ppm is mainly from aromatic and backbone NH protons. Since the N-1 proton of tryptophan residues is in the vicinity of the indole C-2 proton and C-7 proton, large nuclear Overhauser effects (NOEs) among these protons are expected. Thus, NOE experiments were performed in H_2O solution; resonances at around 10 ppm were saturated by selective irradiation and the resulting NOE was extracted by taking difference spectra (fig.1B–E). The irradiation of resonances b–d gives an NOE on singlet resonances at 7.38, 7.22 and 7.17 ppm, respectively. Doublet resonances are also found in the NOE difference spectra at 7.45, 7.49 and 7.40 ppm on irradiation of resonances b–d, respectively. Therefore resonances b–d were assigned to tryptophan N-1 protons and the corresponding singlet and doublet resonances found in the NOE-difference spectra to C-2 and C-7 protons in the same residues. Irradiation of resonance a produces an NOE on slightly broad resonances at 7.21 and 7.09 ppm, and therefore the resonance (1) was tentatively assigned to the N-1 proton of the fourth tryptophan residue. Irradiation of other resonances at around 10 ppm did not produce such NOEs characteristic of tryptophan residues. The pH dependences of the hydrogen exchange rates of these protons, which were monitored as line broadening in the spectra taken in H_2O solution (with presaturation of the solvent signal), also support these assignments (not shown).

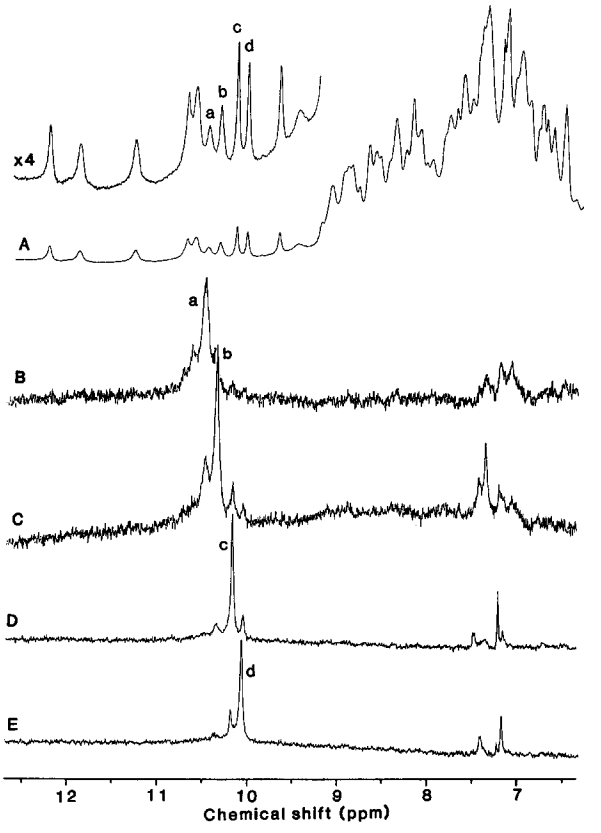

Fig.1. The 400-MHz ^{1}H-NMR spectrum of 1 mM *T. flavoviridis* phospholipase A_2 in H_2O solution (pH 5.5, 0.1 M $CaCl_2$, 40°C) (A) and NOE difference spectra between those with and without preirradiation (0.5 s) of resonances a–d (B–E).

In order to assign further the tryptophan N-1 proton resonances (labeled a–d) to the specific positions in the sequence, phospholipase A_2 derivatives were prepared, in which tryptophan residues were oxidized to varying extents [5], and their ^{1}H-NMR spectra were compared (fig.2). As oxidation proceeded, broad resonances a and b first decreased in intensity and then resonance c followed, and finally resonance d became weak. The chemical shifts of these resonances were not affected by the oxidation. Since it was previously found that Trp-3 and Trp-30 were oxidized first at nearly equal rates and then Trp-68, and finally Trp-108 were oxidized [5], resonances a and b were assigned to Trp-3 or Trp-30, resonance c to Trp-68, and resonance d to Trp-108.

In photo-CIDNP experiments, NMR signals are enhanced only for protons of the aromatic residues

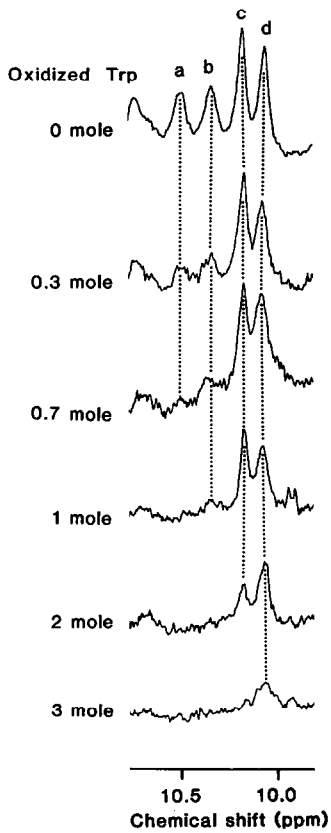

Fig.2. The 270-MHz ^{1}H-NMR spectra of 1 mM *T. flavoviridis* phospholipase A_2 in H_2O solution (pH 5.4, 0.1 M $CaCl_2$, 40°C) with tryptophan residues oxidized to different extents. The numbers of moles of oxidized tryptophan residues per subunit was determined [5] and is indicated.

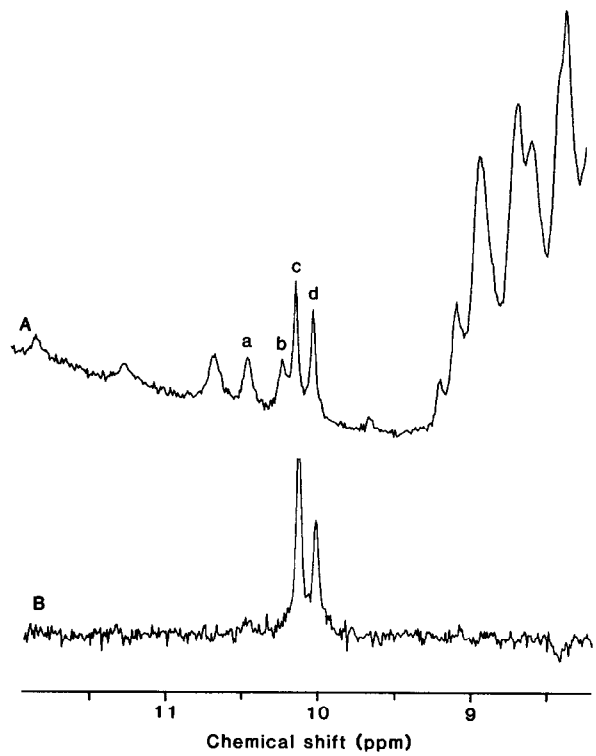

Fig.3. The 360-MHz ^{1}H-NMR spectrum of 1 mM *T. flavoviridis* phospholipase A_2 in H_2O solution (pH 4.7, 0.1 M $CaCl_2$, 40°C) and a photo-CIDNP difference spectrum (B). The latter is the difference between a 'light' and a 'dark' spectrum. For the light spectrum the sample was irradiated for 0.5 s with an argon ion laser in the presence of 0.1 mM flavin.

that are accessible to the photoexcited dye (flavin), so that resonances from surface residues can be detected. Fig.3 shows photo-CIDNP difference spectra ('light' minus 'dark' spectra) of phospholipase A_2 in H_2O solution. The N-1 proton resonances from only Trp-68 and Trp-108 are extracted in the photo-CIDNP spectrum, indicating that these two residues are on the molecule's surface. Trp-3 and Trp-30 are probably buried in the molecule.

The present photo-CIDNP results on *T. flavoviridis* phospholipase A_2 in solution are consistent with the dimer topology found for the *C. atrox* enzyme in the crystal form, where residues 3 and 30 are located at a dimer interface and residues 68 and 108 are exposed [3,4]. On the other hand, the revealed accessibility of tryptophan residues does not readily fit with the NBS-oxidation results: susceptibility to oxidation was found to be in the order, Trp-3 = Trp-30 > Trp-68 > Trp-108 [5]. NBS may perhaps have unusually high affinity for a dimer interface, which contains Trp-3 and Trp-30, of the phospholipase A_2.

The assigned, well-resolved N-1 proton resonances of the 4 tryptophan residues in *T. flavoviridis* phospholipase A_2 will provide useful probes to monitor a change in intersubunit interactions which may permit access of the substrate to the shielded catalytic site.

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