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Note

Application of laser photo-CIDNP for an intact glycoprotein in solution *

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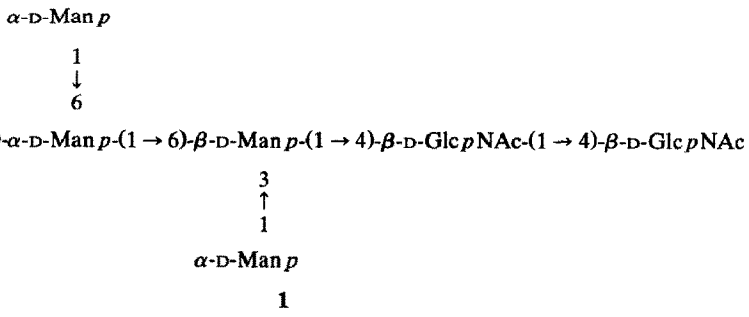
Carbohydrate chains of glycoproteins have been shown to take part in cell–cell interactions, like sperm adhesion to egg[1], in host pathogen interactions, e.g., adhesion of some bacteria to epithelial cells[2], in protein sorting, such as the targeting of lysosomal enzymes[3], and in expression of hormonal activity, which is important for the pituitary glycoprotein hormones, lutropin and follitropin[4]. However, despite the convincing evidence for essential roles for many glycoprotein glycans, relatively little is understood about the physical basis for these functions[5]. Until now, no complete three-dimensional structure of an intact glycoprotein has been reported. As part of our research program, bovine pancreatic ribonuclease B (RNase-B) has been chosen as a model system to study an intact glycoprotein in solution. This glycoprotein consists of 124 amino acids and one N-linked, oligomannose-type of carbohydrate chain, the major component being[6] $Man_5GlcNAc_2$



(1). Larger carbohydrate structures containing one to three additional (1 → 2)-linked α-D-mannose units have been found as minor components. Both the crystal[7]

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and the solution[8,9] structure are known for the nonglycosylated form, ribonuclease A (RNase-A). Thus, this system offers a good opportunity to estimate the influence of the carbohydrate chain on the protein.

Laser, photochemically-induced, dynamic nuclear polarisation (photo-CIDNP) is a powerful method to study the surface properties of an intact protein in solution[10,11], but has never been used for glycoproteins. Here, laser photo-CIDNP is introduced to assess the physical role of a carbohydrate chain on the protein part of a glycoprotein in solution. The surface exposure of aromatic amino acids in RNase-B is compared with that of RNase-A and with that of enzymically deglycosylated RNase-B (dg-RNase-B).

Bovine pancreatic RNase-B and -A contain four histidine, six tyrosine, and three phenylalanine residues as aromatic amino acid residues. The aromatic region of the ^{1}H NMR spectrum of RNase-A has previously been assigned[8,9,12]. Also laser photo-CIDNP studies have been performed on RNase-A[12,13]. In the present study, RNase-A was included in order to be able to directly compare its photo-CIDNP spectrum with those of RNase-B and of dg-RNase-B. The photo-CIDNP difference spectrum of RNase-B is shown in Fig. 1. By comparing the spectrum with that of RNase-A (Fig. 1), two strong emission (negative) lines of the H-3,5 atoms of the aromatic ring of tyrosine residues are assigned to Tyr-76 and Tyr-115 (see ref. 12). One histidine residue (His-119 in the active site) shows enhanced absorption (positive peaks) at the positions of its H-2,4 atoms of the imidazole ring. No other polarised residues can be seen. The photo-CIDNP difference spectrum of dg-RNase-B is shown in Fig. 1. As is the case for RNase-B and -A, polarisation of two tyrosine and one histidine residues is observed. Thus, based on the similarity of the photo-CIDNP difference spectra, it can be concluded that the carbohydrate part of the glycoprotein has no influence on the surface exposure of the aromatic amino acid residues of bovine pancreatic ribonuclease.

The results of the present report are in agreement with previous studies by Puett[14], who has used various other spectral properties [circular dichroism (CD), optical rotatory dispersion, and ultraviolet denaturation difference spectroscopy] to compare bovine RNase-A and -B, and with those of Berman et al.[15], who have applied ^{13}C NMR spectroscopy. However, for porcine pancreatic ribonuclease, Wang and Hirs[16] have shown, using CD measurements and spectrophotometric titrations, that the carbohydrate chains influence both the conformation and the stability of the protein. In contrast to bovine pancreatic RNase-B, the porcine enzyme has three N-linked carbohydrate chains[17]. This stresses the care which has to be taken when trying to generalize the role of glycoprotein glycans.

It should be noted that the laser photo-CIDNP method can be used for quite large systems, which give too complex 2D and 3D NMR spectra for sequence specific resonance assignments, necessary for obtaining distance constraints for deducing a three-dimensional structure[18]. This should allow future laser photo-CIDNP studies on other intact glycoproteins of considerable complexity. It has also to be expected that laser photo-CIDNP could be used to study lectin–carbo-

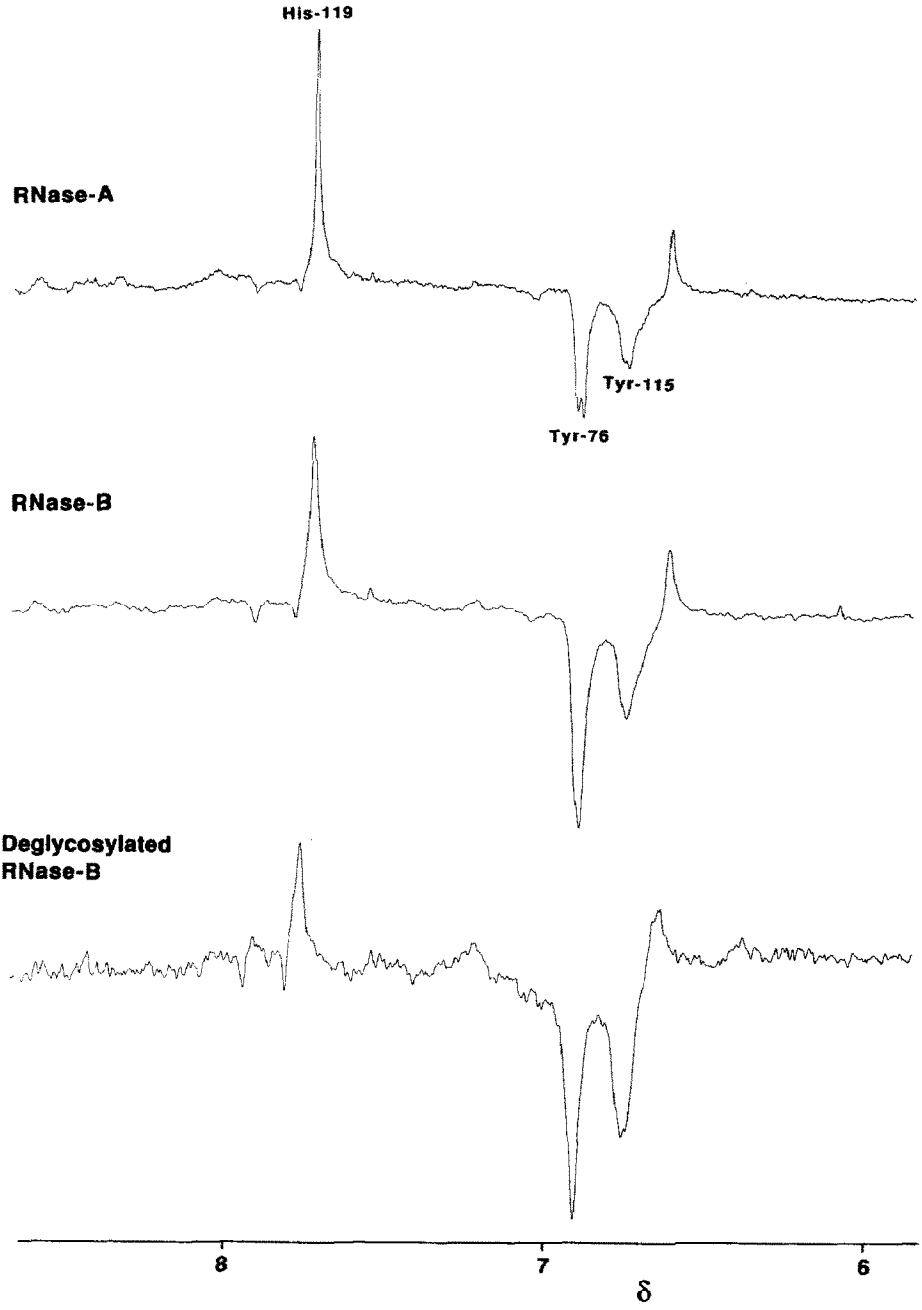

Fig. 1. Photo-CIDNP difference spectra of RNase-A, RNase-B, and deglycosylated RNase-B. For RNase-B and -A 4 "light" scans and 16 "dark" scans were collected, and for dg-RNase-B 16 "light" scans and 64 "dark" scans.

hydrate interactions. Comparison of the polarized amino acid residues of a lectin in the presence and in the absence of a carbohydrate ligand could give information on aromatic amino acid residues important for the carbohydrate–protein recognition process.

EXPERIMENTAL

Materials.—Bovine pancreatic ribonuclease A (RNase-A, type XII A) and B (RNase-B, type XII B) were obtained from Sigma Chemical Co. (St. Louis, MO). Concanavalin A (Con A) Sepharose and agarose–5′-(4-aminophenylphosphoryl)-uridine 2′(3′)-phosphate [AG 2′(3′), 5′-UDP] were purchased from Pharmacia. Peptide-N^4-(*N*-acetyl-β-D-glucosaminyl)asparagine amidase F (PNGase-F) from *Flavobacterium meningosepticum* (EC 3.5.1.52) was from Boehringer Mannheim. Flavin I (*N*-3-carboxymethyllumiflavin, FI) was a gift of Dr. F. Müller (Sandoz, Basel). Deuterium oxide (99.9 and 99.96 atom% D) was obtained from MSD Isotopes (Montreal, Canada).

Purification of ribonuclease B.—The commercial preparation of RNase-B contains significant amounts of RNase-A, as was seen from an extra band on sodium dodecyl sulfate–poly(acrylamide)gel electrophoresis and proven by N-terminal amino acid sequencing (data not shown, cf. Williams and Lennarz[19]). Therefore, the commercial RNase-B sample was purified by lectin affinity chromatography on Con A Sepharose. All purification steps of the (glyco)proteins were carried out at 4°. Commercial RNase-B (50 mg), dissolved in 10 mM Tris · HCl, pH 7.5 (10 mL), containing 0.15 M NaCl and mM each of $CaCl_2$, $MgCl_2$, and $MnCl_2$ (Con A buffer), was loaded on a column (2.5 × 7 cm) of Con A–Sepharose. The column was closed for 30–60 min, and then the contaminating RNase-A was eluted with Con A buffer (50 mL), followed by RNase-B eluted with the Con A buffer containing 0.5 M methyl α-D-mannopyranoside. Salts and methyl α-D-mannopyranoside were removed from purified RNase-B by repeated ultrafiltration under N_2 pressure and dilution with 25 mM NH_4HCO_3, pH 8, in a 10-mL Amicon cell equipped with a Diaflo YM5 membrane. Because sodium dodecyl sulfate poly(acrylamide)gel electrophoresis and N-terminal amino acid sequencing of the salt-free sample showed a 10% contamination with Con A, affinity chromatography on a column of AG2′(3′), 5′-UDP was used to remove Con A from the RNase-B sample[19,20]. To this end, the lyophilized sample was dissolved in 20 mM sodium acetate, pH 5.2, containing 0.1 M methyl α-D-mannopyranoside, and applied to a column (1.2 × 5.5 cm) of AG2′(3′), 5′-UDP. The column was closed for 90 min, and then unbound material was eluted with the same buffer, and active RNase with 20 mM sodium acetate, pH 5.2, containing 4 M NaCl. Salts were removed by repeated ultrafiltration and dilution with 25 mM NH_4HCO_3, pH 8, on a Diaflo YM5 membrane.

Enzymic deglycosylation of purified ribonuclease B.—The N-linked carbohydrate chains of RNase-B were enzymically removed by PNGase-F. The purified glyco-

protein (15 mg) was dissolved in 50 mM Tris · HCl, pH 7.1 (1.5 mL), containing 50 mM EDTA. The solution was incubated overnight at room temperature with 25 units of PNGase-F, then another batch of 25 units of PNGase-F was added, and the incubation was continued for 8 h. To prevent any denaturation of the protein, no detergents or reducing agents were added to the incubation mixture. Deglycosylated RNase-B (dg-RNase-B) was separated from intact RNAse-B by lectin affinity chromatography on Con A Sepharose. Finally, affinity chromatography on a column of AG2′(3′), 5′-UDP was used to remove any residual Con A in the samples.

Laser ^1H photo-CIDNP.—The samples contained 0.2–1 mM protein and 0.4 mM *N*-3-carboxymethyllumiflavin in D_2O, pH 7.2. ^1H Photo-CIDNP experiments were performed at 300 K on a Bruker WM-360 NMR spectrometer essentially as described before[11], except that the light from an Ar-ion laser was introduced into the NMR probe by use of an optical fiber. Difference spectra were obtained by alternately recording "light" and "dark" free-induction decays (fid's) and subtracting the resulting spectra. The "light" fid was acquired after irradiation of the sample for 0.5 s with the Ar-ion laser beam (5 W) prior to data sampling. The residual HOD signal was presaturated during 2 s. All data processing was carried out on a VAXstation 3100 using the TRITON NMR software package, developed at the Department of NMR Spectroscopy, Utrecht University.

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