Spectroscopic and Protein Chemical Analyses Demonstrate the Presence of C-Mannosylated Tryptophan in Intact Human RNase 2 and Its Isoforms†

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ABSTRACT: Recently, the C-mannosylation of a specific tryptophan residue in RNase 2 from human urine has been reported [Hofsteenge, J., et al. (1994) *Biochemistry 33*, 13524−13530; de Beer, T., et al. (1995) *Biochemistry 34*, 11785−11789]. In those studies, identification of this unusual modification was accomplished by mass spectrometric and NMR spectroscopic analysis of peptide fragments. The evidence for the occurrence of C^2-α-mannosyltryptophan [(C^2-Man-)Trp] in the intact protein relied exclusively on the detection of the same phenylthiohydantoin derivatives during Edman degradation. In this paper, we have (1) excluded the possibility that (C^2-Man-)Trp arose artificially under the acidic conditions previously employed for protein and peptide isolation and analysis, by maintaining the pH >5 throughout these procedures, (2) demonstrated the occurrence of (C^2-Man-)Trp in the intact protein, by NMR spectroscopy, (3) showed that (C^2-Man-)Trp is not unique for RNase 2 from urine but that it is also present in the enzyme isolated from erythrocytes, and (4) found also that high-molecular mass isoforms of urinary RNase 2 are *C*-mannosylated. These observations firmly establish C-mannosylation as a novel way of post-translationally attaching carbohydrate to protein, in addition to the well-known N- and O-glycosylations. Furthermore, the NMR data, in combination with molecular dynamics calculations, indicate that in the native protein the mannopyranosyl residue is in a different conformation than in the glycopeptide or denatured protein, due to protein−carbohydrate interactions.

Recently, it was found that Trp-7 in human RNase 2[1] is modified by C-glycosidic attachment of an α-mannosyl residue (Hofsteenge et al., 1994; de Beer et al., 1995). In contrast to the previously known N- and O-glycosidic linkages to asparagine and serine, or threonine, the mannopyranosyl C1′ atom is in this case directly linked to the C2 atom of the tryptophan indole ring. The evidence for this was obtained from mass spectrometric and NMR spectroscopic analyses of peptides isolated from human urinary RNase 2.

The discovery of this unusual modification raises interesting questions about its biosynthesis and function. However, before these can be tackled, two questions related to the authenticity of this modification should be answered. First, is the mannopyranosyl residue on Trp-7 originally present in the protein, or is (C^2-Man-)Trp[2] possibly formed during purification or analysis of the protein and/or its peptides? So far the evidence for the presence of (C^2-Man-)Trp in the intact protein relies on the detection of the same PTH derivative(s) during Edman degradation of the protein and the peptides derived from it (Hofsteenge et al., 1994). However, this procedure employs highly acidic conditions under which the indolic tryptophan side chain can undergo a variety of modifications (Fontana & Tonioli, 1976). Also during isolation and purification, both RNase 2 and its peptides were temporarily exposed to acidic conditions (pH ∼2). From studies on peptide synthesis, it is known that position-dependent, acid-catalyzed carbenium ion reactions at C2 of the indole moiety are common (Barany & Merrifield, 1980; King et al., 1990). Trp-7 in RNase 2 is preceded by Thr-6. If this residue was originally glycosylated, it would be conceivable that an acid-catalyzed reaction causes a migration of the sugar moiety to the neighboring Trp-7 residue.[3] Thus, it is important to verify that no alterations

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[1] RNase 2 is identical with RNase U_s. The nomenclature used here was proposed by Zhou and Strydom (1993).

[2] Abbreviations: 2D, two-dimensional; CID, collision-induced dissociation; ESI, electrospray ionization; EI, electron impact ionization; FID, free induction decay; HSQC, ^{1}H-detected heteronuclear single-quantum coherence spectroscopy; MLEV, composite pulse designed by M. Levitt; NOE/ROE, nuclear Overhauser enhancement/rotating frame NOE; PFG, pulsed field gradient; PTH, phenylthiohydantoin; TFA, trifluoroacetic acid; TOCSY, total correlation spectroscopy; TPPI, time-proportional phase increment; (O^3-Man-)T, O^3-mannosylthreonine; (C^2-Man-)Trp and (C^2-Man-)W, C^2-α-mannopyranosyltryptophan.

[3] We thank one of the referees of one of our previous papers (Hofsteenge et al., 1994) for suggesting this possibility.

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were introduced in the protein or peptides by using acidic conditions during isolation and analysis.

Second, is the modification unique to RNase 2 from urine and the particular isotype studied so far? In human RNase 2 isolated from blood cells (Gleich et al., 1986a; Yasuda et al., 1990a) and several tissues (Sorrentino et al., 1988; Yasuda et al., 1990; Shapiro & Vallee, 1991), the amino acid at position 7 could not be identified by Edman degradation, suggesting a post-translational modification in these proteins as well. However, in none of these cases has the exact nature of the modification been established. The possibility that (C^2-Man-)Trp does not occur in all isoforms of RNase 2 is suggested by the results of Sakakibara et al. (1992). These authors have found a high-molecular mass isoform of RNase 2 (RNase UpI-2) in the urine of pregnant women, which contains larger N-linked oligosaccharides, as well as an extension of the N-terminus with a tetrapeptide. The tryptophan at position 11 in this protein (corresponding to Trp-7 in RNase 2) has been reported not to be modified.

To answer these questions, we have herein (i) analyzed human urinary RNase 2 and its peptides by NMR and MS, respectively, keeping the pH >5 throughout purification and analysis, (ii) examined the effect of acid on the synthetic glycopeptide F(O^3-Man-)TWA, and (iii) investigated RNase 2 from erythrocytes and the high-molecular mass isoform from urine.

MATERIALS AND METHODS

Purification of RNase 2 and High-Molecular Mass Isoenzymes from Urine. RNase 2 obtained using low-pH conditions (lp-RNase 2[4]) was purified by chromatography on SP-Sephadex at pH 3.0, heparin Sepharose, and SP-Sephadex at pH 7.5 and reversed-phase HPLC as described by Hofsteenge et al. (1994). In a separate isolation, the procedure was altered to avoid conditions with low pH, yielding hp-RNase 2. Male human urine was diluted with 1 volume of distilled water (the initial pH was between 6.6 and 6.8 and was lowered to 5.0 with dilute acetic acid) and loaded onto a column of SP-Sephadex equilibrated in 50 mM sodium acetate (pH 5.0). Elution was achieved with 100 mM Tris-HCl (pH 7.5) containing 500 mM NaCl. The fractions containing RNase activity were dialyzed against 20 mM Bistris (pH 6.0) and applied to a Mono S HR5/5 FPLC column (Pharmacia) equilibrated with the same buffer. The maximum amount of protein loaded was 10 mg. Bound protein was eluted with 60 mL of a linear gradient (0 to 500 mM NaCl in the equilibration buffer) at a flow rate of 1 mL/min. The active fractions, eluting between 230 and 265 mM NaCl, were pooled and concentrated to 2 mL by ultrafiltration (DIAFLO ultrafilter YM5; AMICON). Final purification of hp-RNase 2 was achieved by gel filtration on a Sephadex G-75 column [1 × 120 cm; 100 mM $NaHCO_3$ (pH 8.0) containing 250 mM NaCl, at a flow rate of 6 mL/h]. hp-RNase 2 eluted at a volume of approximately 65 mL.

High-molecular mass isoenzymes were isolated from a crude urinary preparation of human chorionic gonadotropin from pregnant or menopause women (a generous gift from AKZO, Diosynth, The Netherlands) by chromatography on heparin Sepharose equilibrated with 10 mM Bistris (pH 6.0). Separation of high- and low-molecular mass isoforms of RNase 2 was achieved by elution with 280 mM NaCl in equilibration buffer, followed by elution with 560 mM NaCl. High-molecular mass isoforms, eluting at the lower NaCl concentration, were extensively dialyzed against 10 mM Bistris (pH 6.0) and subsequently rechromatographed on heparin Sepharose using a gradient of 0 to 350 mM NaCl. Final purification of the active fractions, eluting at approximately 250 mM NaCl, was achieved by reversed-phase HPLC as described for lp-RNase 2 (Hofsteenge et al., 1994).

Purification of RNase HE-1 from Erythrocytes. A human erythrocyte concentrate, derived from 2 L of whole blood, was obtained from the Swiss Red Cross Blood Center, Bern. All procedures were performed at 4 °C, except HPLC which was performed at room temperature. After extraction of the erythrocytes with 0.25 N H_2SO_4 (Yasuda et al., 1990a), the precipitate (30−80% acetone fraction) was dissolved in 10 mM sodium acetate (pH 5.0), dialyzed against the same buffer, and applied to an SP-Sepharose Fast Flow column (5 × 30 cm), equilibrated with the dialysis buffer. The column was washed with 300 mL of the same buffer and eluted stepwise with 300 mL of 300 mM NaCl in 10 mM sodium acetate (pH 5.0), followed by elution with 700 mL of a linear gradient (300 to 500 mM NaCl, in the same buffer) and 800 mL of a linear gradient (500 to 600 mM NaCl, in the same buffer). RNase activity eluted at approximately 360 mM (peak I), 510 mM (peak II), and 550 mM NaCl (peak III). Fractions containing the major amount of activity, peak II, were combined, diluted five times with 10 mM Bistris (pH 6.0), and applied to a heparin Sepharose column (2.5 × 8 cm) equilibrated with 10 mM Bistris (pH 6.0) and 100 mM NaCl. The column was washed with 400 mL of equilibration buffer, with 400 mL of the same buffer containing 280 mM NaCl, and eluted with 800 mL of a linear gradient (280 to 1000 mM NaCl, in the same buffer). Fractions containing RNase activity, eluting at about 500 mM NaCl, were pooled, dialyzed against 50 mM NH_4HCO_3, and freeze-dried. Final purification was achieved by reversed-phase HPLC as described above for RNase 2.

Western Analysis. Specific antibodies to RNase 2 from human urine were raised in rabbits. Approximately 500 μg of purified RNase 2 was injected with Freund's complete adjuvant. Antibodies (9.5 mg) were purified on a column of RNase 2-Sepharose according to the method of Campbell et al. (1951). SDS−PAGE on 12.5% polyacrylamide gels was performed according to the method of Laemmli (1970). Proteins were blotted onto nitrocellulose using 10 mM CAPS (pH 11.0) containing 10% methanol. Probing with antibodies and detection were performed as described in the manual to the ECL Western blotting detection system (Amersham).

Peptide Isolation. Peptides from the N-terminal region of RNase 2 and RNase HE-1 were obtained by digestion of reduced and carboxymethylated protein (Hofsteenge et al., 1991) with thermolysin [3% w/w; in 50 mM NH_4HCO_3 (pH 7.8) containing 10 mM $CaCl_2$ at 37 °C for 3 h], yielding residues 5−10. Subsequent digestion of this thermolytic hexapeptide with elastase [3% w/w; 50 mM NH_4HCO_3 (pH 7.8) at 37 °C for 4 h] yielded residues 5−8. Alternatively, native protein was preincubated for 10 min at 75 °C and digested with thermolysin [3% w/w; 50 mM NH_4HCO_3 (pH 7.8) containing 10 mM $CaCl_2$ at 75 °C for 45 min]. Peptides were purified on a C_{18} reversed-phase HPLC column using a trifluoroacetic acid solvent system (system I; pH 2.1) or

[4] RNase 2 isolated using acidic conditions has been designated lp-RNase 2 to distinguish it from the enzyme isolated at higher pH, hp-RNase 2.

an acetic acid/trimethylamine solvent system (system II; pH 6.0) as described by Hofsteenge et al. (1991).

Peptide Synthesis. F(O^3-Man-)TWA was manually synthesized on poly(ethylene glycol)−poly(dimethylacrylamide copolymer) (PEGA) resin (0.3 g, 0.03 mmol) (Meldal, 1992), derivatized with 4-(hydroxymethyl)phenoxyacetic acid (HMPA) linker (Atherton & Sheppard, 1989) using 2-(1*H*-benzotriazo-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) (Knorr et al., 1989). The first amino acid (Fmoc-Ala-OH) was coupled by the Blankemeyer-Menge procedure (Blankemeyer-Menge et al., 1990). N^{α}-Deprotections were carried out with 20% piperidine in DMF for 10 min, and W and F were incorporated as their N^{α}-fluoren-9-ylmethoxycarbonyl (Fmoc)-protected pentafluorophenyl (Pfp) esters (Kisfaludy & Schön, 1983) (3 equiv) with the addition of 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine (DhbtOH, 1 equiv) (König & Geiger, 1970). The side chain of W was protected with an *N-tert*-butoxycarbonyl (Boc) group, and the coupling times were 2 h. The glycosylated building block N^{α}-FmocThr(α-ManAc$_4$)OPfp (Andrews & Seale, 1993) (2 equiv) was allowed to react overnight. The glycopeptide was cleaved from the resin with a mixture of 92.5% TFA, 2.5% water, 2.5% ethanedithiol, and 2.5% triisopropylsilane (5 mL, 3 h) and purified by reversed-phase HPLC on a C_{18} column before deacetylation with sodium methoxide (pH 11, 35 min). Neutralization with solid CO_2 and purification by reversed-phase HPLC yielded F(O^3-Man-)TWA (15 mg, 73%). Five hundred megahertz ^{1}H NMR spectra of F(O^3-Man-)TWA in 1:1 CD_3COOD/H_2O (pH 1.6) were recorded on a Bruker AM-500 spectrometer: δ_H Phe 7.20−7.08 (5H aromatic), 4.37 (H^{α}), 3.08 (H^{β} and $H^{\beta'}$), Thr 8.22 ($J_{NH\alpha}$ = 8.0 Hz, $N^{\alpha}H$), 4.54 ($J_{\alpha\beta}$ = 4 Hz, H^{α}), 4.13 (H^{β}), 1.18 ($J_{\beta\gamma}$ = 6.5 Hz, γCH_3), Trp 9.84 (s, N1H), 7.95 ($J_{NH\alpha}$ = 7.0 Hz, $N^{\alpha}H$), 7.62 (J_{H4H5} = 8.0 Hz, H4), 7.38 (J_{H6H7} = 8.0 Hz, H7), 7.22 (s, H2), 7.14 (H6), 7.10 (H5), 4.75 (H^{α}), 3.32 ($J_{\alpha\beta}$ = 6.0 Hz, $J_{\beta\beta'}$ = 15.0 Hz, H^{β}), 3.20 ($J_{\alpha\beta'}$ = 7.0 Hz, $H^{\beta'}$), Ala 8.01 ($J_{NH\alpha}$ = 8.0 Hz, $N^{\alpha}H$), 4.37 (H^{α}), 1.36 ($J_{\alpha\beta}$ = 6.5 Hz, βCH3), mannopyranosyl 4.83 (s, H1′), 3.83 (J_{56} = 2.0 Hz, $J_{66'}$ = 12 Hz, H6′), 3.77 ($J_{56'}$ = 5 Hz, H6″), 3.73 (H2′ and H3′), 3.69 (J_{34} = 9.0 Hz, J_{45} = 9.0 Hz, H4′), 3.63 (H5′). ESI mass spectra were recorded in the positive mode on a VG Quattro Mass Spectrometer with 50% aqueous acetonitrile as the liquid phase, yielding $[M + H]^+$ at *m/z* 686.3 and $[M + Na]^+$ at *m/z* 708.4. ($C_{33}H_{43}N_5O_{11}$ requiring M = 685.7). Automated Edman degradation was performed as described (Hofsteenge et al., 1994) and indicated a modified amino acid in position 2, since no PTH derivative was detectable.

Stability of F(O^3-Man-)TWA. A lyophilized sample of F(O^3-Man-)TWA (3 mg) was dissolved, under argon, in neat TFA (1 mL) and heated at 56 °C for 5 min. The sample was dried for 1 h (speedvac, 40 °C), redissolved in water (0.5 mL), and lyophilized overnight. The residue was analyzed by HPLC and eluted at the same time (33.3 min) as the untreated glycopeptide (see above). Furthermore, ESI-MS analysis yielded identical molecular masses for the treated and the untreated sample, whereas automated Edman degradation confirmed the presence of a modified threonine residue in position 2. Finally, the ^{1}H NMR spectrum of the two samples in D_2O (pH adjusted to 7.5 with 0.2 M Na_2CO_3 in D_2O) was recorded.

Mass Spectrometry. Electrospray ionization MS (ESI-MS) and ESI-MS/MS measurements were carried out in the positive ion mode using a PE Sciex API III triple-quadrupole mass spectrometer (Perkin-Elmer Sciex Instruments, Thornhill, Canada). For collision-induced dissociation (CID) of mass-selected ions in ESI-MS/MS experiments, argon was used as a target gas at a density of 2.4×10^4 molecules/cm^2 and the collision energy was set to 30 eV. Samples were dissolved in a 1:1 mixture of methanol/water containing no acidic additive.

NMR Spectroscopy on RNase 2. Protein samples were dissolved in H_2O, in D_2O, or in 6 M guanidinium deuteriochloride, containing 50 mM or 0.1 M sodium chloride. The RNase 2 concentration in the NMR samples was 0.25 or 0.5 mM. The pH was adjusted to 5.1, without correction for the D isotope effect. Most NMR spectra were recorded at 295, 300, or 315 K using Bruker AMX-500 or AMX-600 spectrometers. The 2D ^{1}H NOE spectrum of RNase 2 dissolved in ^1H_2O was recorded with a Varian UnityPlus 750 MHz spectrometer (SON NMR Large Scale Facility, Utrecht University). 1D ^{1}H NMR spectra of RNase 2 were recorded using an NOE sequence collecting 64−512 FIDs of 2K data points each. The 2D ^{1}H NOE spectra (Jeener et al., 1979) were recorded with an NOE mixing time of 150−200 ms. The water resonance was suppressed as follows. Low-power irradiation was applied during the relaxation delay of 900 ms and during the NOE mixing time. In addition, a spoiler sine-bell-shaped pulsed field gradient (PFG) was included in the NOE mixing time. This PFG was applied along the *z*-axis with a strength of 75 G/cm at the center of the sine-bell and was followed by a recovery delay of 500 μs.

2D ^{1}H TOCSY spectra (Griesinger et al., 1988) were recorded using a "clean" MLEV-17 spin-lock sequence with a duration of 35−70 ms (Bax & Davis, 1985; Griesinger et al., 1988). In these experiments, low-power irradiation of the water resonance during the relaxation delay was followed by a 90° (^{1}H; $\phi_1 = x,-x$)−1 μs−90° [^{1}H; $\phi_2 = 2(x),2(-x)$] pulse scheme, a low-power irradiation pulse of 5 ms, and a spoiler PFG as above, before the actual TOCSY pulse sequence was executed. Alternatively, the PFG was replaced by a homo-spoil pulse of 5 ms, followed by a recovery delay of 15 ms. A 2D ^{1}H−^{13}C HSQC spectrum (Braunschweiler & Ernst, 1983) was recorded essentially as described (de Beer et al., 1994), but with the ^{13}C carrier frequency placed at 150.915 875 MHz and a ^{13}C sweep width corresponding to 75 ppm.

Typically, 2D experiments consisted of 400−512 t_1 experiments and 96−320 FIDs of 2K data points each were collected per t_1 experiment. Quadrature detection was achieved using either the TPPI method (Marion & Wüthrich, 1983) or the States−TPPI method (Marion et al., 1989). The NMR data sets were processed as described above or, analogously, by using the Triton NMR software package instead (Bijvoet Center, Utrecht University).

Molecular Dynamics Calculations. The molecular dynamics simulations on the peptide FT(C^2-Man-)WAQW, where (C^2-Man-)Trp is either C^2-α-D- or C^2-α-L-mannosyltryptophan, were performed using the Amber force field [Insight II (95.0), 1200 °C, 50 ps, 50 000 steps]. The initial temperature of the calculation was determined by gradually increasing it until the mannose residue was able to leave the usual $^1C_4$ chair conformation. Then, the starting structure was selected with the mannopyranosyl residue oriented as in the native protein. Every 50th step of the structure was saved for further examination.

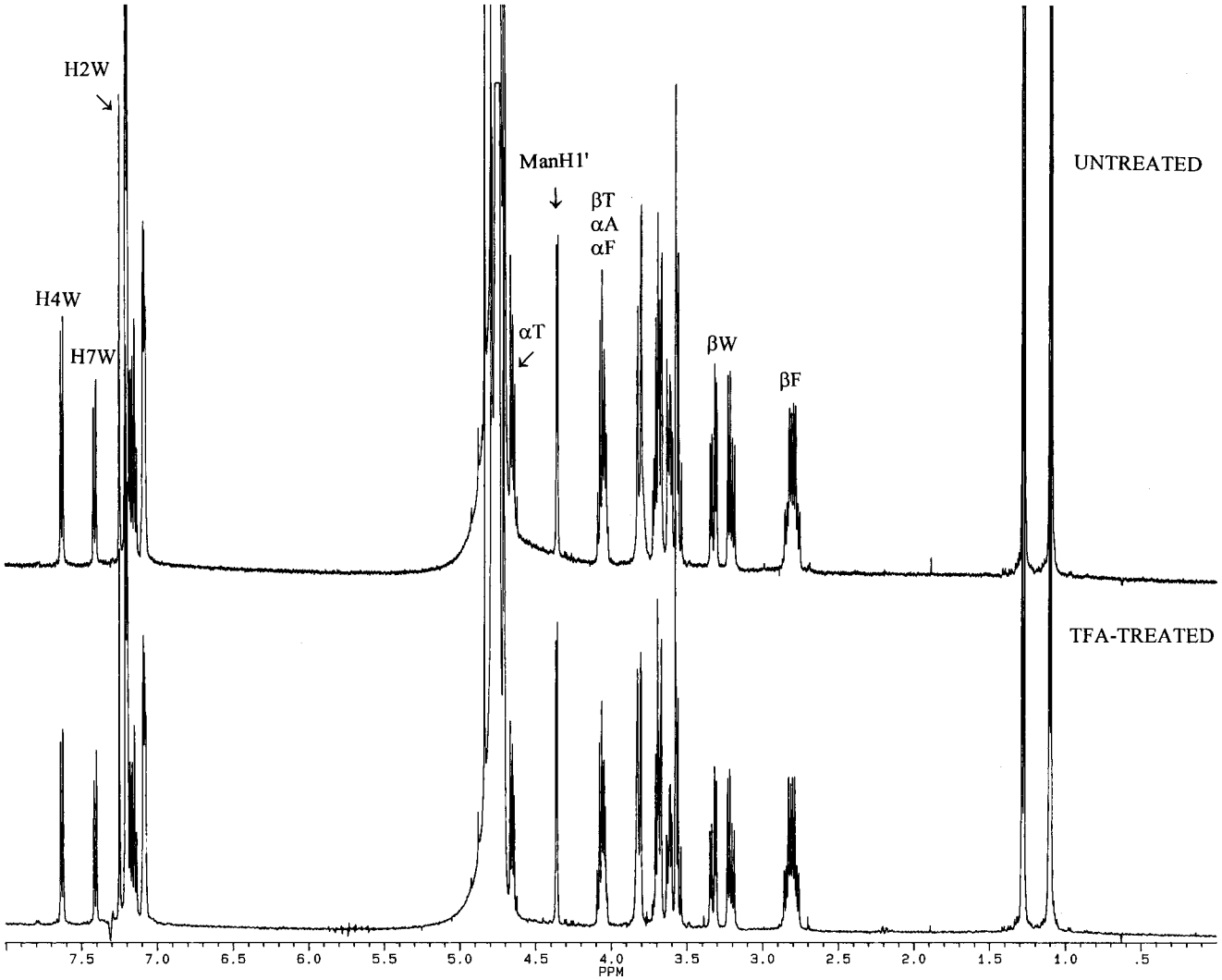

FIGURE 1: ^{1}H NMR spectra of untreated (upper panel) and TFA-treated (lower panel) samples of synthetic F(O^3-Man-)TWA. Spectra were recorded at 500 MHz in D_2O (pH adjusted to 7.5 with 0.2 M Na_2CO_3 in D_2O).

RESULTS AND DISCUSSION

Stability of the O-Mannosylated Synthetic Peptide, F(O^3-Man-)TWA. An interesting hypothesis[3] that could explain the observation of (C^2-Man-)Trp during Edman degradation of RNase 2 is the migration of a glycosyl residue from a neighboring O-glycosylated amino acid to Trp-7, during the acidic cleavage step. Since the study of such a reaction is difficult to perform in the entire protein, a model study was done using the synthetic peptide F(O^3-Man-)TWA. The peptide was treated with anhydrous TFA at 56 °C under argon, to mimic the conditions of the Edman procedure, and analyzed by HPLC, ESI-MS, ^{1}H NMR spectroscopy, and automated Edman degradation. ^{1}H NMR spectra of untreated and TFA-treated synthetic F(O^3-Man-)TWA are presented in Figure 1. The peptide treated with TFA and the untreated one were found to be completely O-mannosylated at the threonine residue and also to be otherwise identical; i.e., there was no indication of C-mannosylation of the tryptophan residue. These results showed that, under strongly acidic conditions known to promote position-dependent carbenium ion reactions at C2 of the indole moiety, no migration of the mannopyranosyl residue from Thr-6 to Trp-7 had occurred. Although unlikely, the possibility remains that in the entire protein an acid-catalyzed migration can occur from a location other than Thr-6.

Protein Chemical and Mass Spectrometric Characterization of hp-RNase 2.[4] In order to exclude the possibility that *C*-mannosylated Trp-7 results from acid-catalyzed reactions in the intact protein, RNase 2 was isolated under conditions milder than those previously used (designated hp-RNase 2). The following changes were made. (i) The first ion-exchange chromatography of the urine was conducted at pH 5.0 rather than at pH 3.0. (ii) The final purification step by HPLC using the trifluoroacetic acid solvent system I (pH 2.1) was replaced by FPLC on a Mono S column (pH 6.0), followed by gel filtration at pH 8.0. (iii) Edman degradation, which exposes the protein or peptide to high concentrations of TFA, was omitted at this stage of analysis. The changes in the purification procedure did not significantly influence the final yield and purification factor of the protein (data not shown).

To avoid acidic conditions also during the HPLC purification of the thermolytic peptide comprising residues 5−10 from hp-RNase 2, solvent system II (pH 6.0) was used. The yield of the peptide was the same as previously obtained with the 0.1% TFA system (data not shown). Its retention time on a C_{18} reversed-phase HPLC column (system II) was identical to that of the peptide obtained from lp-RNase 2 (Table 1), for which the sequence FT(C^2-Man-)WAQW has previously been determined (Hofsteenge et al., 1994).

Table 1: Comparison of lp- and hp-RNase 2 and Their Peptides[a]

	retention time (min)[a]	relative A_{280}[b]	mass ([M + H]$^+$)
lp-RNase 2			
FT(C^2-Man-)WAQW	78.3	nd[c]	1000.4
FT(C^2-Man-)WA	44.1	1.58	686.4
hp-RNase			
FT(C^2-Man-)WAQW	78.1	nd	1000.4
FT(C^2-Man-)WA	44.4	1.62	nd

[a] RNase 2 was digested with thermolysin, yielding the peptide FT(C^2-Man-)WAQW. Digestion of the hexapeptide with elastase yielded the peptides FT(C^2-Man-)WA and QW. All peptides were purified on a reversed-phase HPLC column using solvent system II. [b] The absorbance at 280 nm relative to that of unmodified tryptophan in the dipeptide QW is given. [c] nd represents not determined.

Furthermore, digestion of the thermolytic peptide with elastase yielded the peptides 5−8 and 9−10, whose absorbance ratio at 280 nm was 1.62 and 1.58, for the peptides obtained from hp- and lp-RNase 2, respectively. The value of this ratio was in good agreement with the one previously found for C-mannosylated and unmodified Trp, i.e., 1.56 (Hofsteenge et al., 1994), which suggests that the peptides from both proteins are identical and also that Trp-7 in the peptide from hp-RNase 2 is modified.

In order to confirm this identity, the thermolytic hexapeptides FT(C^2-Man-)WAQW prepared from hp- and lp-RNase 2 were compared by ESI-MS/MS using methanol/water, without acidic modifiers as the spray solvent. ESI-MS analysis of the peptides prepared from hp- and lp-RNase 2 showed that the observed mass for [M + H]$^+$ of both peptides (monoisotopic mass of 1000.4) was 162 Da higher than that expected from the unmodified hexapeptide (monoisotopic mass of 838.4; spectra not shown). In order to compare the site of substitution, as well as its type, mass-selected [M + H]$^+$ ions (*m*/*z* 1000.4) of both peptides have been subjected to low-energy CID. The ESI-MS/MS spectra obtained for the two preparations were practically identical (Figure 2). Nearly complete series of N-terminal b- and C-terminal y-ions in both spectra located the substituent at the Trp-7 residue (b-series shifted by 162 Da beyond b_2, y-series beyond y_3), and the substituted indolylmethyl cation at *m*/*z* 292 restricted the position of the modification to the tryptophan side chain. The loss of 120 Da from all ions containing the mannosylated Trp-7 (e.g., *m*/*z* 1000 → 880, and *m*/*z* 752 → 632) was typical for aromatic C-glycosides and was already observed in the EI-MS/MS spectrum of the side chain fragment *m*/*z* 292 from the isolated PTH derivative of (C^2-Man-)Trp (Hofsteenge et al., 1994). These results allow the conclusion that the acidic conditions previously used to generate the peptides did not cause the C-mannosylation of Trp-7.

Determination of (C^2-Man-)Trp in intact RNase 2. In order to exclude the possibility that (C^2-Man-)Trp was formed during the preparation of the peptide, its presence in intact RNase 2 was investigated using NMR spectroscopy. To reduce the possibility that the three-dimensional structure of the protein affects the conformational features of the (C^2-Man-)Trp moiety, which would hamper a direct comparison with NMR data obtained previously for FT(C^2-Man-)WAQW (Hofsteenge et al., 1994; de Beer et al., 1995), the ^{1}H NMR spectra of RNase 2 denatured in 6 M guanidinium deuteriochloride were recorded. Furthermore, the ^{1}H NOE and ^{1}H TOCSY spectra of denatured RNase 2 display favorable

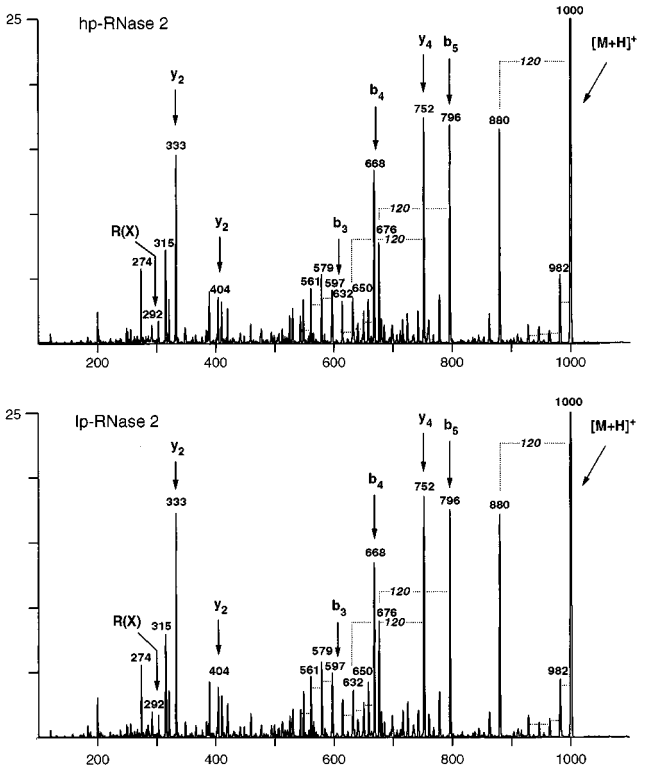

FIGURE 2: ESI-MS/MS spectra of [M + H]$^+$ ions of FT(C^2-Man-)WAQW derived from hp- (upper panel) and lp-RNase 2 (lower panel).

simplifications compared to the corresponding ^{1}H NMR spectra of native RNase 2 (compare Figures 3 and 4), which facilitated the identification of ^{1}H signals stemming from (C^2-Man-)Trp.

In the aromatic region (δ 6.9−7.6), the ^{1}H resonances of the two tryptophan residues of RNase 2, Trp-7 and Trp-10, were identified by well-resolved and characteristic scalar ^{1}H−^{1}H connectivity patterns between tryptophan H4, H5, H6, and H7 (Figure 3). Cross-peaks on the tryptophan H4 track in the ^{1}H NOE spectra subsequently led to the resonance positions of the C$^\alpha$Hs and C$^\beta$Hs (indicated for Trp-7 in Figure 3). Denaturation of RNase 2 also resulted in removal of most overlap between anomeric proton resonances and those of protein C$^\alpha$ protons. On the basis of the ^{1}H−^{1}H connectivity patterns on the anomeric tracks in ^{1}H NOE and ^{1}H TOCSY spectra, most of these spin systems were assigned as stemming from monosaccharides residing in the *N*-glycans of RNase 2. These assignments were confirmed by comparison with ^{1}H chemical shift data of the free oligosaccharide alditols of RNase 2 (Lawrence et al., 1993) and N-linked GlcNAc residues (van Zuylen et al., 1995). An additional anomeric track is located at 5.23 ppm. The cross-peak patterns on this anomeric track in ^{1}H NOE and ^{1}H TOCSY spectra of denatured RNase 2, as well as the locations of these cross-peaks, are similar to those of the Man H1′ tracks in ^{1}H ROE[5] and ^{1}H TOCSY spectra of C-linked Man in FT-(C^2-Man-)WAQW [Figure 3 and Hofsteenge et al. (1994) and de Beer et al. (1995)]. This includes the typical, intense NOE/ROE[5] cross-peak that indicates that Man H1′ and Man H6′ are close in space (Figure 3). A series of ^{1}H−^{1}H NOEs

[5] Note that we only qualitatively compare ROE spectra of FT(C^2-Man-)WAQW with NOE spectra of denatured RNase 2 and native RNase 2.

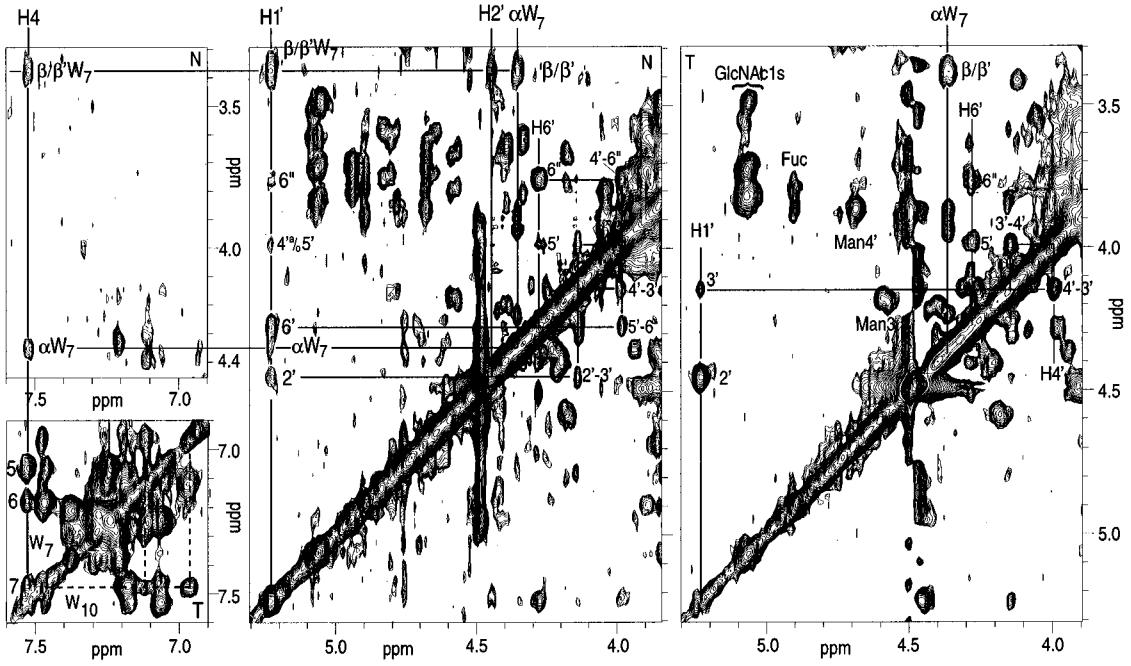

FIGURE 3: Fragments of 2D ^{1}H NOE (N) and ^{1}H TOCSY (T) spectra showing the resonance assignments of the (C^2-Man-)Trp moiety in RNase 2 dissolved in 6 M guanidinium deuteriochloride. W_7 stands for Trp-7. The numbers and Greek symbols for the mannopyranosyl and tryptophan residues have been defined in Hofsteenge et al. (1994) and Wüthrich (1986), respectively, and are shown in Figure 5A. At off-diagonal cross-peaks, they represent a correlation of the corresponding proton and the proton indicated by the vertical line. In some cases, ^{1}H−^{1}H correlations are indicated by a pair of numbers. Cross-peaks stemming from monosaccharide constituents of the *N*-glycans (GlcNAc-**1**s, Fuc, and Man) of RNase 2 have been indicated as well.

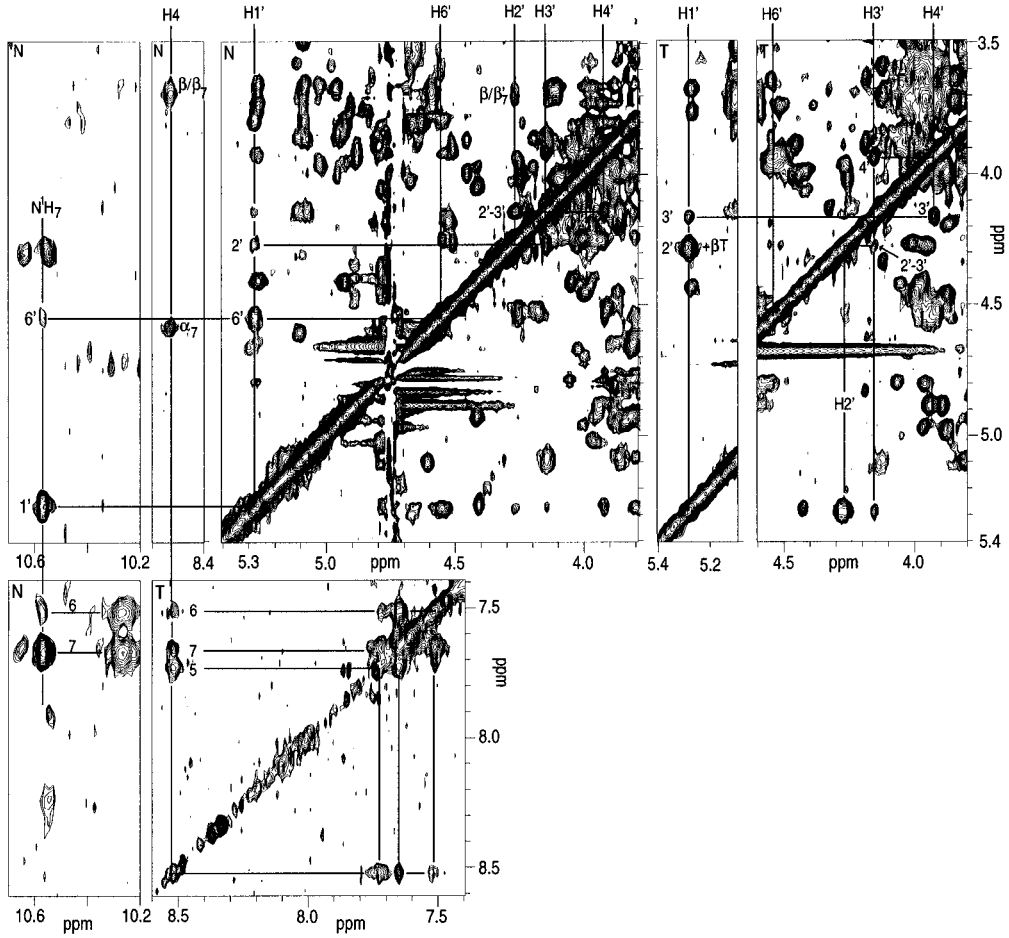

FIGURE 4: Fragments of 2D ^{1}H NOE (N) and ^{1}H TOCSY (T) spectra showing the resonance assignments of the (C^2-Man-)Trp moiety in native RNase 2. For further details, see the legend to Figure 3.

between this Man moiety and the one of the tryptophan residue (thus, Trp-7) substantiate these assignments and simultaneously prove the presence of (C^2-Man-)Trp in intact RNase 2. NOEs are observed between (i) Man H1′ and

Trp-7 C$^{\beta}$Hs, (ii) Man H1′ and Trp-7 C$^{\alpha}$H, and (iii) Man H2′ and Trp-7 C$^{\beta}$Hs (Figure 3).

With the existence of (C^2-Man-)Trp in RNase 2 firmly established, it was worthwhile to examine NMR spectra of native RNase 2. Such an analysis might reveal carbohydrate−protein interactions and constitute a first step toward elucidating the function of C-mannosylation. The assignment of the spin system of the C-linked Man substituent started with the presence of a well-resolved Man H1′−C1′ correlation at δ_H/δ_C 5.27/67.8 in a 2D ^{1}H−^{13}C HSQC spectrum (not shown), its location being very close to that of the Man H1′−C1′ correlation of the C-linked Man residue in FT(C^2-Man-)WAQW (Hofsteenge et al., 1994). Despite the severe crowdedness of cross-peaks in the relevant areas in ^{1}H NOE and ^{1}H TOCSY spectra of native RNase 2, a partial assignment of the Man moiety was achieved, which has been outlined in Figure 4. As exemplified in the ^{1}H NOE spectrum, a cross-peak of high intensity was present on the Man H1′ track, presumably stemming from the proximity of Man H1′ and H6′. The assignment of the Man H6′ resonance position was substantiated by an NOE connectivity between Trp-7 N^{1}H and the Man H6′. The proximity of Man H1′ and H6′ indicates also that in native RNase 2 the Man moiety exists in one or more conformations with a nonequatorial hydroxymethyl group, as previously concluded for the corresponding residue in FT(C^2-Man-)WAQW (Hofsteenge et al., 1994).

The assignment of the spin system of Trp-7 has been included in Figure 4 as well. Some remarkable differences exist between the NOE pattern of (C^2-Man-)Trp in native RNase 2 and the NOE/ROE pattern of (C^2-Man-)Trp in guanidinium deuteriochloride-denatured RNase 2 and in FT-(C^2-Man-)WAQW. (i) In the ^{1}H ROE spectrum of FT(C^2-Man-)WAQW, a connectivity between the Trp-7 indole N^{1}H and Man H2′ was present (Hofsteenge et al., 1994), whereas for (C^2-Man-)Trp in native RNase 2, this particular NOE is absent and instead an NOE between Trp-7 N^{1}H and the H1′ of Man is observed (Figure 4). (ii) In the ^{1}H ROE spectrum of FT(C^2-Man-)WAQW (Hofsteenge et al., 1994) and in the ^{1}H NOE spectrum of guanidinium deuteriochloride-denatured RNase 2 (Figure 3), intense cross-peaks indicated that Man H1′ was located close to the Trp-7 C$^{\beta}$Hs. In the ^{1}H NOE spectrum of native RNase 2, the intensities of the corresponding cross-peaks could not be unambiguously addressed due to overlap with an AMX spin system (Figure 4). Nevertheless, when these ^{1}H NOEs were of equal intensity as observed for this moiety in denatured RNase 2, the corresponding cross-peaks would have been more prominently present than actually observed (compare Figures 3 and 4).

These differences in NOEs indicate that a rotation around the Trp-7 C2−Man C1′ bond has occurred upon denaturation in guanidinium hydrochloride and in the peptides. This shows that the secondary structure and/or the tertiary structure of RNase 2 affects the conformational features of (C^2-Man-)Trp in native RNase 2. In this respect, it is noteworthy that several NOEs have been identified between an amide proton at high field (δ 10.26) and the H6 and H7 of Trp-7 (Figure 4), confirming that other amino acids are close to (C^2-Man-)Trp and could influence its structural properties.

Molecular Dynamics Calculations. Further insight into the different conformations of (C^2-Man-)Trp in native and denatured RNase 2 was obtained by molecular dynamics calculations on the peptide FT(C^2-Man-)WAQW. Since it is not known whether the mannopyranosyl residue in (C^2-Man-)Trp is the L- or D-stereoisomer, calculations were performed for either one. The starting structure of (C^2-Man-)Trp was selected with the mannopyranosyl residue oriented as found in the native protein. After 1.5 ps in the molecular dynamics simulation, the distance between Trp-7 N^{1}H and Man H1' was found to be 2.72 Å for the peptide with α-D-mannosyl-L-tryptophan, and Man H1' is very close to Man H6' (Figure 5B). This is qualitatively in agreement with the observed NOEs in the ^{1}H spectrum of the native protein (with the mannopyranosyl residue in the skew boat conformation). Subsequently, a rotation around the Trp-7 C2−Man C1' bond occurs and the molecule flips over to, and remains in, a conformation in which Trp-7 N^{1}H is close to Man H2′. Typical structures of the trajectory of the molecular dynamics simulation are shown in Figure 5C−F, in which the distances between Trp-7 N^{1}H and Man H2′ are 2.30 Å (C), 2.80 Å (D), 2.18 Å (E), and 3.05 Å (F). In addition, Man H1′ always remains close to the Trp-7 C$^{\beta}$Hs. These findings are qualitatively in agreement with the

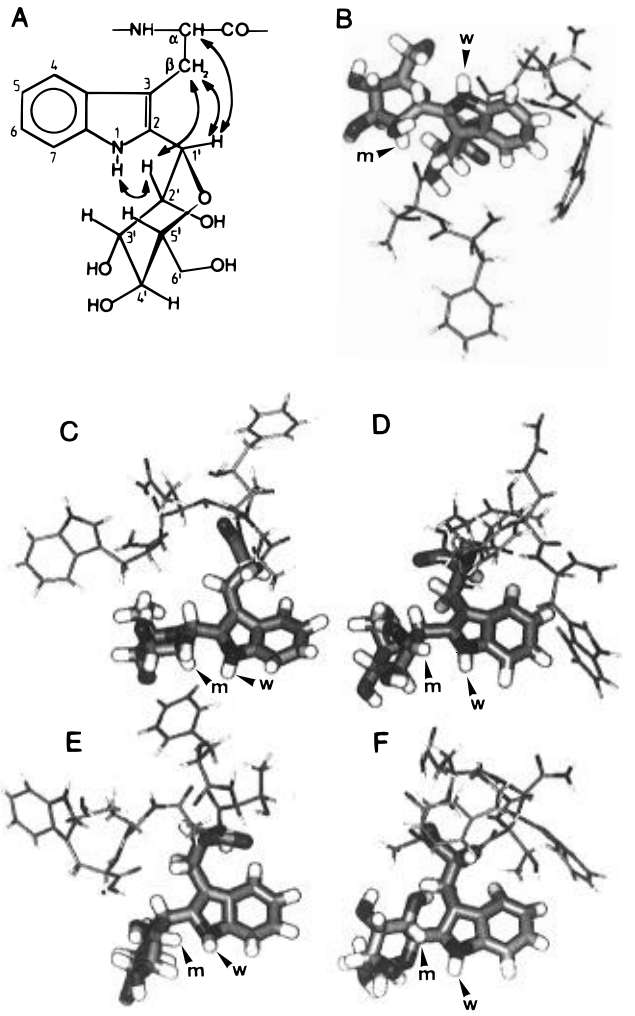

FIGURE 5: (A) Expected NOE correlations in the conformation of denaturated RNase 2 and FT(C^2-Man-)WAQW. For the sake of clarity, Man H6′ and H6″ have been omitted. (B) Structure of FT-(C^2-Man-)WAQW after 1.5 ps in the molecular dynamics simulation, which confirms the NOEs observed in the native protein. Trp-7 N1H and Man H2′ have been indicated with w and m, respectively. (C−F) Typical structures of the trajectory of the molecular dynamics simulation of FT(C^2-Man-)WAQW.

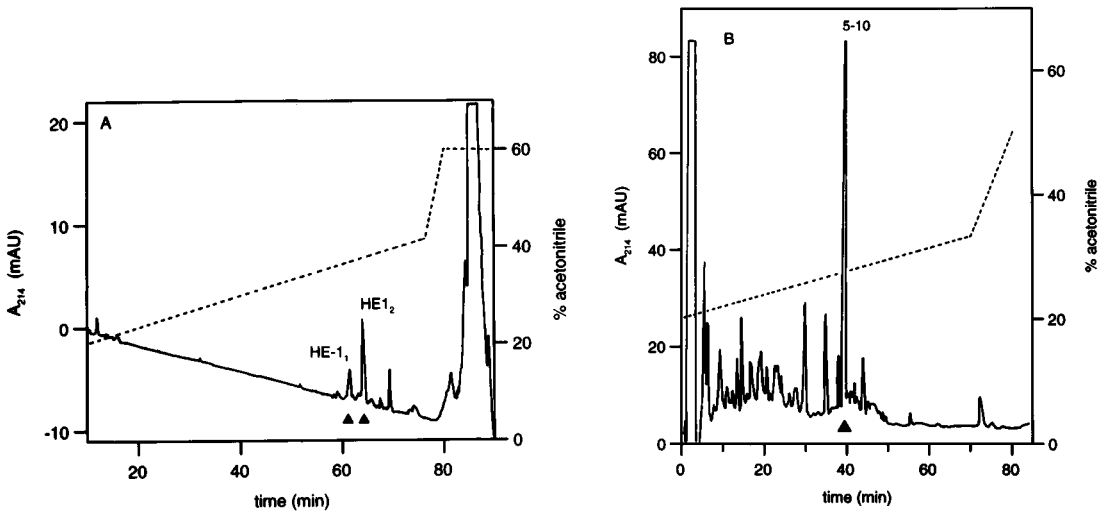

FIGURE 6: Reversed-phase HPLC of RNase HE-1 and its thermolytic peptides. (A) Final purification step of RNase HE-1 on a C_4 HPLC column. The arrowheads indicate the elution position of urinary RNase 2_1 and 2_2. (B) Elution pattern of thermolytic peptides of RNase HE-1. The arrowhead indicates the elution position of the peptide FT(C^2-Man-)WAQW from RNase 2.

observed NOEs in the ^{1}H spectra of denaturated protein and of the peptide FT(C^2-Man-)WAQW, which have been summarized in Figure 5A. During the simulation, the mannopyranosyl residue flips between a chair and a skew boat conformation, but the C-linked indole moiety occupies an equatorial position, which is compatible with the coupling constants observed for the Man residue in FT(C^2-Man-)-WAQW (de Beer et al., 1995). Essentially, the same results were obtained for the peptide containing the α-L-mannosyl stereoisomer.

These results are in agreement with the NMR data and indicate that the conformations found in the denaturated protein and the glycopeptide differ from the one in the native protein. In the latter, the conformation of the C-linked mannopyranosyl residue is stabilized by interactions with the protein. Such protein−sugar contacts are shown by the observation of NOEs between an amide proton and the H6 and H7 of Trp-7 (Figure 4).

C-Mannosylation of RNase 2 from Erythrocytes. From the experiments presented above, it was clear that (C^2-Man-)Trp was originally present in the RNase 2 protein and did not arise during purification or analysis of its peptides. Therefore, standard protocols for the isolation and analysis of RNase 2 from other sources, which often employ acidic conditions, can be applied. Only RNase 2 isolated from urine has been used in the analyses so far. Therefore, it was of interest to determine whether C-mannosylation of Trp-7 also occurs in RNase 2 obtained from cells. RNase 2 from human erythrocytes (RNase HE-1) was isolated using a modification of the method of Yasuda et al. (1990a). The final purification step, C_4 reversed-phase HPLC, yielded two peaks with RNase activity (RNase HE-1_1 and HE-1_2; Figure 6A). Also, reversed-phase HPLC of RNase 2 from urine yielded two peaks, with retention times that were the same as those found for RNase HE-1_1 and HE-1_2, respectively (Figure 6A, Table 2). Analysis by ESI-MS showed that the molecular mass of RNase 2_1 and 2_2 differed by 16 Da. The exact nature of the modification that causes this difference is unclear and is the subject of current investigations. It was found, however, that the N-terminal peptides comprising residues 1−12 obtained from RNase 2_1 and 2_2 were identical and that in both cases Trp-7 was glycosylated (data not shown).

Table 2: Comparison of RNase 2 Isolated from Urine and RNase HE-1 Isolated from Erythrocytes

	retention time of the protein (min)[a]	retention time of peptide 5−10 (min)	PTH-(C^2-Man-)Trp detected[b]	mass of peptide 5−10 ($[M + H]^+$)
RNase 2_1	61.2	38.7	X_1, X_2, X_3 (protein)	1000.4
RNase 2_2	63.9	nd[c]	X_1, X_2, X_3	nd
RNase HE-1_1	61.0	38.6	nd	nd[d]
RNase HE-1_2	63.7	nd	X_1 (protein) X_1, X_2 (peptide)	1000.4

[a] Proteins and peptides were purified on C_4 and C_{18} HPLC columns, respectively, using solvent system I. [b] X_{1-3} refer to the three PTH derivatives of (C^2-Man-)Trp found previously (Hofsteenge et al., 1994). X_1 (ca. 80% of the total signal) was always observed, whereas X_2 (most likely a diastereomer of X_1) and X_3 could only be detected if large amounts of material (>100 pmol) were sequenced. The analyzed material has been indicated in parentheses. [c] nd represents not determined. [d] Insufficient material was available for further analysis.

The thermolytic peptide comprising residues 5−10 isolated from RNase HE-1_1 and HE-1_2 had the same retention time on reversed-phase HPLC as the one isolated from RNase 2 (Figure 6B, Table 2). This suggests also that these peptides contained (C^2-Man-)Trp.

Edman degradation of native RNase HE-1_2 showed its N-terminal sequence (residues 1−16) to be identical to that reported for RNase 2 (Beintema et al., 1988) and RNase HE-1 (Yasuda et al., 1990a). At cycle 7, one PTH-amino acid was observed, eluting at the same time as the major PTH derivative, X_1, of (C^2-Man-)Trp from RNase 2 from urine, described previously (Hofsteenge et al., 1994). Moreover, Edman degradation of the thermolytic peptide comprising residues 5−10 derived from RNase HE-1_2 yielded the sequence FT(C^2-Man-)WAQW (Table 2).

The presence of a C-mannosylated Trp at position 7 in the enzyme from erythrocytes was proven by MS of the thermolytic peptide FT(C^2-Man-)WAQW from RNase HE-1_2. ESI-MS revealed that this peptide had a mass of 999.4 Da, like the thermolytic hexapeptide FT(C^2-Man-)WAQW isolated from urinary RNase 2. In ESI-MS/MS experiments, it also showed the typical fragmentation pattern expected for an aromatic *C*-glycoside, i.e., the loss of 120 Da (data not shown).

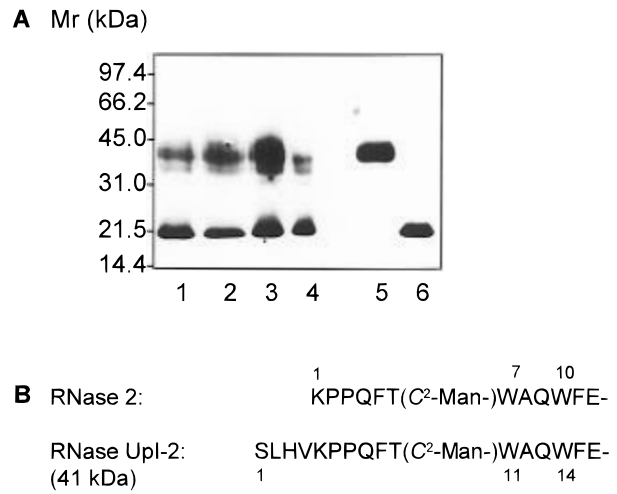

FIGURE 7: (A) Western analysis of RNase 2 and its high-molecular mass isoforms in urine: lane 1, male urine; lane 2, nonpregnant female urine; lane 3, pregnant female urine; lane 4, menopause female urine; lane 5, the purified major high-molecular mass isoform of RNase 2; and lane 6, purified RNase 2 (10 ng). The proteins were separated by SDS−PAGE, and immunoblot analysis was performed using purified anti-RNase 2 antibodies. (B) N-Terminal sequence of RNase 2 (Hofsteenge et al., 1994) and the major isoform of RNase UpI-2, as determined in this paper.

These results show that (C^2-Man-)Trp in RNase 2 from urine does not represent a metabolized form of the protein, because it also occurs in the enzyme isolated from a cellular source.

C-Mannosylation of RNase 2 Isoforms: Immunoblotting. Using polyclonal antibodies raised against RNase 2, the finding of Sakakibara et al. (1992) that the urine from pregnant women contains a high-molecular mass isoform of RNase 2 (RNase UpI-2) could be confirmed. These authors have demonstrated that the difference in apparent molecular mass between RNase 2 (ca. 19 kDa) and RNase UpI-2 (ca. 38 kDa) is mainly due to larger N-linked oligosaccharides. In addition, the N-terminus of RNase UpI-2 was found to be four amino acids longer (Sakakibara et al., 1992). In Figure 7A, it is shown that the antibody raised against RNase 2 bound to several proteins that had apparent molecular masses of approximately 19 (RNase 2), 31, 38, and 41 kDa. The detection of multiple species was most likely due to heterogeneity of N-linked oligosaccharides. In contrast to the results of Sakakibara et al. (1992), small amounts of RNase UpI-2 could also be detected in urine from man and nonpregnant women (Figure 7A). Experiments with individual urine samples suggested significant variation in the total amount of each isoform, as well as in the ratio of the isoforms to each other, depending on, e.g., the stage of pregnancy (data not shown). The complexity of the problem requires more experiments, however, before a correlation with altered physiological conditions or hormone levels can be made.

Amino Acid Sequence Analysis. RNase 2 and its major high-molecular mass isoform were purified from the urine of pregnant women. In agreement with the immunological data (Figure 7A), several isoforms were observed by reversed-phase HPLC (data not shown), but sufficient pure material was only obtained for the major one (apparent molecular mass of 41 kDa). Edman degradation of RNase 2 and RNase UpI-2 confirmed the previously published sequences (Beintema et al., 1988; Sakakibara et al., 1992), except for the residue at position 11 of RNase UpI-2. At this position, PTH-(C^2-Man-)Trp but no PTH-Trp was found (Figure 7B), in contrast to the findings of Sakakibara et al. (1992). The tryptophan at position 14, however, was found to be unmodified.

This result was confirmed by analyzing the thermolytic peptide from RNase UpI-2 [FT(C^2-Man-)WAQW] using LC/MS. The peptide coeluted with the one obtained from RNase 2 and had an identical mass ($[M + H]^+ = 1000.4$). Interestingly, we found a small amount of unmodified peptide FTWAQW ($[M + H]^+ = 838.5$), which had gone undetected during Edman degradation. In contrast, no unmodified Trp-7 was found in the low-molecular mass isoform, RNase 2, from pregnant women or male urine (data not shown).

RNase 2 and UpI-2 were also purified from the urine of women in menopause. Edman degradation of these proteins showed the presence of C-mannosylated tryptophan at position 7 and 11, respectively, whereas those at position 10 and 14 were unmodified.

These results demonstrate that not only RNase 2 but also its major high-molecular mass isoform is (at least partially) C-mannosylated at Trp-11. The findings of Sakakibara et al. (1992) led us to propose the hypothesis that C-glycosylation is linked to other post-translational modifications, like, e.g., N-glycosylation, and is physiologically regulated (Hofsteenge et al., 1994). The results reported here do not support this hypothesis. The absence of (C^2-Man-)Trp in the preparation of RNase UpI-2 of Sakakibara et al. (1992) may be explained by the existence of several high-molecular mass isoforms (Figure 7A), and assuming that some of these are not or only partially C-glycosylated. The detection by LC/MS of a small amount of the peptide FTWAQW in RNase UpI-2 (see above) is in agreement with such an interpretation.

Conclusion. It is concluded that C-mannosylation of Trp-7 in RNase 2 is not a product of the acidic conditions used during isolation and purification of the enzyme and its peptides. The modification is not restricted to the enzyme from urine. The modification reaction can take place in cells, because it was found that the RNase from erythrocytes also contains (C^2-Man-)Trp. Furthermore, it was demonstrated that (C^2-Man-)Trp is also present in the major high-molecular mass isoform of the enzyme.

These observations firmly establish C-mannosylation as a genuine post-translational modification and warrant further studies on its biosynthesis and function.

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