Glycosylation and Specific Deamidation of Ribonuclease B Affect the Formation of Three-dimensional Domain-swapped Oligomers*

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Giovanni Gotta‡, Massimo Libonati‡, and Douglas V. Laurents§¶

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‡ Supported by the Ramón y Cajal Program of the Spanish Ministerio de Ciencia y Tecnología. To whom correspondence should be addressed. Tel.: 34-91-561-9400; Fax: 34-91-564-2431; E-mail: dlaurent@iqfr.csic.es.

RNase A oligomerizes via the three-dimensional domain-swapping mechanism to form a variety of oligomers, including two dimers. One, called the N-dimer, forms by swapping of the N termini of the protein; the other, called the C-dimer, forms by swapping of the C termini. RNase B is identical in protein sequence and conformation to RNase A, but its Asn34* bears an oligosaccharide chain that might affect oligomerization. The ability of RNase B to oligomerize under two sets of conditions has been examined. The amount of oligomers formed via lyophilization was somewhat lower for RNase B than RNase A, and RNase B oligomerized more rapidly in 40% ethanol solution at high temperature than RNase A. The ratio of the N-dimer to C-dimer formed increased with the size of the carbohydrate chain under both sets of conditions. These results suggest that the oligosaccharide chain either favors productive collisions or stabilizes the oligomers, especially the N-dimer. Endoglycosidase H treatment of RNase B partially restored RNase A-like oligomerization. Derivatives of RNase A conjugated at the amine groups to polyethylene glycol chains showed a greatly reduced capacity for oligomerization, suggesting that oligomerization can be impeded sterically. Commercial preparations of RNase B eluted as two major peaks by cation exchange chromatography. Using chromatography, mass spectroscopy, and two-dimensional NMR, the major peak was identified as RNase B selectively deamidated at Asn67 stupidity. This deamidated protein showed a >4 °C drop in thermal stability, disruption of the native structure of residues 67–69, and a decreased ability to oligomerize compared with unmodified RNase B.

In their classic work, Crestfield et al. (1) discovered and characterized the ability of bovine ribonuclease A to dimerize by exchanging segments of secondary structure when lyophilized from 50% acetic acid. Since then, many proteins have been found to form oligomers by the same mechanism, known as three-dimensional domain swapping (Ref. 2; see Ref. 3 for an excellent recent review). This process of oligomerization is being studied as a mechanism for the formation of oligomeric proteins by evolution. Moreover, it has been proposed that three-dimensional domain swapping might lead to inappropriate formation of large aggregates of cross-β-structure, known as amyloid, which have been linked to >23 diseases, including Alzheimer’s, Parkinson’s, and prion-induced diseases (4). This hypothesis is supported by recent findings that the amyloidogenic protein cystatin C (5) and the human prion protein (6) dimerize via three-dimensional domain swapping between monomers.

Modern studies on the oligomerization of bovine RNase A show that this normally monomeric protein can form a variety of dimers, trimers, and larger oligomers (7) via three-dimensional domain swapping of the N-terminal α-helix, the C-terminal β-strand, or both (8–11). The dimers, trimers, and tetramers each form at least two conformational isomers, which can be separated by cation exchange chromatography as a less basic and a more basic oligomer (7). The less basic dimer, formed by swapping of the N termini of the protein, and the more basic dimer, formed by swapping of the C termini, were named N-dimer and C-dimer, respectively (12). Oligomerization of RNase A also occurs in solution at high substrate concentrations (13) or at high temperature (14), and variation of solution and environmental conditions, including temperature, affects the size and amount of oligomer formed (12).

Bovine pancreatic ribonuclease is also produced in vivo in a glycosylated version, ribonuclease B, which bears a single oligosaccharide chain linked to Asn64. The sugar chain cloaks charged residues on the surface of RNase B, causing it to elute before RNase A on cation exchange columns (15). The oligosaccharide chain of RNase B consists of a common trunk of two GlcNAc residues and five to nine mannose residues, whose branch pattern has been determined (16, 17). Species with different sized oligosaccharide chains can be separated by affinity chromatography (18). Bovine RNases A and B share identical amino acid compositions (15) and sequences, including a single Asn67–Gly68 residue pair that was shown to undergo selective deamidation in RNase A (19). The protein moieties of RNases A and B have essentially identical structures as revealed by crystallography (20) and by NMR, both for shorter (21) and longer (22) glycoforms. The global conformational stability of RNase B, as measured by thermal denaturation, is slightly higher than that of RNase A (23). Furthermore, hydrogen exchange measurements reveal that NH groups forming main chain hydrogen bonds show an increased protection of 1.1–2-fold in RNase B versus RNase A (24), consistent with a slight increase in conformational stability. NMR evidence (nuclear Overhauser effect) and molecular dynamics calculations also reveal that the carbohydrate chain is flexible and that its conformation is not restricted by the protein moiety, with the exception of the somewhat rigid GlcNAc residue attached directly to Asn64 (18).

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Because the presence of carbohydrate moieties reduces or prevents aggregation or precipitation for several glycoproteins (25, 26) and because Asn\(^{34}\) and its carbohydrate chain in RNase B are close to the N-terminal \(\alpha\)-helix participating in the three-dimensional domain-swapping oligomerization of RNase A, it is possible that the sugar chain attached to this residue sterically hinders the swapping of this helix and reduces oligomerization. However, a single sugar residue in a human prion protein domain has been found not only to inhibit amyloidogenesis, but also to accelerate it, depending on its position (27). One key objective of this work is to test the ability of RNase B to form domain-swapped oligomers. Both the lyophilization (1) and aqueous ethanol solution (12) protocols were tested using the conditions that produce the highest yields of RNase A oligomers. In the case of the ethanol solution protocol, two different temperatures, 60 and 70 °C, were studied to test whether the different thermal stabilities of RNases A and B affect their oligomerization. Two different fractions of RNase B containing small or large carbohydrate chains were examined. At the start of this work, commercial RNase B was discovered to elute as two main peaks by cation exchange chromatography. Identifying the cause of these two peaks and the characterization of its effect on oligomerization are other key objectives of this work.

Matousek \(\textit{et al.}\) (28) have recently shown that RNase A becomes a potent cytotoxin when coupled at amine groups to polyethylene glycol (PEG)\(^1\) 5000. This cytotoxicity might be due to the PEG chains acting to impede the approach of the cytoplasmic ribonuclease inhibitor. We hypothesize that such steric hindrance might also block the oligomerization of RNase molecules. To check this possibility, multiple PEG (\(M_n = 5000\)) groups were coupled to RNase A lysine residues, and the ability of this material to oligomerize was measured. Finally, as a positive control, we quantified the ability of RNase B to aggregate after most of its carbohydrate chain had been cleaved off with endoglycosidase H.

\(^1\) The abbreviations used are: PEG, polyethylene glycol; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; NOESY, nuclear Overhauser effect correlation spectroscopy; FPLC, fast protein liquid chromatography.

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**FIG. 1.** Representative separation of commercial RNase B into Peaks X and Y by cation exchange chromatography at pH 6.4 using a 40–200 mM sodium phosphate gradient.

**EXPERIMENTAL PROCEDURES**

\textit{Materials—}Deionized, double-distilled water was used for all experiments. All reagents and salts were the highest grade of purity commercially available. RNase B (catalog no. R-7884, lot 060K7850), and RNase A (type XIIa, catalog no. R-5500, lot 104H7110) were obtained from Sigma. Some experiments were repeated using other RNase B samples from Sigma (catalog no. R-5875, lot 115H7041) or highly (catalog no. 8005E, lot 101084) or moderately (catalog no. 104907, lot 63148) active preparations from ICN. Sample purity was checked by cation exchange chromatography. Endoglycosidase H (recombinant from Streptomyces pilatus) was from Sigma (catalog no. A-9810, lot 122K1557). RNase B was either used without further treatment or separated into fractions with short (two GlcNAc + five to six mannose residues) or long (two GlcNAc + seven to nine mannose residues) oligosaccharide chains by affinity chromatography using concanavalin A-Sepharose resin (Sigma) and eluted using a o-methoxyglucosamine (Sigma) as previously described (18). The purified protein was then dialyzed and lyophilized. The number of mannose groups in these fractions was measured using the phenol-sulfuric acid assay (29) and confirmed by MALDI-TOF mass spectroscopy performed in the Instituto de Quimica-Physica Mass Spectroscopy Facility. Amino acid analysis was carried out in triplicate following acid hydrolysis of the amide bonds on an EZCHROM amino acid analyzer at the Protein Chemistry Facility of the Centro de Investigaciones Biologicas, Consejo Superior de Investigaciones Cientificas (Madrid, Spain).

\textit{Assays for Enzymatic Activity—}Samples of RNase B fractions separated by affinity or ion exchange chromatography were assayed for ribonuclease activity on single-stranded baker’s yeast RNA using the procedure of Libonati and Sorrentino (31). All assays were conducted at 25 °C.

**Thermal Denaturation—**Thermal unfolding transitions were monitored by far-UV CD spectroscopy at 219 nm in a 0.1-cm cell using a Jasco J-810 spectrometer equipped with a Peltier temperature control unit. The temperature range followed was 20–80 °C at a heating rate of 1 °C/min. The slit width was 1.0 nm. The protein concentration was 0.7–1.2 mg/ml in 20 mM sodium phosphate buffer (pH 7.0) containing 100 mM KCl. Each experiment was repeated once. Data analyses were carried out applying the two-state approximation as described (32).

**Deamidation Test—**The susceptibility of RNases A and B to deamidation was tested by incubating samples (1–2 mg/ml) at 37 °C in 1% ammonium bicarbonate buffer at pH 8.2 as described (19) or at pH 9.2. Aliquots were taken over the course of 3 weeks and analyzed by ion exchange chromatography. The observed deamidation rates were determined by fitting to a first-order kinetic model using a least-squares algorithm.

**NMR Spectroscopy—**Two-dimensional 1H NOESY spectra (33) were
commercial RNase B causes it to elute off cation exchange columns before RNase A. Because the number of mannose residues present in the oligosaccharide chain of RNase B ranges from five to nine, it is reasonable that the molecules with longer sugar chains might have more surface charges covered and elute before molecules with shorter sugar chains, thus explaining the appearance of Peaks X and Y. However, no significant difference in the ratio of moles of mannose residues to moles of protein for Peaks X and Y was found using the phenol-sulfuric acid assay (data not shown). Moreover, Peaks X and Y, fractionated and desalted, were analyzed by MALDI-TOF mass spectroscopy, and both showed

**TABLE I**

Amino acid composition of RNase B Peaks X and Y

| Residue  | Peak X (%) | Peak Y (%) | Theory (%) |
|----------|------------|------------|------------|
| Asp + Asn| 15.0 ± 0.2 | 14.94 ± 0.04 | 15         |
| Thr      | 10.1 ± 0.2 | 9.8 ± 0.2  | 10         |
| Ser      | 15.0 ± 0.4 | 15.2 ± 0.4 | 15         |
| Glu + Gln| 12.4 ± 0.2 | 12.55 ± 0.10 | 12         |
| Gly      | 2.86 ± 0.09 | 3.3 ± 0.3  | 3          |
| Ala      | 11.3 ± 0.2 | 11.4 ± 0.4 | 12         |
| Cystine  | 3.2 ± 0.5  | 3.59 ± 0.13 | 4          |
| Val      | 8.51 ± 0.14 | 8.4 ± 0.2  | 9          |
| Met      | 4.02 ± 0.10 | 4.01 ± 0.07 | 4          |
| Ile      | 2.01 ± 0.05 | 2.05 ± 0.11 | 3          |
| Leu      | 2.49 ± 0.06 | 2.6 ± 0.2  | 2          |
| Tyr      | 6.2 ± 0.2  | 6.12 ± 0.08 | 6          |
| Phe      | 4.02 ± 0.09 | 4.04 ± 0.13 | 3          |
| His      | 4.7 ± 0.3  | 4.7 ± 0.3  | 4          |
| Lys      | 9.44 ± 0.14 | 9.9 ± 0.3  | 10         |
| Arg      | 3.89 ± 0.13 | 4.1 ± 0.2  | 4          |
| Pro      | 4.3 ± 0.3  | 3.5 ± 0.6  | 4          |

* The means ± S.D. (±1σ) for three trials are reported.

**RESULTS**

**Commercial RNase B Elutes as Two Major Peaks by Cation Exchange Chromatography**—At the start of this investigation, commercial RNase B (Sigma lot 060K7650) was observed to elute as two major peaks when subjected to cation exchange chromatography (Fig. 1). These two peaks (called Peaks X and Y) eluted at ~102 and 120 mM sodium phosphate (pH 6.4), respectively. These same two peaks also appeared in a different sample of Sigma RNase B (lot 115H7041), although their relative amounts were inverted relative to those in lot 060K7650. For RNase B from ICN (catalog no. 8005E), Peak X was found to be only slightly larger than Peak Y (data not shown). We hypothesized that the difference that causes RNase B to elute as two separate peaks might involve the amino acid composition, the oligosaccharide composition or conformation, a chemical alteration, or some combination of these factors. To investigate the nature of these two peaks, they were first separated by cation exchange chromatography and subjected to amino acid analysis. As shown in Table I, Peaks X and Y have very similar if not identical amino acid compositions, although acid hydrolysis of the polypeptide chain also converted Asn and Gln to Asp and Glu, respectively. Some residues such as cystine and Ile were found to be in slightly lower amounts compared with the amino acid composition expected from the sequence. This is likely due to destruction of cystine and the resistance of Ile peptide bonds to hydrolysis, as observed previously for RNase B (15) and other proteins.

Screening of surface charges by the oligosaccharide chain of RNase B causes it to elute off cation exchange columns before RNase A (15). In fact, the small peak eluting at ~142 mM sodium phosphate in Fig. 1 corresponds to RNase A. Because the number of mannose residues present in the oligosaccharide chain of RNase B ranges from five to nine, it is reasonable that the molecules with longer sugar chains might have more surface charges covered and elute before molecules with shorter sugar chains, thus explaining the appearance of Peaks X and Y. However, no significant difference in the ratio of moles of mannose residues to moles of protein for Peaks X and Y was found using the phenol-sulfuric acid assay (data not shown). Moreover, Peaks X and Y, fractionated and desalted, were analyzed by MALDI-TOF mass spectroscopy, and both showed


Glycosylation and Deamidation Affect RNase B Oligomerization

The masses are given in daltons, and the relative peak heights are shown in parentheses, with the largest peak in each spectra assigned a value of 100. Peak X is N67D,RNase B, and Peak Y is unmodified RNase B. NO, none observed.

|        | RNase A |        | RNase B |        |
|--------|---------|--------|---------|--------|
|        | Five mannoses | Six mannoses | Seven mannoses | Eight mannoses | Nine mannoses |
| Theory | 13,681 | 14,898 | 15,060 | 15,222 | 15,384 | 15,546 |
| Pre-X  | 13,687 (4) | 14,904 (100) | 15,066 (78) | 15,227 (33) | 15,390 (61) | 15,552 (29) |
| X1     | NO      | 14,903 (100) | 15,065 (75) | 15,228 (31) | 15,390 (57) | 15,552 (29) |
| X2     | NO      | 14,901 (100) | 15,063 (83) | 15,226 (37) | 15,388 (56) | 15,551 (21) |
| X3     | NO      | 14,906 (100) | 15,068 (55) | 15,230 (10) | 15,393 (8)  | 15,556 (3)  |
| Y1     | 13,689 (28) | 14,906 (100) | 15,068 (85) | 15,230 (42) | 15,393 (66) | 15,555 (46) |
| Y2     | 13,686 (20) | 14,903 (100) | 15,068 (95) | 15,230 (47) | 15,393 (62) | 15,555 (21) |
| Y3     | 13,686 (33) | 14,901 (100) | 15,064 (65) | 15,227 (12) | 15,388 (7)  | NO          |

* Theory was calculated based on the masses of RNase A, mannose (180.1 Da), and N-acetylglucosamine (221.1 Da), subtracting the mass of the water (18.02 Da) released upon forming the glycoside bond and adding 1 Da to account for the H⁺ ion acquired by protein molecules in the MALDI-TOF mass spectroscopy method.

* Pre-X is a small peak eluting before Peak X. It might be RNase B containing multiple deamidated Asn residues. X1-X3 and Y1-Y3 correspond to the left, center, and right of Peaks X and Y, respectively.

several peaks (Fig. 2, A and B); their masses correspond, within the experimental uncertainty of ±0.5 Da, to those expected for RNase B molecules with five, six, seven, eight, and nine mannose residues (Table II). Interestingly, the fractions from both the right limbs (X3 and Y3) (Fig. 1) of Peaks X and Y were depleted in the amount of long chain RNase B molecules present. This suggests that RNase B molecules carrying oligosaccharide chains with seven, eight, or nine mannose residues bound slightly less well to the cation exchange resin and eluted just before the short chain RNase B molecules in both Peaks X and Y. The right limbs of Peaks X and Y (X3 and Y3) show a distinct gel filtration elution profile, viz. the Peak X shoulder consistently eluted 0.3 ml before the Peak Y shoulder. This behavior may be due to a conformational difference in the surface loop containing residues 67–69 (see below) or, less probably, to the very small amount of RNase A present in this shoulder as detected by mass spectroscopy.

Affinity Chromatography—RNase B molecules with different sized sugar chains can be fractionated by affinity chromatography. Three peaks (peaks I–III) were separated as shown in a typical chromatogram (Fig. 3), with peak I eluting without binding to the column. Similar peaks were previously observed and identified as RNase A (peak I); RNase B with five or six mannose residues (peak II); and RNase B with seven, eight, or nine mannose residues (peak III) (18). Quantification of the amount of sugar residues by the phenol-sulfuric acid assay revealed that peak II contains 5.6 ± 1.1 mol of mannose/mol of protein and that peak III contains 8.7 ± 1.0 mol of mannose/mol of protein. Analysis of the composition of these fractions by mass spectroscopy revealed that five and six mannose-containing oligosaccharide chains are predominant in peak II, whereas peak III contains mostly oligosaccharide chains with eight mannoses, with some chains of seven or nine mannoses also being present (Fig. 4, A and B; and Table III). Mass spectroscopy also showed that the oligosaccharide compositions of the left and right halves of peak II appear to be similar to each other and that both halves of peak III have a similar content of sugars. It is noteworthy that, when subjected to cation exchange chromatography, peak II eluted as two peaks (Peaks X and Y), as did peak III (data not shown).

Incubation under Deamidation Conditions—Asn⁶⁷ of RNase A has been shown to be susceptible to deamidation in vitro under mild conditions (pH 8.2, 37 °C), and the separation of native and deamidated RNase A by cation exchange chromatography (19) produces peaks reminiscent of Peaks X and Y observed in this work. As a test, samples of Peaks X and Y, previously separated by ion exchange chromatography, were incubated under these conditions. Over the course of 3 weeks, the Peak X sample remained largely unchanged, whereas in the Peak Y sample, Peak Y gradually disappeared, and a new peak, whose elution volume corresponded to that of Peak X, appeared and progressively increased in size (Fig. 5). As a control, a sample of RNase A was incubated in parallel and also showed the appearance and growth of a new peak, and the difference in elution position of the new and original peaks of RNase A was the same as for Peaks X and Y of RNase B (16–17 min sodium phosphate). The amplitudes of these reactions obtained from the least-squares fit of the data to a first-order kinetic equation suggest that Peak Y is quantitatively converted to Peak X. The observed first-order rates of the decrease in Peak Y of RNase B and of RNase A observed here and by Di Donato et al. (19) are all very similar to each other and to the observed rates of the growth of Peak X and the RNase A peak corresponding to Peak X (Table IV). At pH 9.2, similar results were observed, except that the rates of Peak Y decrease and Peak X increase were both 3.2-fold faster relative to pH 8.2 (Fig. 5 and Table IV).

NMR Spectroscopy—Regions of the two-dimensional ¹H NOESY NMR spectra of Peaks X and Y are shown in Fig. 6. In general, the spectra are very similar to each other and to previously assigned spectra for RNases A (34) and B (18, 21). In the spectrum of Peak Y, the backbone connectivities can be traced, and many side chain resonances can be identified using the assignments of RNase A or B for residues 61–73. Moreover, the pattern of nuclear Overhauser effects for Asn⁶⁷ is essentially identical to that of RNase B, and a nuclear Overhauser
effect resonance between the side chain amide protons of Asn$^{67}$ is prominent in the spectrum of Peak Y, as it is in RNase B spectra. In contrast, this resonance is missing in the spectrum of Peak X, and the backbone and side chain chemical shifts are different. The resonance between the side chain amide protons of Asn$^{67}$ of RNase B Peak X, and the backbone and side chain chemical shifts are different. The resonance is missing in the spectrum of Peak X, and the backbone and side chain chemical shifts are different. The resonance is missing in the spectrum of Peak X, and the backbone and side chain chemical shifts are different. The resonance is missing in the spectrum of Peak X, and the backbone and side chain chemical shifts are different. The resonance is missing in the spectrum of Peak X, and the backbone and side chain chemical shifts are different. The resonance is missing in the spectrum of Peak X, and the backbone and side chain chemical shifts are different. The resonance is missing in the spectrum of Peak X, and the backbone and side chain chemical shifts are different. The resonance is missing in the spectrum of Peak X, and the backbone and side chain chemical shifts are different. The resonance is missing in the spectrum of Peak X, and the backbone and side chain chemical shifts are different. The resonance is missing in the spectrum of Peak X, and the backbone and side chain chemical shifts are different. The resonance is missing in the spectrum of Peak X, and the backbone and side chain chemical shifts are different. The resonance is missing in the spectrum of Peak X, and the backbone and side chain chemical shifts are different. The resonance is missing in the spectrum of Peak X, and the backbone and side chain chemical shifts are different. The resonance is missing in the spectrum of Peak X.
MALDI-TOF mass spectra of RNase B fractionated by affinity chromatography

The masses are given in daltons, and the relative peak heights are shown in parentheses, with the largest peak in each spectra assigned a value of 100. NO, none observed.

|        | RNase A |        | RNase B |        |        |        |
|--------|---------|--------|---------|--------|--------|--------|
|        | Five mannos | Six mannos | Seven mannos | Eight mannos | Nine mannos |
| Theory\(^a\) | 13,681 | 14,898 | 15,060 | 15,222 | 15,384 | 15,546 |
| IIa\(^b\) | NO | 14,898 (100) | 15,061 (76) | 15,220 (12) | 15,384 (100) | 15,546 (76) |
| IIb | NO | 14,896 (100) | 15,061 (69) | 15,223 (16) | NO | NO |
| IIIa | NO | 14,900 (2) | 15,062 (5) | 15,225 (27) | 15,387 (100) | 15,550 (40) |
| IIIb | NO | 14,902 (6) | 15,064 (8) | 15,227 (44) | 15,390 (100) | 15,552 (68) |

\(^a\) Calculated based on the masses of RNase A, mannose (180.1 Da), and N-acetylglucosamine (221.1 Da) subtracting the mass of the water (18.02 Da) released upon forming the glycoside bond and adding 1 Da to account for the H\(^+\) ion acquired by protein molecules in the MALDI-TOF method.

\(^b\) IIa, the left half of peak II; IIb, the right half of peak II; IIIa, the left half of peak III; IIIb, the right half of peak III (as shown in Fig. 4).

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Glycosylation and Deamidation Affect RNase B Oligomerization

The key objective of this paper was to study the effects of an oligosaccharide chain on oligomer formation via the three-dimensional domain-swapping mechanism. Early in this investigation, commercial RNase B was found to elute as two main peaks, called here Peaks X and Y, when applied to cation exchange chromatography. The cause of this different chromatographic behavior might also affect the oligomerization of the protein. Therefore, another key objective here was to identify this cause and to determine how it affects the structure and oligomerization of the protein.

**Nature of Peaks X and Y**—It is hypothetically possible that one of these peaks is due to a variant RNase B that carries a difference in the number or type of charged residues. This possibility was previously addressed and rejected by Eaker et al. (38) to explain the appearance of two novel forms of commercial RNase A on the basis that ribonuclease is prepared industrially from large pools of animals whose genotypes are not likely to vary significantly from batch to batch. The deslysylglutamyl- and deslysylpyroglutamyl-RNases A isolated by Eaker et al. (38) cannot be the cause of the Peaks X and Y observed here, as they would have produced peaks in the mass spectra and differences in the amino acid composition that were not observed.

Another explanation for Peaks X and Y is that RNase B molecules with longer oligosaccharide chains may sterically mask protein surface charges. This hypothesis predicts different oligosaccharide chain lengths for Peaks X and Y. However, Peaks X and Y were found to contain the same average number of sugar residues. Moreover, RNase B could be purified by affinity chromatography to yield fractions II and III, which are highly enriched in short and long oligosaccharide chains, and these fractions continued to produce Peaks X and Y when subjected to cation exchange chromatography. The observation by mass spectroscopy that, for both Peaks X and Y, the left and right limbs are enriched and depleted, respectively, in the longer sugar chains shows that the chain length has a minor effect on the elution position of RNase B, but not enough to cause the separation of Peak X from Peak Y. Kawasaki et al. (17) identified multiple branch patterns for the oligosaccharide chains of RNase B by electrospray ionization mass spectroscopy. The appearance of Peaks X and Y might be explained if a charged residue were covered by oligosaccharide chains with

significantly affected by the presence of either short or long sugar chains; both fractions II and III of RNase B oligomerized to approximately the same degree as RNase A (Table VIII). In contrast, the rate of oligomer formation and the ratio of C-dimer to N-dimer formed were strongly affected by the presence of the oligosaccharide chain. The early formation of oligomers was more rapid for RNase B (both fractions II and III) than for RNase A, as detected by the greater drop in the amount of monomer and the higher amount of oligomers present after 5 min of incubation under oligomerization conditions (Table VIII). The delay in RNase A oligomer formation was observed at 60 °C as well as at 70 °C. Additional experiments
one kind of branch pattern, but not the other. However, RNase B eluted as two peaks even after most of the oligosaccharide chain had been cleaved off by endoglycosidase H. Thus, it is unlikely that Peaks X and Y result from a difference in the number of sugar residues or their branch pattern in the oligosaccharide chain of RNase B.

The strong similarity between the rates and amplitude of the conversion of Peak Y to Peak X found here and the deamidation at Asn67 of RNase A observed under equivalent solution conditions and temperature by Di Donato et al. (19) strongly suggests that Peak Y is unmodified RNase B and that Peak X is RNase B that has been deamidated at Asn67. The deamidation of Asn proceeds through a two-step reaction mechanism (39, 40). The reaction is highly specific for Asn-Gly pairs due to the extraordinary conformational freedom of glycine. Of the 10 Asn residues present in RNase A or B, Asn67 is the only one followed by Gly. The cyclic intermediate formed in the first step is resolved in the second reaction step to form Asp or iso-Asp depending on which carbonyl is attacked. The iso-Asp product, which introduces an extra methylene group into the backbone of the protein, has been predicted on chemical grounds (39) and has been found experimentally in the case of RNase A to be the major product (19). Both Asp and iso-Asp products would carry an extra negative charge at neutral pH relative to unmodified protein and thus could account for the earlier elution of Peak X relative to Peak Y from the cation exchange column. Deamidation can be acid- or base-catalyzed (39, 40), and the conversion of Peak Y to Peak X is found to be accelerated at a higher pH. The fact that the acceleration is only 3.2-fold and not 10-fold at pH 9.2 where [OH\(^{-}\)] is 10-fold higher relative to pH 8.2 is probably due the limited mobility of Asn\(^{67}\) and Gly\(^{68}\) within the folded structure (41, 42).

Because the hydrolysis step used in the amino acid analysis

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**TABLE IV**

| Sample                  | pH    | \(k_{obs} a\) | \(A^\infty\) | R²   |
|-------------------------|-------|---------------|--------------|------|
| RNase B Peak Y          | 8.2   | \((4.1 ± 0.6) \times 10^{-3}\) | 0.01 ± 0.03  | 0.993|
| RNase A                 | 8.2   | \((4.3 ± 0.5) \times 10^{-3}\) | 0.009 ± 0.03 | 0.997|
| RNase A\(^d\)           | 8.2   | \((3.4 ± 0.2) \times 10^{-3}\) | 0.008 ± 0.009 | 0.999|
| RNase B de novo Peak X\(^e\) | 8.2   | \((4.0 ± 0.5) \times 10^{-3}\) | -0.011 ± 0.03 | 0.995|
| Asn67-deamidated RNase A | 8.2   | \((4.3 ± 0.4) \times 10^{-3}\) | -0.008 ± 0.002 | 0.998|
| RNase B Peak Y          | 9.2   | \((1.29 ± 0.11) \times 10^{-2}\) | -0.015 ± 0.02 | 0.997|
| RNase B de novo Peak X\(^e\) | 9.2   | \((1.27 ± 0.08) \times 10^{-2}\) | 0.014 ± 0.02  | 0.999|

\(a\) \(k_{obs}\), the observed rate constant, and \(A^\infty\), the absorbance at infinite time, and their respective uncertainties as obtained from fitting the data to \(A^\infty = A^\infty + At\) for Peak Y and RNase A, and \(1 - e^{-kt} + A^\infty\) for the growth of Peak X or Asn67-deamidated RNase A, using a least-squares fitting algorithm.

\(b\) \(R\), the correlation coefficient.

\(c\) This work.

\(d\) Fit of the data adapted from Di Donato et al. (19).

\(e\) The peak appearing in the position of Peak X upon incubation of purified Peak Y of RNase B with time.
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TABLE V
Lyophilization-induced oligomerization of RNase B, RNase A, and deglycosylation controls

| Oligomeric state | RNase A | RNase B Peak X$^a$ | RNase B Peak Y$^a$ | Deglycosylated RNase B Peak X | Deglycosylated RNase B Peak Y |
|------------------|---------|-------------------|-------------------|-----------------------------|-----------------------------|
| Monomer          | 7       | 7                 | 7                 | 3                           | 3                           |
|                  | % average (S.D.)$^b$ | 66.0 (2.7) | 75.3 (3)          | 71.6 (2.1)                  | 72.3 (2.4)                  |
| N-dimer          | 7       | 7                 | 7                 | 3                           | 3                           |
|                  | % average (S.D.)$^b$ | 4.6 (1.0)   | 5.5 (1.0)         | 6.3 (0.4)                   | 6.4 (0.3)                   |
| C-dimer          | 7       | 7                 | 7                 | 3                           | 3                           |
|                  | % average (S.D.)$^b$ | 19.0 (2.2)  | 13.8 (1.6)        | 14.5 (1.4)                  | 14.6 (1.5)                  |
| Trimer           | 7       | 7                 | 7                 | 3                           | 3                           |
|                  | % average (S.D.)$^b$ | 6.4 (0.6)   | 4.7 (1.6)         | 5.1 (0.9)                   | 4.0 (0.2)                   |
| Tetramer         | 7       | 7                 | 7                 | 3                           | 3                           |
|                  | % average (S.D.)$^b$ | 2.1 (0.6)   | 1.2 (0.6)         | 1.6 (0.6)                   | 1.1 (0.2)                   |

$^a$ Peak X of RNase B was separated by cation exchange chromatography; this peak was later identified as N67D,D-RNase B (RNase B selectively deamidated at Asn$^{67}$ containing a mixture of Asp and iso-Asp at position 67).

$^b$ Percent average is the amount of the oligomer form relative to the total. The S.D. (1σ) is given in parentheses.

TABLE VI
Lyophilization-induced oligomerization of RNase B dependence on oligosaccharide size and Peak X versus Peak Y

| Oligomeric state | RNase A | RNase B Peak X$^a$ (right half; five to six mannoses)$^b$ | RNase B Peak Y$^a$ (right half; five to six mannoses)$^b$ | RNase B II (five to six mannoses)$^c$ | RNase B III (seven to nine mannoses)$^d$ |
|------------------|---------|----------------------------------------------------------|----------------------------------------------------------|----------------------------------------|----------------------------------------|
| Monomer          | 3       | 7                                                        | 7                                                         | 3                                      | 3                                      |
|                  | % average (S.D.)$^b$ | 67.5 (0.8)   | 72.6                                                     | 69.2                                   | 73.3 (1.0)                             |
| N-dimer          | 3       | 2                                                        | 2                                                         | 3                                      | 3                                      |
|                  | % average (S.D.)$^b$ | 5.9 (0.5)   | 7.9                                                     | 8.2                                    | 7.6 (0.2)                             |
| C-dimer          | 3       | 2                                                        | 2                                                         | 3                                      | 3                                      |
|                  | % average (S.D.)$^b$ | 17.9 (0.6)  | 12.4                                                     | 13.7                                   | 12.1 (0.4)                            |
| Trimer           | 3       | 2                                                        | 2                                                         | 3                                      | 3                                      |
|                  | % average (S.D.)$^b$ | 5.1 (0.2)   | 4.8                                                     | 6.0                                    | 4.5 (0.1)                             |
| Tetramer         | 3       | 2                                                        | 2                                                         | 3                                      | 3                                      |
|                  | % average (S.D.)$^b$ | 2.5 (0.3)   | 1.7                                                     | 2.7                                    | 1.6 (0.1)                             |

$^a$ The right half of Peak X that eluted from the cation exchange column (Peak X3) (Fig. 1), contained mostly shorter glycoforms. Peak X was later identified as N67D,D-RNase B.

$^b$ The right half of Peak Y that eluted from the cation exchange column (Peak Y3) (Fig. 1) contained mostly shorter glycoforms. Peak Y was later identified as unmodified RNase B and contains Asn at position 67.

$^c$ Deglycosylated RNase B fraction II was obtained by affinity chromatography and contained short oligosaccharide chains of five to six mannoses.

$^d$ Deglycosylated RNase B fraction III was obtained by affinity chromatography and contained larger oligosaccharide chains of seven to nine mannoses.

$^e$ % average is the amount of the oligomer form relative to the total. The S.D. (1σ) is given in parentheses.

converts Asn and Gln to Asp and Glu, respectively, this would account for why this method is unable to distinguish Peak X from Peak Y. At neutral pH, the substitution of Asn for Asp or iso-Asp should produce changes (≪ 1 Da) that are too small to detect by MALDI-TOF, but that are easy to see by NMR. The NOESY spectrum of Peak Y is essentially equivalent to spectra of RNase B recorded previously under similar experimental conditions. In particular, peaks arising from the side chain amide protons of Asn$^{67}$ are clearly visible. In contrast, these resonances are absent in the spectrum of Peak X, and the backbone resonances of Asn$^{67}$, Gly$^{68}$, and Gln$^{69}$ are significantly altered. Taking these findings together with the evidence presented above, we conclude that Peak Y is wild-type unmodified RNase B$^2$ and that Peak X consists of RNase B deamidated at Asn$^{67}$, carrying instead a mixture of Asp (D) and iso-Asp (D) at position 67. Henceforth, RNase B Peak Y will be referred to as RNase B, and Peak X will be called N67D,D-RNase B. The results of earlier studies (18, 20–24) suggest that the RNase B studied contained insignificant amounts of deamidated RNase B. It is possible that the N67D,D-RNase B identified here formed during the alkaline pH steps of the RNase B purification procedure (15).

A comparison of NMR spectra of RNase B and N67D,D-RNase B indicates that the backbone and side chain conformations of Asn$^{67}$, Gly$^{68}$, and Gln$^{69}$ are altered in the deamidated protein. These changes can be attributed to the loss of the hydrogen bonds formed between the side chains of Asn$^{67}$ and Gln$^{69}$ and the peptide groups of Cys$^{65}$ and Gly$^{68}$ present in unmodified RNase B as well as the backbone strain induced by the insertion of an extra methylene group in N67D-RNase B. The similarity of the NMR spectra indicates that the strong electrostatic interac-
tions between Lys66 and Asp121 are maintained in N67D,D-RNase B, causing the backbone distortion induced by the additional -CH2- group to be propagated in the C-terminal rather than the N-terminal direction. These results are comparable to those detected by x-ray crystallography for N67D-RNase A (43, 44) and for deamidated bovine seminal RNase (45), in which distortions or multiple conformations were reported.

In the NMR spectrum of N67D,D-RNase B, the appearance of two resonances from a single methyl group of Val63 and from the amide proton of Lys66 is good evidence for conformational or chemical heterogeneity; it is likely that one peak arises from N67D-RNase B and the other from N67D-RNase B. The surface loop bounded by the Cys53–Cys72 disulfide bond has been proposed to be an initiation site for ribonuclease folding (46). The

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**TABLE VII**

Oligomerization of RNase A conjugated to PEG 5000

| N-terminally Conjugated RNase A | Lyophilized | Not lyophilized |
|--------------------------------|--|---|
| Smaller PEG-RNase A conjugates | | |
| (22 kDa; two PEG chains) | | |
| (19 kDa; one PEG chain) | | |
| Agg. 1 | (TT) | Agg. 2 | (T) | Agg. 3 | (D) | Monomer | Agg. 1 | (TT) | Agg. 2 | (T) | Agg. 3 | (D) | Monomer |
| % | | | | | | | | | | | | | |
| A | 1.0 | 4.9 | 9.0 | 84.8 | NO* | | | | | | | | |
| B | 0.5 | 3.2 | 14.1 | 82.7 | NO* | | | | | | | | |
| Larger PEG-RNase A conjugates | | |
| (60 kDa; nine PEG chains) | | |
| (70 kDa; 11 PEG chains) | | |
| Agg. 4 | | Agg. 5 | | Monomer | Agg. 4 | | Agg. 5 | | Monomer |
| % | | | | | | | | | | | | |
| A | 1.4 | 5.1 | 93.5 | 1.1 | 1.4 | 97.5 | |
| B | 0.2 | 5.6 | 94.2 | 0.2 | 5.6 | 94.2 | |

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**TABLE VIII**

RNase oligomerization upon incubation in 40% ethanol and 60% water at 60 or 70 °C

| Incubation time | M RNase A (60 °C) | M RNase B II (60 °C) | M RNase B III (60 °C) | M RNase A (70 °C) | M RNase B II (70 °C) | M RNase B III (70 °C) |
|----------------|------------------|---------------------|----------------------|------------------|---------------------|----------------------|
| 5 min | % | | | | | |
| 5 | 86.5 | 76.1 | 69.6 | 94.1 | 86.3 | 91.0 |
| 15 | 63.9 | 64.8 | 64.0 | 92.1 | 66.0 | 69.6 |
| 60 | 66.5 | 63.9 | 67.0 | 65.1 | 64.7 | 62.0 |
| 120 | 64.9 | 71.8 | 65.9 | 70.2 | 64.0 | 52.7 |

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**Footnotes:**

a These were lyophilized from 40% acetic acid to induce oligomerization.

b These are the unlyophilized controls.

c The amounts of aggregates formed as a percent of the total; based on their molecular masses as estimated from their elution volumes from gel filtrations, the aggregates were tentatively identified as follows: aggregate (Agg.) 1, tetramer (TT); aggregate 2, trimer (T); and aggregate 3, dimer (D).

d The molecular masses of the derivatives and the number of attached PEG chains were estimated from their elution volumes from gel filtrations.
e NO, none observed.
f The molecular mass of this conjugate is particularly approximate.
g The identities of aggregates 4 and 5 are undetermined because of their very large molecular masses.
Glycosylation and Deamidation Affect RNase B Oligomerization

finding here by NMR that the disruption of this loop structure upon deamidation of Asn67 occurs not only in the crystal, but also in solution, is consistent with this proposal and with evidence that N67D-RNase A refolds more slowly than RNase A (19).

The strong activity against single-stranded (yeast) RNA and the weak anti-double-stranded poly(A)-poly(U) action observed here for fractions II and III of RNase B and of N67D,D-RNase B are typical for mammalian pancreatic type RNases with charge properties similar to those of RNase A and are conclusive that all the various fractions and peaks of RNase B studied here adopt native conformations in solution. The slightly higher activity for the longer glycoform is consistent with previously reported results for glycosylated mammalian ribonucleases (47, 48), and the slightly lower activity of N67D,D-RNase B might be due to a decreased electrostatic attraction for substrate due to the presence of a new negative charge or ascribable to an alteration of the B2 binding subsite, as was observed in crystal structures of N67D-RNase A (43, 44). N67D-RNase A and N67D,RNase A also show enzymatic activities similar to those of the parent protein (19).

RNase B was found by thermal denaturation to have a slightly higher stability ($\Delta T_m = 0.97 ^\circ C$, $\Delta G = +0.23$ kcal/mol) than RNase A. This result is consistent with earlier studies based on thermal unfolding (23) or hydrogen exchange near ambient temperature (21). The stabilizing effect is due to the sugar residues closest to the protein chain because a short chain provides the same stability increase (49) and because little or no change in stability occurs when the oligosaccharide chain is pruned back by glycosidases (50). The large drop in stability ($\Delta T_m = -4.4 ^\circ C$, $\Delta G = -1.1$ kcal/mol) upon deamidation is due to the loss of two hydrogen bonds, the appearance of a new negative charge, and backbone distortion due to the inserted -CH$_2$- group. These results are consistent with the stability measured for N67D-RNase A and N67D-RNase A by Catanzano et al. (51) and their interpretation based on the beautiful crystal structures of Mazzarella and co-workers (43, 44).

Oligomerization—A key objective of this study was to examine the effect of glycosylation on the formation of three-dimensional domain-swapped oligomers. This process requires at least two reversible steps: 1) a partial unfolding event in which the swap domain and the flexible hinge loop detach from the monomer core to form a swap-competent “unhinged” intermediate and 2) the binding of swap domains to different protein cores (52). For RNase A, the picture is more complex because both the N-terminal $\alpha$-helix and the C-terminal $\beta$-strand act as swap domains, giving rise to several oligomers (7–11). The results reveal that a covalently bound oligosaccharide chain affects the amount, rates, and types of oligomers formed.

This is the first investigation on how carbohydrate chains affect the formation of domain-swapped oligomers. Many other mammalian pancreatic ribonucleases, including those from human (53), ox, sheep, hamster, roe deer, and giraffe, are also glycosylated at Asn, whereas guinea pig ribonuclease carries two chains, and the pig and horse proteins carry three oligosaccharide chains (47, 54, 55). Based on their homology to the bovine RNase B studied here, the oligomerization of these glycoproteins will probably be significantly altered compared with that of RNase A.

PEG is frequently employed as a crowding agent to favor the formation of protein crystals. The ability of crowding agents to accelerate the formation of amyloid by human apolipoprotein C-II has been recently demonstrated (56). The results presented here indicate, however, that attaching PEG chains covalently to the protein produces the opposite effect, viz. the aggregation of protein molecules is blocked because the PEG chains impede their mutual approach. As PEG chains are highly soluble and do not stimulate the immune system, we propose that their attachment to amyloidogenic polypeptides might lead to a general method for reducing the formation of amyloid.

Selective deamidation at Asn67 of RNase B was found to decrease slightly the formation of oligomers induced by lyophilization from 40% acetic acid. If oligomers of N67D,D-RNase B were more unstable than those of RNase B relative to the monomer, this may explain why the former form less efficiently. Alternatively, the reduced formation of oligomer might be due to changes in the contacts and structure of the C terminus induced by deamidation, as detected by x-ray crystallography (44).

Two very different sets of conditions were used to promote oligomerization. Very high protein concentrations that favor oligomerization are attained by lyophilization from 40% acetic acid solution, the low pH of which destabilizes the protein electrostatically, favoring its partial unfolding. N67D,D-RNase B may be relatively less unstable in 40% acetic acid, where the Asp or iso-Asp residues at position 67 will be in the neutral state and therefore more similar to Asn. In contrast, the conditions of thermal aggregation (high temperature and 40% ethanol) likely act to preferentially weaken hydrophobic interactions between protein groups. Rapid cis/trans-isomerization of proline residues may add to the flexibility of the backbone at high temperatures, but is too slow to play a role under lyophilization conditions (57, 58). This effect may be especially relevant for Pro114, located in the C-terminal hinge loop.

The overall extent of oligomerization induced by thermal aggregation (60 ^\circ C and 40% ethanol) was found to be the same for RNase A versus RNase B, whereas oligomerization by lyophilization was lower for RNase B. This difference could be due to the higher protein concentrations attained during lyophilization, where the steric inhibition of the carbohydrate chain upon association is greater; the stabilizing effect of the carbohydrate chain against acid unfolding (50); or both. Despite this difference, it was found under both sets of conditions that 1) glycosylation favors the N-dimer, and 2) this preference increases with the length of the carbohydrate chain. The fact that these results were observed under both sets of conditions is evidence for their generality.

Following the progress of oligomerization at high temperature in 40% ethanol with time reveals insights into the kinetics of the process. Oligomerization was surprisingly found to proceed more rapidly in the presence of the carbohydrate chain than in its absence. All of the forms of RNase B studied showed a more rapid initial aggregation than RNase A, so the increased rate seems to be independent of the size of the carbohydrate chain or deamidation. Although we do not completely understand how this rate enhancement is achieved, it is unlikely to be directly related to the relative stability of the protein monomers and therefore to their ability to rapidly form an unhinged intermediate because both unmodified RNase B (more stable) and N67D,D-RNase B (less stable) oligomerized more rapidly than RNase A. We suggest two possible mechanisms to account for these results: 1) the oligosaccharide chain may operate kinetically by favoring productive collisions between unhinged intermediates; and 2) the oligosaccharide chain may stabilize the oligomers over the monomer by forming interactions with the hinge loop, by altering the solvation conditions, or by the excluded volume effect because oligomers are more compact.

3 The dielectric constant decreases as the temperature is raised. The dielectric constant is further reduced by the presence of ethanol. A smaller dielectric constant will act to strengthen electrostatic interactions. Moreover, ethanol is a less efficient competitor for peptide hydrogen bonds than water, so protein–protein hydrogen bonds will become stronger in 40% ethanol. In contrast, ethanol is more hydrophobic than water, and its presence weakens the stabilizing contribution of hydrophobic interactions.
than monomers. The N-dimer, being more compact than the C-dimer (8, 9), would be favored by the excluded volume effect. Both mechanisms could also account for why the N-dimer is favored over the C-dimer as the size of the carbohydrate chain increases. To test these possible mechanisms, we will measure the relative stabilities of the various dimer and monomer forms of RNases B and A and determine the solution structure of their dimers by NMR to identify any stabilizing interactions.

Comparison with Other Amyloid-forming Polypeptides—Our findings present interesting similarities and contrasts to the pathological oligomerization of amyloid-forming polypeptides. The formation of amyloid fibrils and plaques by the Alzheimer’s disease Aβ peptide (59) and the human prion protein appears to be extremely favorable thermodynamically (4), whereas the oligomers of RNases B and A formed by domain swapping have only marginally favorable thermodynamics. Besides the energetics of the process, evidence is building that there are different pathways, not just one, for forming amyloid (60) because some small peptides appear to pass directly from the unfolded to the amyloid state (61, 62), whereas the Alzheimer’s disease Aβ peptides can pass through a series of intermediate oligomeric states before forming amyloid (59). For many proteins, the first step in amyloid formation is likely the formation of domain-swapped oligomers (5, 6, 8–11), whereas the last hurdle appears to often be a very large kinetic barrier (4). The results presented here suggest that the formation of three-dimensional domain-swapped oligomers is less sensitive to glycosylation or deamidation than later stages in amyloid formation. Recently, the addition of a single sugar residue has been found to greatly accelerate or inhibit amyloid formation by a highly amyloidogenic human prion domain, depending on its preparation (83). The deamidation of Asn67. This modification reduces the oligomerization of the protein by 30–50% (58). To test these possible mechanisms, we will measure the stabilities and solution structures of these oligomers as being undertaken to understand the mechanism(s) by which glycosylation and deamidation affect oligomerization of bovine pancreatic ribonuclease.

Conclusions—An important finding of this work is that large fractions of the molecules in all four commercial RNase B preparations tested are selectively deamidated at Asn67. This modification reduces the oligomerization of the protein by three-dimensional domain swapping induced by lyophilization. Another key and unexpected finding is that the presence of Asn67 enhances both the rate and amount of N-dimer formed while reducing the formation of the C-dimer. This result is surprising because the oligosaccharide chain is positioned to sterically hinder swapping of the N-terminal domain. Experiments to determine the stabilities and solution structures of these oligomers are being undertaken to understand the mechanism(s) by which glycosylation and deamidation affect oligomerization of bovine pancreatic ribonuclease.

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Glycosylation and Specific Deamidation of Ribonuclease B Affect the Formation of Three-dimensional Domain-swapped Oligomers
Giovanni Gotte, Massimo Libonati and Douglas V. Laurents

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