Ribonucleases of Human Cerebrospinal Fluid: Detection of Altered Glycosylation Relative to Their Serum Counterparts†

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ABSTRACT: The ribonucleases (RNases) of human cerebrospinal fluid (CSF) have been characterized by phosphocellulose chromatography and glycosidase digestion in conjunction with activity staining following electrophoresis in sodium dodecyl sulfate–polyacrylamide gels. CSF was found to contain the major RNases present in serum plus additional species specific to CSF. The CSF RNases have been classified into six groups corresponding to the alkaline serum RNases 1–5 and the leukocyte RNase-like enzyme of serum. Molecular weights displayed by CSF RNases on gels are 29 700 (RNase 1), 26 000 (RNase 2.0), 24 500 (RNase 2.1), 22 900 (RNase 2.2), 19 700 (RNase 3.0), 19 200 (RNase 3.1), 18 700 (RNase 3.2), 16 000 (RNase 4), and 13 900 (RNase 5). RNases 3.1 and 3.2 are found in CSF but not in serum. Optimum resolution of both CSF and serum RNases was obtained in 15% polyacrylamide gels cast with poly(cytidylic acid) as substrate. Addition of 50 μg/mL bovine serum albumin to gels greatly increased the sensitivity of detection. Glycosidase digestion followed by electrophoresis and activity staining, which was also applied to bovine pancreatic RNases B, C, and D to provide internal controls, indicates that the human RNase 1, 2, and 3 groups are glycoproteins containing complex-type oligosaccharides. RNase 3.2 was found to lack the glycosidase-sensitive, terminal sialic acid and penultimate galactose residues present in RNase 3.0; RNase 3.1 lacks some but not all of these residues. These findings suggest that the CSF-specific RNases may arise from serum RNases by modification upon transport into the CSF.

Cerebrospinal fluid (CSF)[1] is separated from the blood by the blood–brain barrier, thus allowing a discrete internal environment to be maintained for the brain. The composition of CSF, including protein content, is precisely regulated at the primary site of CSF secretion, the choroid plexus (Rapoport, 1976). The protein concentration of CSF is only 0.5% that of blood plasma, the source of most CSF protein (Lewin & Tradatti, 1976). However, the serum (or plasma) RNase/CSF RNase concentration ratios for normal individuals range from 4.9:1 to 20:1 (Rabin et al., 1977a), demonstrating that RNase activity represents a larger proportion of the total protein of CSF than of plasma.[2]

Human CSF RNases have not been investigated as thoroughly as those of human blood serum or urine. Rabin et al. (1977b) reported that three incompletely separated peaks of RNase activity observed upon Sephadex G-100 chromatography of human CSF contain proteins which are antigenically related to a purified human urine RNase. These authors suggested that CSF RNase activity is derived from the blood plasma RNases. Recently, we have achieved visualization of the CSF RNases by activity staining following electrophoresis in RNA-cast $NaDodSO_4$–polyacrylamide gels and demonstrated that CSF contains RNases with mobilities comparable to those of serum RNases 1–5, as well as additional species not found in serum (Blank & Dekker, 1981). These results raise questions concerning the origin of CSF RNase activity, particularly the relationship between the RNases specific to CSF and those present in both CSF and serum.

The nomenclature used in this work for the CSF RNases is based on the system devised for the serum RNases by Akagi et al. (1976). These authors designated as RNases 1–5 the five peaks of activity separable on phosphocellulose columns; RNases 1–5 were subsequently found to correspond to electrophoretically distinct species or groups of species (Blank & Dekker, 1981). This nomenclature has been extended in the present work by the use of a decimal system to identify multiple species within an RNase group. The use of improved gel techniques providing greater sensitivity and resolution has permitted the detection in serum of two RNase species, named here 2.1 and 2.2, previously observed only in CSF. The letter "L" has been used to designate RNases displaying properties similar to those of leukocyte RNase.

The RNases of CSF have been examined by phosphocellulose and molecular sieve chromatography in conjunction with activity staining following $NaDodSO_4$–polyacrylamide gel electrophoresis. Gel techniques have been used to demonstrate the presence of a leukocyte RNase-like activity and to visualize the results of glycosidase digestion. In general, the CSF RNases have been found to consist of the RNases present in serum plus additional species lacking certain glycosidase-sensitive, terminal sialic acid and galactose residues present in their serum counterparts.

Materials and Methods

Materials. CSF and serum were obtained from untreated normal volunteers. Phosphocellulose (Selectacel phosphate type 40, 1.02 mequiv/g, lot 2973) was purchased from Brown Co., Sephadex G-75 was from Pharmacia, endoglycosidase D and crystallized bovine albumin were from Miles, bovine pancreatic RNases A (type I-A) and B (type XII-B), neuraminidase (type X), β-galactosidase (jack bean), β-*N*-acetylglucosaminidase (jack bean), and α-mannosidase (jack bean) were from Sigma, and sodium dodecyl sulfate was from Bio-Rad. Endoglycosidase H [purified by the procedure of Tarentino & Maley (1974)] was the gift of Drs. Clinton E.

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[1] Abbreviations: CSF, cerebrospinal fluid; $NaDodSO_4$, sodium dodecyl sulfate; BSA, bovine serum albumin; poly(C), poly(cytidylic acid); Tris, tris(hydroxymethyl)aminomethane.

[2] Using an alternate assay (Blank & Dekker, 1981), we find ratios in the range 1:2 to 2:1 for serum/CSF RNase activity (G. L. Schieven and A. Blank, unpublished experiments).

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Ballou and W.-j. Zhang. Bovine pancreatic RNases C and D were the gift of Dr. Thomas H. Plummer, Jr. RNase U_1 was purified in this laboratory (Kenney & Dekker, 1971).

RNase Assay. RNase activity was assayed by the method of Blank & Dekker (1981).

Electrophoresis and Activity Staining. Electrophoresis in either 12.5% or 15% $NaDodSO_4$–polyacrylamide slabs was carried out at 4 °C (or at room temperature for the gels of Figure 4) as described by Blank & Dekker (1981). Separating gels were cast with 0.3 mg/mL wheat germ rRNA or with 0.3 mg/mL poly(cytidylic acid) [poly(C)] (P-L Biochemicals) as the RNase substrates. BSA was also cast into certain separating gels as described under Results. Electrophoresis was carried out for 90 min for 9-cm 12.5% gels, 180 min for 9-cm 15% gels, and 14 h for 36-cm 15% gels.[3] $NaDodSO_4$ was then removed from the gels by incubation with gentle stirring for 15 min each in two 0.5-L successive portions of 25% 2-propanol–0.01 M Tris-HCl, pH 7.4 (Blank et al., 1982), followed by two 0.5-L portions of 0.01 M Tris-HCl, pH 7.4, buffer. Enzymatic digestion of the embedded substrate was then allowed to proceed at 37 °C for 24 h (unless otherwise specified) in 0.1 M Tris-HCl, either pH 7.4 or pH 7.5, as specified under Results. The gels were then stained with Toluidine Blue O (Eastman) as described by Blank & Dekker (1981).

Densitometry. Densitometry of activity-stained gels was carried out at 520 nm by using a Kratos SD3000 spectrodensitometer at a scanning rate of either 5 or 10 mm/min. The mobilities of RNase species on gels were determined by the use of a Hewlett-Packard 3380A integrator to locate the point of maximum intensity of each band to within 0.1 mm. Since a reference dye front was not available on all gels[3] for calculation of relative mobilities, RNase 4 intrinsic to the samples was used as a marker in each lane of each gel to align the densitometer scans. RNase 4 was chosen as a marker because serum and CSF RNase 4 have the same mobility on gels and because its mobility is unaffected by glycosidase digestion.

Molecular Weight Determination. Molecular weights were determined from a series of gels by using a modification of the procedure described by Weber & Osborn (1969). Ovalbumin, glyceraldehyde-3-phosphate dehydrogenase, α-chymotrypsinogen, trypsin, avidin, and equine cytochrome *c* were used as molecular weight standards. Reduced samples (prepared by heating in 2% $NaDodSO_4$ and 5% mercaptoethanol for 2 min at 100 °C) of protein standards and CSF RNases from unfractionated CSF and from phosphocellulose fractions such as those seen in Figure 2 were run on the same 9-cm 12.5% or 36-cm 15% gel; all gels were RNA-cast $NaDodSO_4$–polyacrylamide slabs. After electrophoresis, the dye front was marked in each lane, and the lanes containing standards were cut from the slab and stained for protein with Coomassie Brilliant Blue (Bio-Rad) (Fairbanks et al., 1971), while the CSF RNases were detected on the remainder of the slab by activity staining. Molecular weights of CSF RNases reported in Table I were determined by using this procedure. The apparent molecular weights of the serum RNases reported in Table I and shown in Figure 1E, and of the glycosidase products shown in Figure 4, were determined under nonreducing conditions as follows. A standard curve was prepared for each gel with CSF RNases 2.0, 2.1, 2.2, 3.0, 3.2, and 4 serving as molecular weight standards. The mobilities of these RNases were plotted vs. their log molecular weights previously established under reducing conditions (see above). The molecular weights of various other RNase species were read from this standard curve. The mobilities of all RNase bands were determined by densitometry as described above. The use of primary protein standards to calculate molecular weights was precluded due to the absence of a dye marker band on 9-cm 15% gels.[3]

Phosphocellulose Chromatography. Phosphocellulose resin was washed with acid and base (Peterson & Sober, 1962), and the fine particles were decanted prior to use. Five milliliters of CSF containing 1400 units of RNase activity was dialyzed at 4 °C for 20 h vs. two 1-L volumes of 0.01 M sodium phosphate buffer, pH 6.7, and applied to a 1.0 × 17.3 cm phosphocellulose column equilibrated with the same buffer. The column was washed with 38 mL of equilibration buffer, and a linear gradient of 0.2–1.8 M NaCl in 120 mL of the same buffer was applied. Fractions of 0.58 mL were collected in siliconized tubes. The NaCl concentration in the fractions was determined by measurement of conductivity. Chromatography of serum was performed as described for CSF by using 6.5 mL of serum which was dialyzed 18 h vs. two 130-mL volumes of equilibration buffer and then centrifuged.

Molecular Sieve Chromatography. A 2.5-mL sample of CSF was made 2.5 M in NaCl and loaded with Blue Dextran (Pharmacia) as a marker onto a 1.4 × 101 cm G-75 Sephadex column equilibrated with 0.5 M NaCl–0.02 M Tris-HCl, pH 7.4. The flow rate was 8.2 mL/h. Fractions of 2 mL were collected in siliconized tubes and assayed to determine elution volumes. The RNases comprising each peak of activity were identified by activity-stained $NaDodSO_4$–polyacrylamide gels.

Molecular weights were determined by using BSA, ovalbumin, myoglobin, and RNase U_1 (M_r 11 100) as standards. The elution volumes were determined from the absorbance at 280 nm for BSA and ovalbumin and at 410 nm for myoglobin and by measurement of RNase activity for RNase U_1.

Glycosidase Digests. Methods used for glycosidase digestion are modified from those previously employed in this laboratory for digestion of human RNases.[4] For exoglycosidase digests, 0.4 unit of CSF RNases partially purified by phosphocellulose chromatography was digested in a solution containing 0.15 mg/mL BSA, 0.01% sodium azide, and 0.075 M sodium citrate, pH 4.5, plus one or more of the following: 0.05 unit of neuraminidase, 0.063 unit of β-galactosidase, 0.35 unit of β-*N*-acetylglucosaminidase, and 0.30 unit of α-mannosidase; total volume was 150 μL. Digestion was allowed to proceed for 60 h at 37 °C. Aliquots containing 0.1 unit of CSF RNases treated with neuraminidase, β-galactosidase, and β-*N*-acetylglucosaminidase were adjusted to pH 6.5 with NaOH and incubated for 60 h at 37 °C with 0.01 unit of endoglycosidase D in a total volume of 65 μL. Aliquots containing 0.4 unit of untreated CSF RNases were digested in a solution containing 0.15 mg/mL BSA, 0.01% sodium azide, 0.10 M sodium citrate, pH 5.0, and 0.01 unit of endoglycosidase H; total volume was 200 μL. Digestion was allowed to proceed for 60 h at 37 °C. For glycosidase digestion of bovine RNases B, C, and D, 1 μg of each was treated as described above.

Results

Electrophoretic Pattern of Unfractionated CSF and Serum RNases. Serum and CSF RNases exhibit some similarities

[3] The 3-h electrophoretic runs utilized for enzyme separation on the 9-cm 15% gels resulted in migration of the bromophenol blue marker band off the lower end of the gel.

[4] Sugiyama, R. H. (1981) Ph.D. Dissertation, University of California, Berkeley, CA.

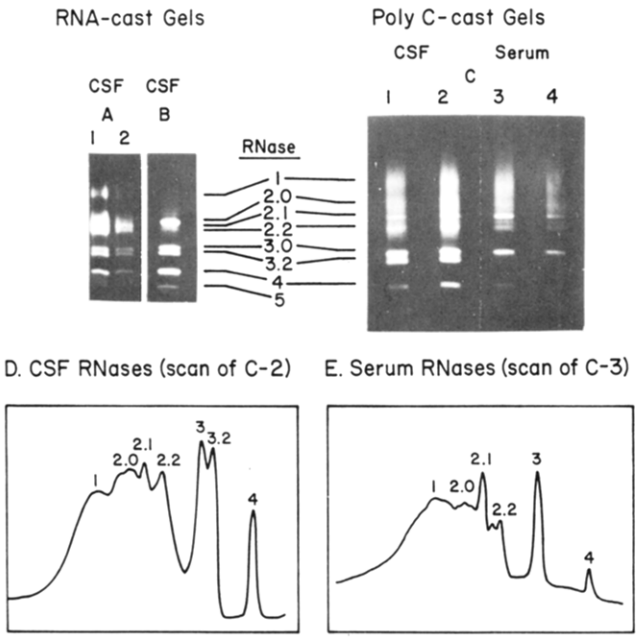

FIGURE 1: Electrophoretic patterns of CSF and serum RNases from the same individual. (A) Samples containing 21.6 (well 1) and 14.4 μL (well 2) of CSF were heated at 100 °C for 2 min in the presence of 2% $NaDodSO_4$ and 5% mercaptoethanol prior to electrophoresis in an RNA-cast 12.5% $NaDodSO_4$–polyacrylamide gel. The gel was incubated for 24 h at 37 °C in 0.1 M Tris-HCl, pH 7.4, to permit the digestion of the embedded RNA and stained for activity as described in the text. (B) A sample containing 3.3 μL of CSF was heated at 100 °C for 2 min in the presence of 2% $NaDodSO_4$ prior to electrophoresis in a 12.5% polyacrylamide gel cast with RNA and 10 μg/mL BSA. The gel was incubated for 2.5 h at 37 °C in 0.1 M Tris-HCl, pH 7.4, and stained for activity as described in the text. (C) Samples of CSF and serum were heated for 2 min at 100 °C in 1% $NaDodSO_4$ and then loaded on a 15% $NaDodSO_4$–polyacrylamide gel cast with poly(C) and 50 μg/mL BSA. Following electrophoresis and removal of $NaDodSO_4$, the gel was incubated for 24 h at 37 °C in 0.10 M Tris-HCl, pH 7.5, and then stained to reveal RNase activity, as described in the text. Wells 1 and 2 contain 0.005 and 0.010 μL of CSF; wells 3 and 4 contain 0.010 and 0.005 μL of serum. (D) Densitometry scan of well 2 of (C). (E) Densitometry scan of well 3 of (C).

in their electrophoretic patterns under a variety of experimental conditions, as is illustrated in Figure 1 by utilizing samples of CSF and serum from the same individual. Bands corresponding to RNases 1, 2.0, 3.0, and 4 are found in both serum and CSF, as has been previously reported (Blank & Dekker, 1981). The improved sensitivity and resolution offered by 15% $NaDodSO_4$–polyacrylamide gels cast with poly(C) and 50 μg/mL BSA (Figure 1C) relative to 12.5% $NaDodSO_4$–polyacrylamide gels cast with RNA (Figure 1A,B) permit the detection of the RNase 2.1 and 2.2 species in serum. Bands corresponding to these species had been previously reported only in CSF (Blank & Dekker, 1981). RNase 5, a minor component of both serum and CSF, has little activity toward poly(C) as substrate (Akagi et al., 1976) and thus is not visible in the gel of Figure 1C on which 5–10-nL samples of CSF or serum were loaded. With RNA as substrate and a 3.3-μL sample of CSF loaded, RNase 5 is readily apparent (Figure 1B). RNase 1 is most clearly seen in samples heated in the presence of 2% $NaDodSO_4$ and 5% mercaptoethanol prior to electrophoresis in RNA-cast $NaDodSO_4$–polyacrylamide gels (Figure 1A), but it is also prominent in the densitometer scans (Figure 1D,E). It should be noted that the densitometer scans show greater detail than do the photographic reproductions of the gels. Leukocyte RNase-like activity common to both samples does not appear in Figure 1, but evidence for its presence is discussed later (Figure 3). However, there are

Table I: Apparent Molecular Weights of RNase Species

RNase	mol wt ($\times 10^{-3}$) CSF (G-75)	CSF (gel)	serum (gel)
1	ND[b]	29.7	29.1
2.0	28	26.0	25.9
2.1		24.5	24.2
2.2		22.9	22.7
3.0	20	19.7	19.6
3.1		19.2	NP[a]
3.2		18.7	NP
4	15.5	16.0	16.0
5	ND	13.9	13.9
L	15.5	17.0	17.0

[a] NP = not present. [b] ND = not determined.

important differences between the serum and CSF patterns as well. RNase 3.2, a major peak in the CSF pattern, is absent in serum. RNase 3.1 (shown in Figure 2C), a minor species which runs between RNases 3.0 and 3.2, is also absent in serum. Diffuse activity staining is seen between RNases 2.2 and 3.0 in CSF, but not in serum (Figure 1C). The serum samples display a small peak between RNases 2.1 and 2.2 when poly(C) is used as a substrate, whereas the CSF samples show only diffuse activity in this region (Figure 1C–E).

The apparent molecular weights of the major CSF and serum RNase species are listed in Table I. Molecular weights were reproducible from gel to gel to within ±1% when the mobilities of the species were determined by densitometry; the differences between the weights of the CSF RNases and their serum counterparts lie within the experimental error and thus are not significant. The small peak between serum RNases 2.1 and 2.2 in Figure 1E displayed a molecular weight of 23 400.

Phosphocellulose Chromatography. The elution profile of human CSF RNase activity observed upon phosphocellulose chromatography is shown in Figure 2A; the peaks have been identified from the corresponding electrophoretic patterns of individual column fractions shown in Figure 2C. The general order of elution of the CSF RNases with increasing salt concentration, namely, RNases 1, L, 2, 3, 4, and then 5, is the same as that shown for the serum RNases (Figure 2B,D). However, the resolution of CSF RNase species by phosphocellulose chromatography is much poorer than that of serum RNases, and a 18–30% higher NaCl concentration is required for elution of CSF RNases relative to their serum counterparts. This may be due in part to the presence of 200 times more protein in serum than in CSF (Lewin & Tradatti, 1976). The RNase 3 group was found to give rise to the largest activity peak in both serum and CSF, followed by the RNase 2 group and then RNase 4. RNases 1 and 5 were found in relatively lesser amounts in CSF than in serum. Similar results were found in phosphocellulose chromatography of CSF under conditions where the ratio of RNase activity to bed volume was higher, except that poorer resolution was observed (data not shown).

Leukocyte RNase-like Activity in CSF. We have found that human leukocyte RNase displays a characteristic difference in mobility on $NaDodSO_4$–polyacrylamide gels depending upon whether it is exposed to 2% $NaDodSO_4$ at 25 °C prior to loading or is heated in 2% $NaDodSO_4$ for 2 min at 100 °C (Blank & Dekker, 1981). Figure 3A shows the results of such treatment of phosphocellulose and G-75 Sephadex column fractions containing CSF RNase L activity, and of human leukocyte RNase. In each case, the CSF RNase L species displayed the expected change in mobility while the other CSF

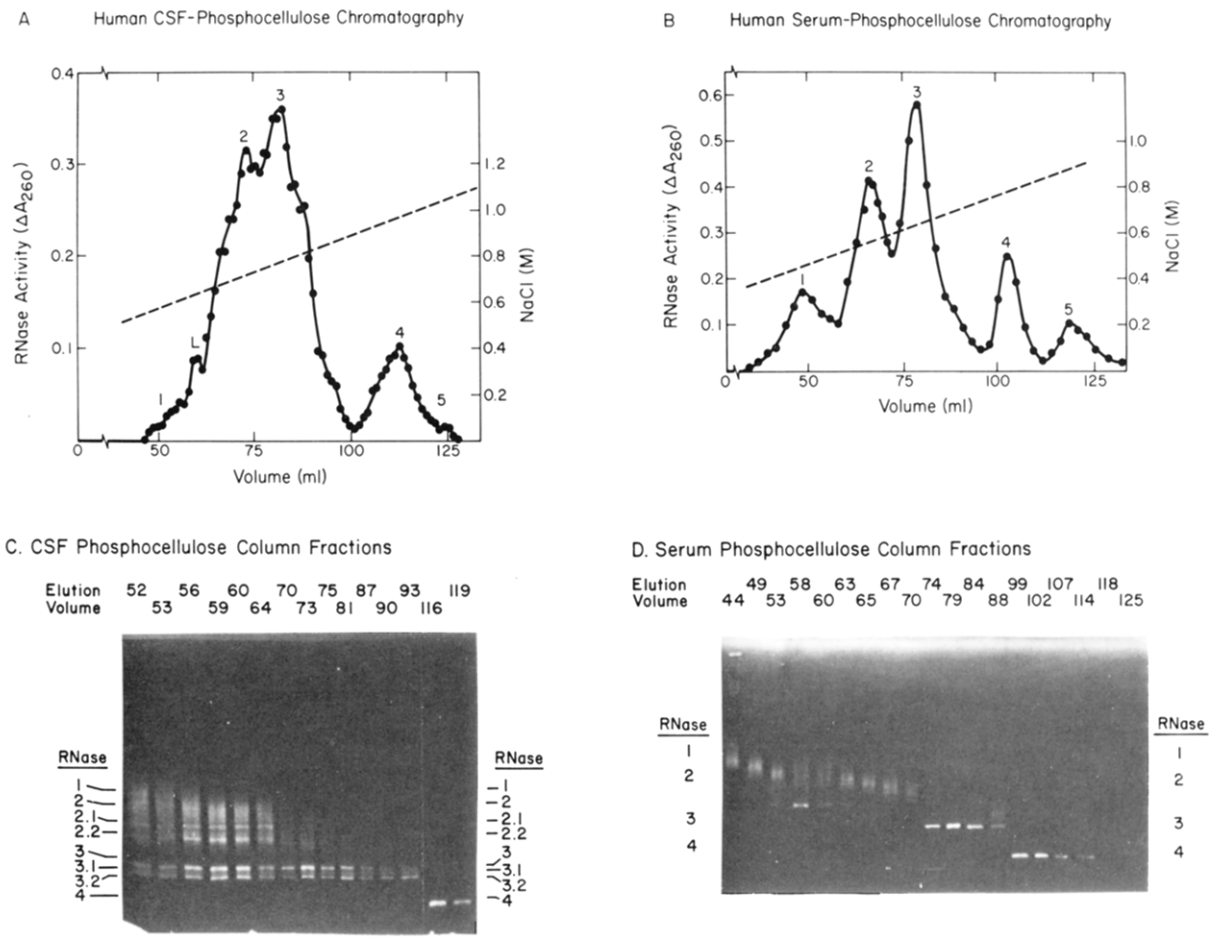

FIGURE 2: Chromatographic profiles of human CSF and serum ribonuclease with electrophoretic patterns of individual column fractions. CSF (A) and serum (B) were chromatographed on phosphocellulose, and 10-μL aliquots of column fractions were assayed for RNase activity at pH 8.5. Aliquots of column fractions from CSF (C) and serum (D) chromatography were heated at 100 °C for 2 min in the presence of 1% $NaDodSO_4$ prior to electrophoresis in a poly(C)- and 10 μg/mL BSA-cast 15% $NaDodSO_4$–polyacrylamide gel. Each well was loaded with 0.024 unit of RNase activity. The gel was incubated for 24 h at 37 °C in 0.1 M Tris-HCl, pH 7.5, to permit digestion of the embedded poly(C) and stained for activity as described in the text.

RNases examined (2.0, 2.1, 2.2, 3.0, 3.2, and 4) did not change in mobility.

The pH optimum for digestion of RNA by leukocyte RNase activity has been reported to be 6.5 (Silber et al., 1967; Akagi et al., 1978), whereas the optimum for serum RNases 1–5 ranges from pH 7.5 to 8.5 (Akagi et al., 1976). Figure 3B compares the results of incubation at pH 6.5 and 8.5 for phosphocellulose column fractions containing CSF RNases. Only the CSF RNase L band displayed higher activity at pH 6.5 than at pH 8.5 when visualized on gels by activity staining.

Glycosidase Digestion. Human CSF RNase groups 2, 3, and 4, partially purified by phosphocellulose chromatography, were subjected to glycosidase digestion and then visualized on 15% $NaDodSO_4$–polyacrylamide gels cast with poly(C) as substrate. Bovine pancreatic RNases B, C, and D were also subjected to glycosidase digestion as internal controls. Glycosidase digestion of the RNases was detected by observing increases in mobility on the $NaDodSO_4$–polyacrylamide gels, a method previously employed in the analysis of purified proteins such as yeast invertase by using protein-stained gels (Trimble & Maley, 1977).

Figure 4 shows the results of glycosidase digestion of bovine pancreatic RNases B, C, and D, all of which have the same polypeptide as bovine pancreatic RNase A but vary in the extent and nature of their glycosylation. RNase B contains a single high mannose type oligosaccharide chain N-linked to Asn-34 (Plummer & Hirs, 1964). The major RNase B species present in the Sigma product contains an oligosaccharide with the composition $Man_5GlcNAc_2$, while three minor species have one, two, and three additional mannose residues, respectively (Liang et al., 1980). The Sigma product also contains an RNase A-like activity, designated RNase A′, which is not glycosylated and which elutes before RNase A upon CM-cellulose chromatography (Berman et al., 1981). We have found that RNase A′ displays the same mobility on $NaDodSO_4$–polyacrylamide gels as RNase A (data not shown). As expected for high mannose type glycoproteins, only α-mannosidase and endoglycosidase H displayed activity against RNase B (Figure 4A). Bovine pancreatic RNases C and D contain a single complex type oligosaccharide chain N-linked to Asn-34, with the composition $GlcNAc_4Man_4Fuc_1Gal_2SA_2$ reported for RNase C and $GlcNAc_4Man_3Fuc_1Gal_2SA_4$ reported for RNase D (Plummer, 1968). Parts B and C of Figure 4 display the increases in mobilities of RNases C and D, respectively, due to removing sequentially sialic acid, galactose, *N*-acetylglucosamine, and α-linked mannose residues, and to endoglycosidase D digestion. Results are in accord with those expected for complex type glycoproteins. Treatment with β-galactosidase, β-*N*-acetylglucosaminidase, or α-mannosidase alone had no effect, as would be expected if there were no terminal galactose, *N*-acetylglucosamine, or α-linked mannose residues.

Figure 5 shows the results of densitometry of gels loaded with CSF RNases treated with glycosidases. No significant difference was seen between samples treated once with glycosidase(s) (Figure 5) and samples treated under the same

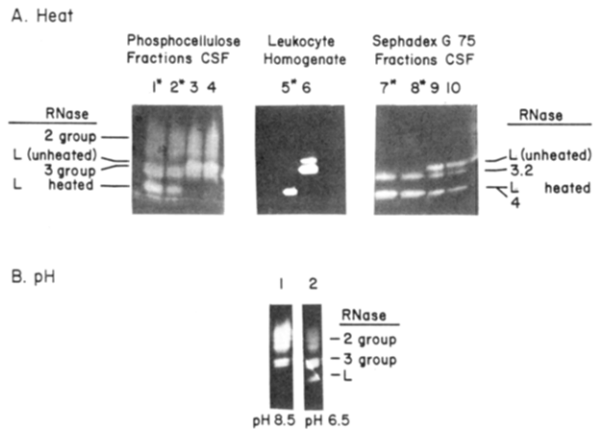

FIGURE 3: Presence of leukocyte RNase-like activity in CSF. (A) Differences in the electrophoretic mobility of CSF RNase L with heating (indicated by asterisks) and without heating at 100 °C prior to loading on RNA-cast 12.5% $NaDodSO_4$–polyacrylamide gels. Before being loaded on gels, unheated samples were allowed to stand at 25 °C in the presence of 2% $NaDodSO_4$, whereas heated samples were heated at 100 °C for 2 min in the presence of 2% $NaDodSO_4$. In each case, 0.12 unit of activity was loaded. Samples are from a single phosphocellulose column fraction of CSF (wells 1–4), a leukocyte homogenate as a control (wells 5 and 6), and a single G-75 Sephadex column fraction of CSF (wells 7–10). The gels were incubated for 24 h in 0.1 M Tris-HCl, pH 7.4, in the course of activity staining. (B) Effect of the pH of incubation on the activity staining of CSF RNases. A single RNA-cast 12.5% $NaDodSO_4$–polyacrylamide gel was loaded with a phosphocellulose column fraction heated for 2 min at 100 °C in the presence of 2% $NaDodSO_4$. After electrophoresis, the gel was cut into two sections. Section 1 was incubated in 0.035 M Tris-HCl–0.15 M NaCl, pH 8.5, for 24 h, while section 2 containing the same amount of the same sample was incubated in 0.035 M imidazole hydrochloride–0.15 M NaCl, pH 6.5, for 24 h. Both sections were then stained together.

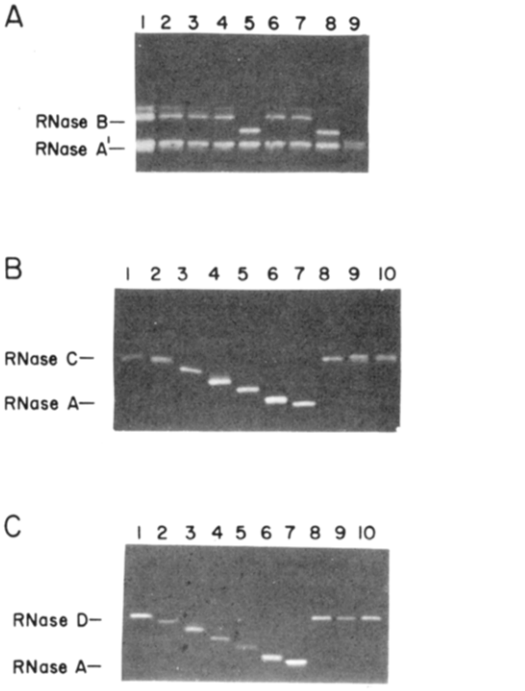

FIGURE 4: Bovine pancreatic RNases were subjected to glycosidase digestion as described in the text. Samples containing approximately 1–10 pg of RNase were heated at 100 °C for 2 min in the presence of 1% $NaDodSO_4$ and loaded on poly(C)-cast 15% $NaDodSO_4$–polyacrylamide gels. The gels were stained for activity as described in the text following incubation for 24 h in 0.10 M Tris-HCl, pH 7.5. (A) RNases A′ and B: (1) untreated; (2) neuraminidase; (3) neuraminidase + β-galactoside; (4) neuraminidase + β-galactosidase + β-*N*-acetylglucosaminidase; (5) neuraminidase + β-galactosidase + β-*N*-acetylglucosaminidase + α-mannosidase; (6) β-galactosidase; (7) β-*N*-acetylglucosaminidase; (8) α-mannosidase; (9) endoglycosidase H. (B) RNase C: (1–5) same treatment as in (A); (6) neuraminidase + β-galactosidase + β-*N*-acetylglucosaminidase + endoglycosidase D; (7) RNase A; (8) β-galactosidase; (9) β-*N*-acetylglucosaminidase; (10) α-mannosidase. (C) RNase D: (1–10) same treatment as in (B).

conditions with three sequential aliquots of glycosidase, indicating that no further digestion was possible under the conditions used. Parts B–E of Figure 5 display, respectively, the results of removing sialic acid, galactose, *N*-acetylglucosamine, and, finally, α-linked mannose residues from the CSF RNase 2 and 3 groups shown intact in Figure 5A.

Prior to glycosidase treatment, the CSF RNase 3 group displays two major peaks (Figure 5A), RNases 3.0 and 3.2, with apparent molecular weights of 19 700 and 18 700. The minor component, RNase 3.1 (Figure 5I), displays an apparent molecular weight of 19 200. After treatment with neuraminidase (Figure 5B), the RNase 3.0 peak shifts to a molecular weight of 19 200. Although RNase 3.2 now appears as a shoulder, its mobility on the gel remains unaffected. After treatment with both neuraminidase and β-galactosidase, the CSF RNase 3 group forms a single peak with an apparent molecular weight of 18 700 (Figure 5K) to 18 800 (Figure 5C). This is within experimental error of the molecular weight of 18 700 displayed by RNase 3.2 (Figure 5A,I), suggesting that RNase 3.2 might differ from RNase 3.0 only in lacking terminal sialic acid and galactose residues, in which case *N*-acetylglucosamine would be expected in the nonreducing terminal position from the general structure of complex carbohydrates (Kornfeld & Kornfeld, 1980). RNase 3.2 is shown to have *N*-acetylglucosamine present in the nonreducing terminal position by its susceptibility to digestion by β-*N*-acetylglucosaminidase alone (Figure 5G), whereas no other RNase species is affected. The peak with a molecular weight of 17 800, representing the product of β-*N*-acetylglucosaminidase digestion of RNase 3.2 (Figure 5G), has the same weight as the product of combined β-*N*-acetylglucosaminidase, β-galactosidase, and neuraminidase digestion of RNase 3.0 (Figure 5D,L). Thus, RNase 3.2 appears to be quite similar to RNase 3.0, except that RNase 3.2 lacks the glycosidase-sensitive terminal sialic acid and penultimate galactose residues present in RNase 3.0. After neuraminidase digestion, the RNase 3.0 peak shifts to a molecular weight of 19 200 (Figure 5B), the same as that displayed by RNase 3.1. However, the RNase 3.1 peak remains after treatment with β-galactosidase (cf. Figure 5I,J), indicating that RNase 3.1 is not the asialo form of RNase 3.0. Treatment with both neuraminidase and β-galactosidase eliminates the RNase 3.1 peak, shifting it and the other member of the RNase 3 group to a peak with a molecular weight of 18 700 (Figure 5K). Thus, RNase 3.1 appears to have some, but not all, of the sialic acid and galactose residues present in RNase 3.0.

In Figure 5G, the RNase 2 group shows no change after treatment with β-*N*-acetylglucosaminidase alone, suggesting that there are no *N*-acetylglucosamine-terminated oligosaccharides available for digestion. However, treatment with β-galactosidase alone (Figure 5H) generates a series of small new peaks, indicating the presence of some exposed terminal galactose residues available for digestion. It should be noted that the original RNase 2.1 and 2.2 peaks remain, indicating that these major species do not have terminal galactose residues. The heterogeneity exhibited by the RNase 2 group is not diminished until after treatment with a combination of neuraminidase, β-galactosidase, β-*N*-acetylglucosaminidase, and either α-mannosidase (Figure 5E) or endoglycosidase D (Figure 5F).

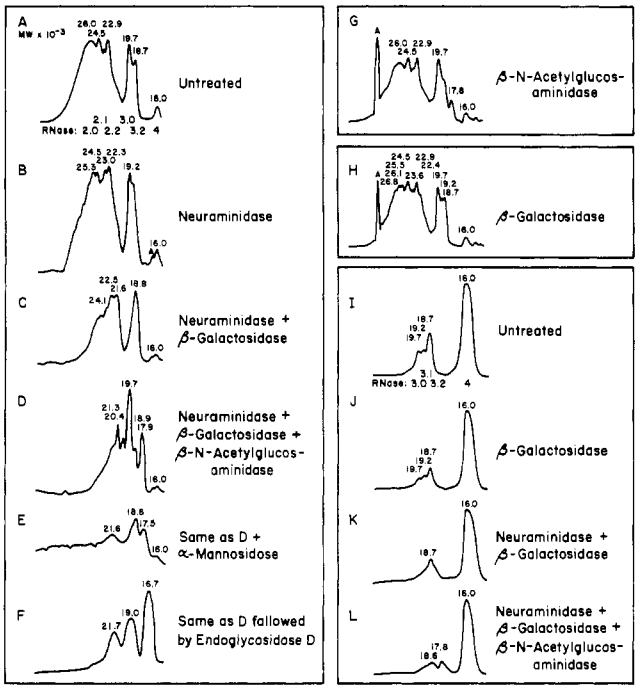

FIGURE 5: CSF RNases partially purified by phosphocellulose chromatography were subjected to glycosidase digestion as described in the text. A phosphocellulose column fraction enriched in CSF RNase groups 2 and 3 was used for (A–H). A phosphocellulose column fraction enriched in CSF RNase groups 3 and 4 was used for (I–L). Aliquots containing 12.5 μL of sample were heated at 100 °C for 2 min in the presence of 1% $NaDodSO_4$ and loaded on poly(C)-cast 15% $NaDodSO_4$–polyacrylamide gels. All samples were run on the same gel except for (E) and (F), which were each run on separate gels. The gels were stained for activity as described in the text following incubation for 24 h in 0.10 M Tris-HCl, pH 7.5. The gels were then scanned by the densitometer at a rate of 10 mm/min (A–H) or 5 mm/min (I–L) as described in the text. A relatively low gain setting was used to scan the gel containing sample (E), resulting in lower peak heights. As mentioned under Discussion, peak A in (G) and (H) arises from a protein contaminant(s) in the jack bean β-*N*-acetylglucosamine and β-galactosidase preparations which enhances (enhance) renaturation of *comigrating* RNase activity. (A) Untreated; (B) neuraminidase; (C) neuraminidase + β-galactosidase; (D) neuraminidase + β-galactosidase + β-*N*-acetylglucosaminidase; (E) neuraminidase + β-galactosidase + β-*N*-acetylglucosaminidase + α-mannosidase; (F) neuraminidase + β-galactosidase + β-*N*-acetylglucosaminidase + endoglycosidase D; (G) β-*N*-acetylglucosaminidase; (H) β-galactosidase; (I) untreated; (J) β-galactosidase; (K) neuraminidase + β-galactosidase; (L) neuraminidase + β-galactosidase + β-*N*-acetylglucosaminidase.

RNase 4 was not affected by treatment with any of the above-mentioned glycosidases. Furthermore, none of the RNases was affected by treatment with α-mannosidase alone, or with endoglycosidase H, indicating that no oligosaccharides of the high mannose type found in bovine RNase B are present in the human CSF RNase 2, 3, or 4 groups. RNase 1 was found to be sensitive to neuraminidase digestion (not shown).

Discussion

The technique of activity staining after $NaDodSO_4$–polyacrylamide gel electrophoresis for the analysis of RNase isozyme mixtures in the presence of other proteins (Blank & Dekker, 1981) has been improved and extended by several modifications. A comparison of parts B and C of Figure 1 reveals that use of a homopolymer-cast 15% gel run for 180 min rather than an RNA-cast 12.5% gel run for 90 min substantially improved the separation and resolution of species which differ only slightly in molecular weight. For example, in Figure 4A, lane 9, the product of endoglycosidase H digestion of RNase B is distinguishable from RNase A, even though they are identical except for the presence of one *N*-acetylglucosamine residue on the former. Similarly, the four major species composing the Sigma RNase B product are readily distinguishable from one another on the original gel or with the aid of densitometry (not shown) even though they differ from one another only by single mannose residues. Casting BSA into the gel enhances the recovery of enzymatic activity following electrophoresis under denaturing conditions (Lacks & Springhorn, 1980), permitting much smaller samples to be used; this enhancement has been attributed to promotion of renaturation (Lacks & Springhorn, 1980) and may also involve mitigation of the deleterious effects of contaminants in the $NaDodSO_4$ used for electrophoresis (Thelen et al., 1982). The use of BSA also diminishes the intensity of spurious bands arising from the enhancement of activity by contaminating proteins which comigrate with and promote renaturation of RNases.[5] Such spurious bands show a relative decrease in intensity when the concentration of BSA in the gel is raised, whereas the relative intensity of genuine bands increases. It should be noted that the same sample can give rise to different electrophoretic patterns under different conditions of electrophoresis and activity staining, as is illustrated in Figure 1. For example, RNase 1 displays greater activity relative to the other RNases after electrophoresis in the presence of mercaptoethanol or when poly(C) is used as a substrate, whereas RNase 5 displays decreased activity relative to the other RNases under such conditions.

Apparent molecular weights determined by $NaDodSO_4$ gel electrophoresis or molecular sieve chromatography of glycoproteins tend to be greater than the true molecular weights (Leach et al., 1980a,b). Thus, the true molecular weights of the glycosylated RNases are likely to be lower than the apparent molecular weights reported here.

The ratio of the concentration of a protein in serum to the concentration of the same protein in CSF has been reported to be a function of the protein's hydrodynamic radius (Felgenhauer, 1974). Larger proteins were found to have higher serum to CSF concentration ratios than smaller proteins, as would be expected if the process of filtration dominated the transport of proteins from plasma to CSF. However, such a correlation does not hold true for the CSF RNases. The smallest species, RNase 5, is present in a lesser amount in CSF than in serum relative to the other RNases (cf. parts A and B of Figure 2), whereas the relative abundance of the larger RNase 2 and 3 groups is not diminished in CSF relative to serum. Thus, the CSF RNases do not appear to arise solely by a filtration mechanism.

The heterogeneity displayed by the CSF RNases appears to be due in part to heterogeneity in glycosylation. Although CSF RNase 1 displays a broad diffuse band on $NaDodSO_4$ gels, treatment with neuraminidase results in the appearance of a series of sharp, well-defined bands (data not shown). This indicates that RNase 1 is glycoprotein in nature and that the broad diffuse band on gels is due at least in part to heterogeneity in glycosylation.

The heterogeneity within the CSF RNase 2 group was not substantially diminished until after treatment with neuraminidase, β-galactosidase, β-*N*-acetylglucosaminidase, and either α-mannosidase or endoglycosidase D. This suggests that

[5] G. L. Schieven and C. A. Dekker, unpublished experiments. For example, such spurious bands appear in Figure 5G,H (peak A), where proteins contaminating the β-*N*-acetylglucosaminidase and β-galactosidase preparations comigrate with RNase activity. Peak A does not appear in Figure 5C,D, or in other samples where these glycosidase preparations were used, because there is no significant RNase activity at the locus of the contaminating proteins.

the heterogeneity within this group is, in part, at the level of oligosaccharide chain branching. After treatment with endoglycosidase D, the major peak of activity displayed a molecular weight of 16700, close to the molecular weight of RNase 4. Given the inherent difficulty in the enzymatic removal of carbohydrate from intact glycoproteins (Li & Li, 1977), the two smaller peaks remaining after treatment with endoglycosidase D may represent incomplete digestion products.

The heterogeneity within the RNase 3 group also appears to be due to differences in glycosylation. The results of glycosidase digestion indicate that RNase 3.2 is quite similar to RNase 3.0 except that RNase 3.2 lacks the glycosidase-sensitive, terminal sialic acid and penultimate galactose residues present on RNase 3.0, leaving *N*-acetylglucosamine exposed in the terminal position. RNase 3.1 appears to be an intermediate between RNases 3.0 and 3.2, lacking some but not all of the sialic acid and galactose residues present in RNase 3.0. RNase 4, which is common to both CSF and serum, does not appear to contain either typical high mannose or complex type oligosaccharides, since it was not affected by any glycosidase treatment.

The human CSF RNases thus appear to be similar to those mammalian pancreatic RNases which occur as glycoproteins with *N*-asparagine-linked, complex type oligosaccharides or as nonglycosylated forms (Beintema et al., 1976). No species corresponding to bovine RNase B with high mannose type oligosaccharides was found.

Although we have used bovine pancreatic RNases B, C, and D as internal standards to establish the activity and specificity of the glycosidase preparations employed, it should be noted that only the oligosaccharide component of the B species is totally characterized structurally. Analysis of the carbohydrate components and of the linkage point of the C and D species of Plummer (1968) suggests that these species have complex type, asparagine-linked oligosaccharides. Our gels confirm the homogeneity of the Plummer preparations and are consistent with a structure in which the outer chains have the sequence (sialic acid)$_n$→β-Gal→β-GlcNAc and are linked to a core of two α-mannose residues attached to a β-mannosyl-di-*N*-acetylchitobiose unit. Our data provide no evidence as to the location of the fucose residue of the C or D species, or of the additional mannose residue suggested by the analytical data (Plummer, 1968) for species C.

The altered glycosylation of members of the CSF RNase 3 group relative to serum raises the question of whether other CSF glycoproteins might be similarly modified. Parker & Bearn (1961) reported that human CSF transferrin consists of two major species. One species has the four sialic acid residues found in serum transferrin while the other, named τ, lacks all four sialic acid residues. Transferrin species with an intermediate number of sialic acid residues were found to be very minor components of CSF. No determination of the galactose content of τ-transferrin was made. Thus, the CSF transferrins are similar to the CSF group 3 RNases in that two major types of glycoprotein are present, one fully glycosylated form common to serum and one form unique to CSF lacking specific terminal carbohydrate residues. This parallelism suggests that τ-transferrin may also lack the galactose residues present in its serum counterpart. In contrast to transferrin and the RNase 3 group, it has been reported that CSF ceruloplasmin, haptoglobin, and orosomucoid do not lack the sialic acid residues found in their serum counterparts (Stibler, 1978). Thus, only some CSF glycoproteins display altered glycosylation.

The function of the modified glycosylation found in the RNase 3 group and its utility in the study of the pathobiology of the central nervous system remain unknown. One possibility is that this modification has a role in the recognition and uptake of these glycoproteins. A mannose/*N*-acetylglucosamine recognition system which mediates the binding of glycoproteins having *N*-acetylglucosamine as the terminal nonreducing sugar has been demonstrated in reticuloendothelial cells (Stahl et al., 1978). A similar system might be present in central nervous system tissues.

Any hypothesis as to the origin of the CSF RNases must account not only for the modified glycosylation but also for the higher specific activity of RNase in CSF relative to that in serum. One possibility is mediated transport of serum RNases across the blood–brain barrier accompanied by modification of the carbohydrate portion of some of the RNases. Enhanced transport of transferrin and α-fetoprotein relative to serum albumin into the CSF has been reported in 60-day-old sheep fetuses which possess intact blood–brain barriers (Saunders, 1977). It is likely that such a transport and modification mechanism would apply to other CSF glycoproteins as well. A second possibility is that CSF RNases are synthesized within the central nervous system. In this case, some would be synthesized with a different pattern of glycosylation than that found in plasma RNases. Such alternate glycosylation might then be expected in other glycoproteins synthesized within the central nervous system as well.

Finally, we would emphasize that the examination of glycoprotein structure by visualizing products of glycosidase digestion on activity-stained gels offers several benefits. First, the sensitivity of the technique (picogram levels of enzymes and below) allows examination when only very small samples are available as is the case for human CSF or tissue specimens obtained by biopsy. Second, the glycoprotein of interest does not have to be fully purified since most impurities will not be revealed on an activity-stained gel. Third, samples containing more than one glycoprotein species can be analyzed simultaneously, as is illustrated in the present example. At the current time, only a few enzymes can be visualized by activity staining after $NaDodSO_4$–polyacrylamide gel electrophoresis. However, it is anticipated that, as our understanding of the factors governing reversible denaturation of proteins increases (Blank et al., 1982) and specific chemical methods for enzyme detection on gels evolve, glycoproteins other than RNases will be analyzable by the methods described herein.

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Folding of Ribonuclease A from a Partially Disordered Conformation. Kinetic Study under Folding Conditions†

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ABSTRACT: Bovine pancreatic ribonuclease A (RNase) was partially disordered with 3.5 M $LiClO_4$ (pH 3.0). The conformation of this partially disordered material was studied by circular dichroism and Raman spectroscopy. Although the partially disordered protein appears to have a lower β-structure content and disordered tyrosyl side chains, compared to native RNase, it seems to retain some ordered backbone structure that is suggested to be α helix. The kinetics of folding of $LiClO_4$-denatured RNase was studied by means of absorption and circular dichroism measurements. For comparison, the kinetics of folding of urea-denatured RNase (which is completely devoid of ordered structure) was examined with the same techniques. Since the kinetics of folding of both denatured species are found to be similar, it appears that the ordered structure present in $LiClO_4$-denatured RNase plays no role in determining the folding pathway. Also, the change in the circular dichroism at 220 nm showed that some of the ordered structure in $LiClO_4$-denatured RNase becomes disordered in the early stages of folding. This implies that all ordered structures in RNase are not equivalent in their influence on the folding pathway; some can play an essential role and some may not.

Konishi et al. (1982b) proposed two types of pathways for protein folding. One is designated as a growth-type pathway in which the nucleation sites are folded in the rate-limiting step and other parts of the polypeptide chain fold around the nucleation sites. In this pathway, native interactions play a significant role in influencing folding. In the second, a rearrangement-type pathway, some nonnative interactions are essential for folding, and the disruption or rearrangement of these interactions to native ones constitutes the rate-limiting step. In this paper, we examine the role or effect of ordered structure in partially disordered RNase[1] (which is disordered by a high concentration of $LiClO_4$) on the proposed folding pathways.

Konishi et al. (1982a) have pointed out that, in studies of the folding pathways of proteins, analysis of the preequilibrium state (as, for example, in the regeneration of RNase) gives

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[1] Abbreviations: RNase, bovine pancreatic ribonuclease A; N-RNase, native RNase; D(urea)-RNase, RNase denatured by urea (the denaturant is enclosed in parentheses); I(urea)-RNase, intermediate in the folding/unfolding of RNase by urea; A_t and A_∞, absorbance at time t and infinite time, respectively, in the kinetic experiments; CD, circular dichroism; UV, ultraviolet; Gdn·HCl, guanidine hydrochloride; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol.

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