The success of all crosslinking reagents is idiosyncratic. In crosslinking experiments with bBBr, the bromomethyl group that remains in an activated monomer is a target for both intramolecular and intermolecular nucleophiles. Fortunately, thiolate is by far the most reactive biomolecular nucleophile toward bBBr (2). Indeed, the high specificity of bBBr for reaction with cysteine residues may make this reagent a particularly effective companion to cysteine-scanning mutagenesis analyses (8). Then, bBBr could be used to probe a complex mixture of biomolecules for otherwise noncovalent interactions. Detection by ultraviolet illumination after gel electrophoresis (as in Fig. 1B) would make such an association apparent. Additional properties of the resulting crosslinked molecules could be studied by fluorescence anisotropy (9). We thus present bBBr as a fluorescent crosslinking reagent of broad utility.

Acknowledgments. We thank D. M. Nierengarten for assistance in the preparation of C31S BS-RNase. J.-S.K. is a Steenbock predoctoral fellow. R.T.R. is a Presidential Young Investigator (NSF), Searle Scholar (Chicago Community Trust), and Shaw Scientist (Milwaukee Foundation). This work was supported by Grant GM44783 (NIH).

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The Extent to Which Ribonucleases Cleave Ribonucleic Acid

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Received September 19, 1994

Ribonucleases (RNases)[2] catalyze the cleavage of the P–$O^{5'}$ bond of single-stranded RNA. These enzymes are used to degrade RNA in procedures that include the ribonuclease protection assay (1) and the isolation of DNA from cellular extracts. These procedures are improved as the extent of RNA cleavage is increased. To enhance the extent of cleavage, biochemists often combine RNases of differing substrate specificity. For example, RNase A from bovine pancreas cleaves RNA after C and U residues, and RNase T1 from *Aspergillus orysae* cleaves after G. Thus, a combination of RNase A and RNase T1 leaves intact only the P–$O^{5'}$ bonds after A residues. Although RNase I from *Escherichia coli* can in theory cleave every P–$O^{5'}$ bond in RNA, this enzyme is inhibited by common impurities in nucleic acid preparations. In contrast, RNase A and RNase T1 are not sensitive to such impurities.

We have created a mutant of RNase A (T45G RNase A) that displays an expanded substrate specificity (2,3). T45G RNase A cleaves poly(A), poly(C), and poly(U) efficiently. Replacing Thr45 with a glycine residue results in only a modest compromise to the thermal stability of RNase A, with the T_m in 0.1 M Mes·HCl buffer, pH 6.0, containing NaCl (0.1 M) decreasing from 63 to 53°C (4). Hence, T45G RNase A and RNase T1 are stable enzymes that together can cleave all four homoribonucleotide polymers. Here, we determine the extent to which various RNases, including T45G RNase A, cleave RNA heteropolymers containing all four ribonucleotides.

We use ^{31}P NMR spectroscopy to assess RNA cleavage. As RNA is hydrolyzed, the chemical state of its phosphoryl groups changes: acyclic diester (NpN) → cyclic diester (N > p) and [ultimately (5)] cyclic diester → monoester (Np). This change can be monitored by ^{31}P NMR spectroscopy because the three phosphorous species (NpN, N > p, and Np) have distinct chemical shifts (2,3,5–7).

Materials and methods. Total RNA from yeast (Sigma Chemical, St. Louis, MO) and various RNases were prepared as described (2,4). RNA (6 mM) and RNase (RNase T1, 3 μg; RNase A, 5 μg; T45G RNase A, 60 μg; or RNase I, 3 μg) were incubated for 36 h at 25°C in 2.0 ml of 0.1 M Mes·HCl buffer, pH 6.0, containing NaCl (0.1 M). Reaction products were analyzed in 10-mm NMR tubes having D_2O inserts. Free induction decays were recorded with a Bruker AM400 spectrometer and were processed with the program FELIX (Hare Research, Bothel, WA). The parameters used to acquire spectra were as reported previously (3), except for the relaxation delay (here, 10 s) and the number of free induction decays recorded for each incubation (here, 100).

Results. Spectra recorded after 24 h of incubation were indistinguishable from those recorded after 36 h. Also, the spectra did not change when fresh RNase was added after 36 h of incubation (data not shown). Each cleavage reaction was therefore complete by 36 h, and the spectra at that time are shown in Fig. 1. The normal-

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[2] Abbreviation used: RNase, ribonuclease.

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0003-2697/95 $6.00

ized integration of each spectral peak from Fig. 1 is given in Table 1.

Discussion. The spectra in Fig. 1 show that RNases cleave RNA to different extents. The most thorough degradation by a single RNase was that catalyzed by RNase I followed in order by T45G RNase A, RNase A, and RNase T1 (Table 1). The T45G mutation increases by 47% the extent to which RNase A cleaves RNA. Of the mixtures tested, T45G RNase A + RNase I provides the most extensive degradation of RNA. Also, the T45G RNase A + RNase T1 mixture, but not the RNase A + RNase T1 mixture, cleaves RNA as extensively as does RNase I alone.

The data in Table 1 indicate that the extent of RNA cleavage increases with the fraction of residues expected to be susceptible to cleavage. Still, the extent to which an RNA heteropolymer is cleaved by an RNase is affected by attributes other than its composition. For example, RNase I can in theory cleave every P–$O^{5'}$ bond in RNA. Yet at 25°C in 0.1 M Mes·HCl buffer, pH 6.0, containing NaCl (0.1 M), RNase I leaves 11% of these bonds intact (Table 1).

^{31}P NMR spectroscopy reveals an important chemical property of degraded RNA. As shown in Fig. 1, RNase A, T45G RNase A, and RNase T1 are able not only to cleave RNA, but also to hydrolyze the cyclic phosphodiester product. Approximately 70% of the residues cleaved by RNase A, T45G RNase A, or RNase T1 were also hydrolyzed to form a phosphomonoester (Table 1). In contrast, only 2% of the residues cleaved by RNase I were hydrolyzed. At neutral pH, the hydrolysis of a phosphodiester to a phosphomonoester imparts additional negative charge on the phosphoryl group. Thus, most of the products of cleavage by RNase A, T45G RNase A, or RNase T1 differ from intact RNA by having both fewer residues and greater charge per residue. This additional charge could be useful in the separation of cleaved RNA from intact RNA or DNA.

A major goal of protein engineering is to create new enzymes that have useful properties. Previously, we showed that changing Thr45 to a glycine residue broadens the specificity of RNase A for homoribonucleotide polymers (2,3). Here, we have used ^{31}P NMR spectroscopy to demonstrate that this broadened specificity results in an enhanced ability to degrade RNA heteropolymers. Using T45G RNase A alone or in combination with RNase T1 can improve procedures that rely on the complete degradation of single-stranded RNA.

TABLE 1

Products of the Exhaustive Cleavage of Yeast RNA by Various Ribonucleases

RNase	Nucleotide phosphoester species (%)[a]			Extent of cleavage (%)[b]
	NpN	N>p	Np	
(None)	100	0	0	0
T1	82	7	12	18
A	55	14	31	45
T45G	34	23	43	66
I	11	87	2	89
A + T1	23	29	48	77
T45G + T1	10	42	48	90
T45G + I	4	51	45	96

[a] The relative amount of each RNA species was determined (±3%) from integration of the peaks in the ^{31}P NMR spectra shown in Fig. 1.

[b] Extent of cleavage refers to the percentage conversion of acyclic phosphodiester (NpN), which is 100% of the material at the initiation of the enzymatic reaction, to cyclic phosphodiester (N>p) and monoester (Np).

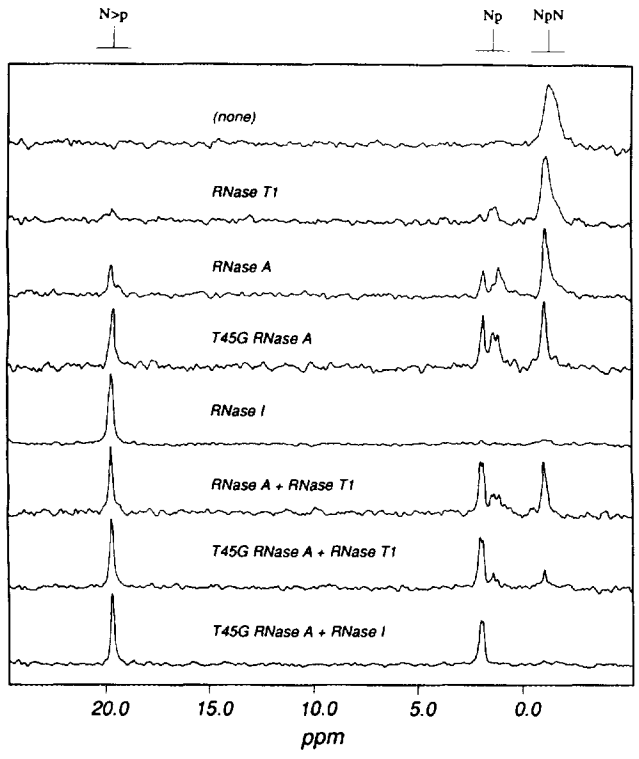

FIG. 1. ^{31}P NMR spectra showing the products of RNA cleavage by various RNases. Chemical shift values are reported relative to aqueous H_3PO_4 (0.1 M).

Acknowledgments. This work was supported by Grant GM44783 (NIH) and made use of the NMRFAM, which was supported by Grant RR02301 (NIH). S.B.delC is a Procter & Gamble predoctoral fellow. R.T.R. is a Presidential Young Investigator (NSF), Searle Scholar (Chicago Community Trust), and Shaw Scientist (Milwaukee Foundation).

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Fluorometric Assay of GTPase Activity: Application to the Couple Elongation Factor eEF-2-Ribosome

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Received September 28, 1994

Elongation factor eEF-2, as several other translation factors, belongs to the super family of GTP binding proteins. These proteins have in common the need to be activated by the binding of GTP and, in addition, to be able to hydrolyze this nucleotide. GTPase activation results from an interaction with another compound, which, in the case of the translation factors, is located on the ribosome itself. A precise measurement of the GTPase activity of these proteins is essential for their study.

Two types of methods have been used for this measurement. The first one derives from that of Conway and Lipmann (1) and uses [γ-^{32}P]GTP. The labeled phosphate released by the reaction combines with molybdate, and phosphomolybdate is extracted with a benzene/isobutanol mixture and counted. This method is sensitive but has some disadvantages, the main one being that the added [γ-^{32}P]GTP is rapidly consumed and the released GDP inhibits the reaction (see Results and Discussion). The second type of method does not require the use of radioactive nucleotide. The amount of GDP released is measured by a cascade of reactions using three enzymes successively: nucleosidediphosphate kinase, pyruvate kinase, and lactate dehydrogenase. Oxidation of NADH into NAD^+ is followed by photometry. This method described by Näslund *et al.* (2) was adapted from the ATPase assay of Pullman *et al.* (3). Its main advantage is that the concentration of the substrate (GTP) remains constant, and GDP does not accumulate. But there are some disadvantages too: the method is significantly less sensitive than the preceding one, and measurements of absorbency are difficult in the presence of very absorbing material like ribosomes, but the main disadvantage comes from the use of ATP. This method is not specific for GTPase: it measures the sum of all ATPase and GTPase activities present in the reaction mixture with no possibility to discriminate between them. Thus, in the case of elongation factor eEF-2, GTPase activity must be measured in the presence of ribosomes, which possess an ATPase activity (4).

To overcome these difficulties, we have developed an assay which is both very sensitive and specific for GTPase without using radioactive GTP. It derives from the second type of method with two important modifications: the first one takes advantage of the fact that pyruvate kinase can phosphorylate GDP in the presence of phospho*enol*pyruvate directly, and, therefore, addition of nucleosidediphosphate kinase and ATP can be omitted, which makes the assay more specific. The second modification consists of using fluorometric measurements, which makes the assay considerably more sensitive.

Materials

Pyruvate kinase (EC 2.7.1.40) and lactate dehydrogenase (EC 1.1.1.27), GTP, phospho*enol*pyruvate, and NADH were from Boehringer, [γ-^{32}P]GTP from DuPont NEN.

Preparations

Rat liver eEF-2 (>95% pure) and ribosomes were prepared as previously described (5,6).

Fluorometric assay of GTPase activity. In a quartz cell (1-cm path length) stirred with a magnetic bar and maintained at 37°C, the complete system was contained in 1 ml: 8 mM $MgCl_2$, 10 μM EDTA, 30 mM triethanolamine–HCl, pH 7.6, 40 mM KCl, 8% (v/v) glycerol, 0.7 mM 2-mercaptoethanol, 16 μM NADH, 3 μM GTP, 0.25 mM phospho*enol*pyruvate, 15 U pyruvate kinase, and 11 U lactate dehydrogenase. The reaction mixture was incubated at 37°C for 15 min to remove the small amount of GDP and pyruvate contaminating the preparations of GTP and phospho*enol*pyruvate. Small particles in suspension which could prevent an accurate measurement of NADH fluorescence were removed by filtration with a HV 45 Millipore filter. The fluorescence baseline was recorded during 5 min with an SLM Aminco 8000 C spectrofluorometer, operated in the corrected ratio mode. The excitation wavelength was set to 340 nm and the emission wavelength to 460 nm (excitation and emission bandwidths set to 4 nm). The course of the reaction was followed in real time with the computer of the system. GDP was added to test the activity of the enzymatic system and to calibrate the drift of the signal. Thus, the decrease of NADH fluorescence emission could be quantitatively related to the rate of GTP hydrolysis into

ANALYTICAL BIOCHEMISTRY **225,** 178–180 (1995)
0003-2697/95 $6.00