μg of plasmid DNA, which is comparable to the efficiency of bacteriophage lambda infection. Alternatively, cells can be stored frozen in 10% glycerol, although glycerol reduced transformation efficiency to ~30% (data not shown). Freezing and thawing of glycerol-treated cells did not result in any further loss of transformation efficiency (data not shown).

This study showed that it is crucial to inactivate the T4 DNA ligase prior to electrotransformation of ligated DNA, which can be ensured by the introduction of a simple heat inactivation step, increasing the number of transformants by 260-fold. Although this paper focuses on the use of *E. coli* MC1061/p3, the experiments were repeated with a different plasmid in the parental strain *E. coli* MC1061 and showed the same result (data not shown). It is therefore likely that electrotransformation of *E. coli* in general is inhibited by T4 DNA ligase activity. Heat inactivation of ligated material might be recommended as a standard procedure whenever high-transformation efficiency is essential.

With the present protocol for electrotransformation of *E. coli* MC1061/p3, it has been possible to generate plasmid cDNA libraries from limiting amounts of tissues for subsequent screening in eucaryotic cells, with efficiencies similar to that of lambda cloning vectors.

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REFERENCES

1. Henikoff, S. (1984) *Gene* **28,** 351–359.
2. Chen, E. Y., and Seeburg, P. H. (1985) *DNA* **4,** 165–170.
3. Craig, D., Howell, M. T., Gibbs, C. L., Hunt, T., and Jackson, R. J. (1992) *Nucleic Acids Res.* **20,** 4987–4995.
4. Okayama, H., and Berg, P. (1983) *Mol. Cell. Biol.* **3,** 280–289.
5. Scott, D. M., Dyson, P. J., and Simpson, E. (1992) *Immunogenetics* **36,** 86–94.
6. Jelinek, L. J., Lok, S., Rosenberg, G. B., Smith, R. A., Grant, F. J., Biggs, S., Bensch, P. A., Kuijper, J. L., Sheppard, P. O., Sprecher, C. A., *et al.* (1993) *Science* **259,** 1614–1616.
7. Seed, B., and Aruffo, A. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 3365–3369.
8. Aruffo, A., and Seed, B. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 8573–8577.
9. Hanahan, D. (1983) *J. Mol. Biol.* **166,** 557–580.
10. Hanahan, D. (1985) *in* DNA Cloning Techniques: A Practical Approach (Glover, D. M., Ed.), p. 109, IRL Press, Oxford.
11. Dower, W. J., Miller, J. F., and Ragsdale, C. W. (1988) *Nucleic Acids Res.* **16,** 6127–6145.
12. Huang, H. V., Little, P. F. R., and Seed, B. (1988) *in* Vectors: A Survey of Molecular Cloning Vectors and Their Uses (Rodriguez, R. L., and Denhardt, D. T., Eds.), pp. 269–283, Butterworth, Stoneham.
13. Heilig, J. S., Lech, K., and Brent, R. (1994) *in* Current Protocols in Molecular Biology (Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K., Eds.), pp. 1.7.1–1.7.8, Wiley, New York.
14. Helfman, D. M., Fiddes, J. C., and Hanahan, D. (1987) *in* Methods in Enzymology: Guide to Molecular Cloning Techniques (Berger, S. L., and Kimmel, A. R., Eds.), p. 355, Academic Press, San Diego.

Dibromobimane as a Fluorescent Crosslinking Reagent

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Fluorescent labeling is a useful tool for analyzing the *in vitro* and *in vivo* behavior of biological molecules (1). Kosawer and Kosawer have demonstrated the utility of bromobimanes as small, thiol-specific reagents to produce relatively photostable fluorescent products (2). Here, we establish that dibromobimane [bBBr[2]; 4,6-bis-(bromomethyl)-3,7-dimethyl-1,5-diazabicyclo[3.3.0]-octa-3,6-diene-2,8-dione; see Scheme 1] can be used to crosslink two protein molecules rapidly and in high yield. We also show that the resulting crosslinked dimer retains native activity, is not converted to monomers by added thiols, and is highly fluorescent.

Bromobimanes have been used to attach a fluorescent label to a variety of proteins (2–4) and to introduce an intramolecular crosslink into myosin subfragment 1 (5). A protein labeled with bBBr has new ultraviolet absorption with λ_{max} = 385 nm ($\epsilon_{385\ nm}$ = ~4000 M^{-1} cm^{-1}) and fluorescence emission with λ_{max} = 477 nm (ϕ_F = ~0.25–0.33) (2). The reaction of bromobimanes most often occurs by the S_N2 displacement of a bromide ion by a thiolate anion from a cysteine residue. Since bBBr has two equivalent bromomethyl groups, we reasoned that bBBr could also be used as an *inter*molecular crosslinking reagent. To test this hypothesis, we attempted to form a dimeric protein crosslinked by bBBr.

Previously, we developed methods to produce and purify bovine seminal ribonuclease (BS-RNase) from *Escherichia coli* (6). BS-RNase is a homodimeric protein in which the two subunits are connected by two disulfide bonds between Cys31 of one subunit and Cys32 of the other. These intersubunit disulfide bonds are easily reduced *in vitro* [by a 10-fold molar excess of dithiothreitol

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[2] Abbreviations used: bBBr, dibromobimane; BS-RNase, bovine seminal ribonuclease; DTT, dithiothreitol; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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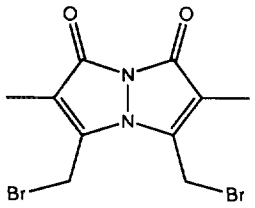

bBBr

SCHEME 1

(DTT)] and probably *in vivo* (by the high reduction potential in the mammalian cytosol) (7). The reduction of native BS-RNase leads to its dissociation into monomers. Here, we describe a BS-RNase dimer in which the monomers are crosslinked by reaction with bBBr.

Materials and methods. To favor the formation of intermolecular rather than intramolecular crosslinks, we performed our reactions with a mutant BS-RNase in which Cys31 was changed to a serine residue. C31S BS-RNase was purified to homogeneity as described (7). bBBr (as "Thiolyte DB") was obtained from Calbiochem (La Jolla, CA). Prior to reaction, the preparation of C31S BS-RNase was treated for 20 min with a 10-fold molar excess of dithiothreitol (3.5 mM) in 10 mM Tris–HCl buffer, pH 8.5. The buffer was then changed by gel filtration chromatography to 10 mM sodium phosphate, pH 7.4, containing NaCl (100 mM) and EDTA (1 mM). The reduced protein was incubated at 25°C with a 0-, 0.5-, 1-, 5-, or 20-fold molar excess of bBBr. After various times, the reaction was quenched by adding DTT to a final concentration of 20 mM. [For the large-scale preparation of crosslinked protein, reduced C31S BS-RNase (20 mg; 0.37 mM) in 10 mM sodium phosphate, pH 7.4, containing NaCl (100 mM) and EDTA (1 mM) was incubated at 25°C for 10 min with bBBr (0.19 mM).] The extent of crosslinking was monitored by SDS–PAGE in the presence of β-mercaptoethanol. Crosslinked dimer was purified by gel filtration chromatography using conditions described previously (7).

Results. Dibromobimane was an extremely effective reagent for producing crosslinked dimers of BS-RNase. The maximum yield of dimeric protein was obtained by reaction with a 0.5-fold molar excess of bBBr (Fig. 1A). This reaction was complete after 15 min (data not shown). Under these conditions, approximately 70% of the monomeric protein was converted to a crosslinked dimer (Fig. 1A, lane 1), which was isolatable by gel filtration chromatography in 60% overall yield (Fig. 2). The crosslinked dimer had enzymatic activity comparable to that of dimeric BS-RNase (data not shown). The crosslinked dimer was highly fluorescent and could be observed by ultraviolet illumination after SDS–PAGE (Fig. 1B) and by fluorescence spectroscopy (Fig. 2).

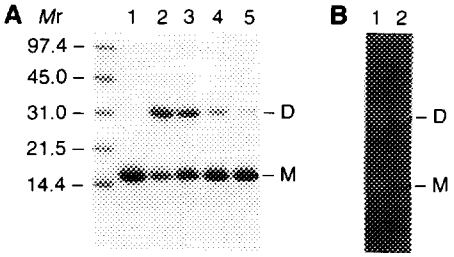

FIG. 1. (A) Products of the reaction of C31S BS-RNase with various concentrations of bBBr. Protein was stained with Coomassie brilliant blue after SDS–PAGE. Lane 1, 0-fold molar excess of bBBr; lane 2, 0.5-fold; lane 3, 1-fold; lane 4, 5-fold; lane 5, 20-fold. (B) Fluorescence of C31S BS-RNase after reaction with a 0.5-fold molar excess of bBBr. Protein was visualized by long-wave ultraviolet illumination after SDS–PAGE. Lane 1, before gel filtration chromatography; lane 2, after gel filtration chromatography.

Discussion. We have demonstrated that bBBr can be used as an effective intermolecular crosslinking reagent for thiol-containing proteins. As far as we know, no other thiol-specific crosslinking reagents are available that also provide a fluorescent label. We found that in the preparation of homodimers, the ratio of bBBr to thiol group is critical—substoichiometric amounts of bBBr are necessary to produce maximum yield. Further, our results suggest that bimane-crosslinked heterodimers can be prepared in a three-step procedure. First, a thiol-containing molecule is reacted with a $\geq$20-fold molar excess of bBBr to produce activated monomer. Under these conditions, only a small amount of homodimer will be produced (Fig. 1A, lane 5). Then, the activated monomer is separated from unreacted bBBr. Finally, another thiol-containing molecule is added to the activated monomer.

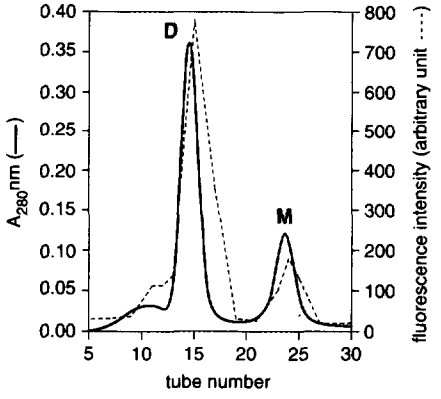

FIG. 2. Purification of crosslinked BS-RNase dimer. C31S BS-RNase was reacted with a 0.5-fold molar excess of bBBr and subjected to gel filtration chromatography. Fractions were assayed for absorbance (280 nm) and fluorescence (385 nm excitation; 477 nm emission). The two peaks correspond to monomeric (M) and dimeric (D) BS-RNase.

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.

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REFERENCES

1. Lakowicz, J. R. (1983) Principles of Fluorescence Spectroscopy, Plenum, New York.
2. Kosower, N. S., and Kosower, E. M. (1987) *Methods Enzymol.* **143,** 76-84.
3. Fahey, R. C., and Newton, G. L. (1987) *Methods Enzymol.* **143,** 85-96.
4. Lundblad, R. L. (1991) Chemical Reagents for Protein Modification, CRC Press, Boca Raton, FL.
5. Ue, K. (1987) *Biochemistry* **26,** 1889-1894.
6. Kim, J.-S., and Raines, R. T. (1993) *J. Biol. Chem.* **268,** 17392-17396.
7. Kim, J.-S., Souček, J., Matoušek, J., and Raines, R. T. (1994) Unpublished results.
8. Flitsch, S. L., and Khorana, H. G. (1989) *Biochemistry* **28,** 7800-7805.
9. LeTilly, V., and Royer, C. A. (1993) *Biochemistry* **32,** 7753-7758.

The Extent to Which Ribonucleases Cleave Ribonucleic Acid

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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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