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Purification of Bleomycin Hydrolase with a Monoclonal Antibody and Its Characterization†

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ABSTRACT: We established a hybridoma clone that produced anti-bleomycin hydrolase antibody. The subclass of the monoclonal antibody was immunoglobulin M. The antibody significantly reacted with bleomycin hydrolase from rabbit tissues, mouse livers, sarcoma 180, and adenocarcinoma 755 but not significantly with that from MH 134 and Ehrlich carcinoma. The enzyme from L5178Y cells showed an intermediate reactivity. Bleomycin hydrolase was purified from rabbit liver by immunoaffinity with the monoclonal antibody and DEAE gel chromatography. Approximately 1300-fold-purified bleomycin hydrolase was obtained. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and isoelectric focusing on a polyacrylamide slab gel of purified bleomycin hydrolase showed a single band with an apparent M_r of 48K and an isoelectric pH of 5.2. The molecular weight of bleomycin hydrolase determined on gel filtration high-performance liquid chromatography was ca. 300K, suggesting a hexameric enzyme. The enzyme showed an optimum pH of 6.8–7.8 and gave a V_{max} value of 6.72 mg min^{-1} mg^{-1} for peplomycin and 9.24 mg min^{-1} mg^{-1} for bleomycin B_2 and a K_m value of 0.79 mM for both substrates. The enzyme was inhibited by E-64, leupeptin, *p*-tosyl-L-lysine chloromethyl ketone, *N*-ethylmaleimide, Fe^{2+}, Cu^{2+}, and Zn^{2+} but was enhanced by dithiothreitol. The results suggest that bleomycin hydrolase is a thiol enzyme.

Bleomycin (BLM)[1] is a group of glycopeptide antibiotics, which differ from one to another in the terminal amine moiety. The drug displays remarkable therapeutic activity for squamous cell carcinoma and malignant lymphoma. The molecular target of BLM is DNA. The antibiotic binds to double-stranded DNA, causing a single strand scission. Peplomycin (PEP) is an analogue of BLM.

Human and animal tissues contain BLM hydrolase (BLMase), which hydrolyzes the carboxamide bond in the

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[1] Abbreviations: BLM, bleomycin; BLMase, bleomycin hydrolase; EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography; Ig, immunoglobulin; NEM, *N*-ethylmaleimide; PEP, peplomycin; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TLCK, *p*-tosyl-L-lysine chloromethyl ketone.

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pyrimidoblamic acid moiety. The hydrolyzed antibiotic shows little antitumor and DNA-cleaving activities. Squamous cell carcinoma induced in mouse skin by 20-methylcholantrene shows higher sensitivity to BLM and lower activity of BLMase than sarcoma induced in the subcutaneous tissue by the same agent (Umezawa et al., 1972). Some BLM-resistant cells contain a higher level of BLMase than sensitive cells (Miyaki et al., 1975; Akiyama et al., 1981; Ozawa et al., 1985). Therefore, the level of BLMase seems to be related to the antitumor spectrum and drug resistance. Since BLMase has not been purified because of its lability, the enzyme has not previously been studied in detail. We have immunized BALB/c mice with partially purified BLMase from rabbit liver and obtained a hybridoma clone that produces antibody against BLMase. We have purified the enzyme from rabbit liver by immunoaffinity with the monoclonal antibody and DEAE-Toyopearl chromatography and studied its characteristics.

Experimental Procedures

Materials

PEP and BLM B_2 were kindly provided by Nippon Kayaku Co., Tokyo, Japan. Sephacryl S-300, protein A–Sepharose, Polybuffer anion exchanger 94, Polybuffer 74, Pharmalyte 3-10, and an electrophoresis calibration kit were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden, and DEAE-Toyopearl was from Toyo Soda Co., Tokyo, Japan. P3-X63-Ag8-6.5.3 mouse myeloma cell line was kindly provided by Prof. T. Tada, Faculty of Medicine, University of Tokyo, Tokyo, Japan. RPMI 1640 medium and fetal calf serum were supplied by GIBCO Laboratories, Chagrin Falls, OH, and *Staphylococcus aureus* and goat anti-mouse immunoglobulin (Ig) M were supplied by Zymed Laboratories, San Francisco, CA. The monoclonal typing kit and the mouse Ig kit were purchased from Miles Scientific, Naperville, IL, and the protein assay dye reagent was from Bio-Rad Laboratories, Richmond, CA. Leupeptin, phosphoramidon, E-64, bestatin, antipain, and amastatin were obtained from the Peptide Institute, Osaka, Japan, and *p*-tosyl-L-lysine chloromethyl ketone (TLCK) and pepstatin from Boehringer Mannheim, West Germany. *N*-Ethylmaleimide (NEM) was supplied by Nakarai Chemicals, Kyoto, Japan, poly(ethylene glycol) 4000 was supplied by E. Merck, Darmstadt, West Germany, and molecular weight markers for gel filtration and p*I* markers for isoelectric focusing were supplied by Sigma Chemical Co., St. Louis, MO. Ag-Stain "Daiichi" was a product of Daiichi Pure Chemicals Co., Tokyo, Japan. The Aquasil SS-352N column was provided by Senshu Scientific Co., Tokyo, Japan, and the Shodex protein WS-803 column by Showa Denko Co., Tokyo, Japan.

Methods

Assay of BLMase by High-Performance Liquid Chromatography (HPLC). The enzyme sample, containing PEP (1.6 mg/mL) in 60 μL, was incubated for 2 h at 37 °C. The reaction was terminated by the addition of 180 μL of ice-cold methanol and 0.6 mg of cupric carbonate in an ice bath. The mixture was centrifuged for 5 min at 2300*g*. PEP and desamido-PEP were separated by HPLC (Akiyama et al., 1981; Lazo et al., 1982). The supernatant (20 μL) was injected into an Aquasil SS-352N HPLC column (4.6 × 250 mm), which was then eluted with a mobile phase consisting of methanol–acetonitrile–20% ammonium acetate–acetic acid (560:440:100:0.5). Two peaks were found in the eluate, and they were identified with PEP and desamido-PEP: retention times were 7 and 10 min (flow rate 1.5 mL/min), respectively. The amounts of PEP and desamido-PEP were determined by UV absorption at 290 nm. One unit of BLMase hydrolyzes 1 μg of PEP/min at 37 °C.

Partial Purification of BLMase from Rabbit Liver. Liver from rabbit was homogenized with 50 mM phosphate buffer (pH 7.4) and centrifuged at 105000*g* for 1 h. The enzyme in the supernatant was precipitated by 30–60% saturation of ammonium sulfate, and the precipitate was dissolved in a minimal volume of 50 mM phosphate buffer (pH 7.4). The solution was applied to Sephacryl S-300 column (3.2 × 90 cm), and the column was developed with 50 mM phosphate buffer (pH 7.4). The active fractions were pooled and applied to a DEAE-Toyopearl column (1.6 × 28 cm). The column was developed with a linear gradient of NaCl (0–200 mM) in 50 mM phosphate buffer (pH 7.4). The 22-fold purified enzyme was obtained and used as the antigen. All experiments were performed at 4 °C.

Immunization of Mice with Partially Purified BLMase. Female BALB/c mice (8 weeks old) were immunized with the partially purified enzyme preparation at 2-week intervals as follows: (1) 170 μg (0.1 mL) with 0.1 mL of Freund's complete adjuvant, subcutaneously; (2) 100 μg, subcutaneously; (3) 40 μg, intravenously. Spleen cells were taken from the immunized mice 4 days after the final immunization.

Production of Hybridoma. Hybridomas were prepared by the method of Galfre and Milstein (1981) with some modifications. Fusion of 1.8×10^8 spleen cells and 0.6×10^8 myeloma cells, P3-X63-Ag8-6.5.3, was performed in serum-free RPMI 1640 medium containing 43% poly(ethylene glycol) 4000 and 13% dimethyl sulfoxide. The cell mixtures were suspended in RPMI 1640 supplemented with 20% fetal calf serum, 2 mM L-glutamine, and 1 mM sodium pyruvate. After 18 h, the cells were transferred to RPMI 1640 medium containing 100 μM hypoxanthine, 0.4 μM aminopterine, and 16 μM thymidine (HAT medium) and aliquoted in 120 μL on 20 96-well plates with 10^6 cells/well thymocytes taken from female BALB/c mice. Fresh medium was added to each well every 3 days. Approximately 2 weeks after the cell fusion, hybridomas in each well were transferred to fresh medium in 24-well plates. When the hybridomas in 24-well plates reached confluence, the culture fluid was assayed for anti-BLMase antibody by the immunoprecipitation method described below. Positive hybridomas were cloned by the limiting dilution technique.

Assay of Monoclonal Antibody against BLMase by Immunoprecipitation. Monoclonal antibody in the hybridoma culture fluid was first adsorbed on *Staphylococcus aureus* coated with anti-mouse IgM. Namely, 50 μL of a 10% suspension of *S. aureus* was incubated with 10 μL of goat anti-mouse IgM serum in 100 μL of 10 mM phosphate buffer (pH 7.4) containing 100 mM NaCl for 1 h at 4 °C, sedimented, and resuspended in 300 μL of the hybridoma culture fluid (Tanaka et al., 1982; Matsukage et al., 1982). The mixture was incubated for 1 h at 4 °C, centrifuged for 5 min at 8000*g*, and washed twice. The 105000*g* supernatant of rabbit liver homogenate, containing ca. 7.2×10^{-2} unit of BLMase, was incubated with the monoclonal antibody and adsorbed on *S. aureus* for 1.5 h at 4 °C. After sedimentation, BLMase remaining in the supernatant was assayed as described above.

Characterization of the Monoclonal Antibody. The subclass of the monoclonal antibody was identified by use of a monoclonal typing (Ouchterlony immunodiffusion) kit, and the monoclonal antibody was quantified by use of a mouse Ig (radial immunodiffusion) kit (Fahey & Mckelvey, 1965).

Purification of BLMase from Rabbit Liver Using the Monoclonal Antibody. BLMase was purified by immunoaffinity and DEAE-Toyopearl chromatography. Anti-BLMase monoclonal antibody in 40 mL of the hybridoma culture fluid was incubated with goat anti-mouse IgM (0.5 mg) for 1 h and then bound on a protein A–Sepharose column (1.3 × 3.5 cm). The column was equilibrated with 10 mM phosphate buffer (pH 7.4) containing 100 mM NaCl. The 105000*g* supernatant (145 mg) of rabbit liver homogenate was then applied to the column. The column was washed with 10 mM phosphate buffer (pH 7.4) containing 100 mM NaCl: 15 mL with 0.05% Tween 20 and then 15 mL without the detergent. BLMase was eluted with 10 mL of the buffer with PEP (1.6 mg/mL) and dithiothreitol (2 mM) at room temperature. The eluate was applied to a DEAE-Toyopearl column (0.8 × 4 cm), and the column was washed with 20 mL of the buffer containing 100 mM NaCl and 2 mM dithiothreitol. BLMase was eluted with a linear gradient of NaCl (100–200 mM) in the buffer containing 2 mM dithiothreitol. All experiments except BLMase elution from the immunoaffinity column were performed at 4 °C.

Protein Content Determination. The content of protein was determined by the dye binding method (Bradford, 1976), with bovine serum albumin as a standard.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS–PAGE). SDS–10% PAGE was carried out as described by Laemmli (1970). The gel (1 mm thick) was fixed in 5% trichloroacetic acid for 1 h and stained with silver (Oakley et al., 1980). Molecular weight markers were used as the standard.

Gel Filtration HPLC. Purified BLMase or molecular mass standards were chromatographed on a Shodex protein WS-803 column (8 × 500 mm). The column was equilibrated and eluted at 1 mL/min with 10 mM phosphate buffer (pH 7.4) containing 150 mM NaCl and 2 mM dithiothreitol. The active fraction of BLMase was determined as described above.

Chromatofocusing. Enzyme partially (6-fold) purified by ammonium sulfate precipitation and Sephacryl S-300 was applied to a chromatofocusing column (1.8 × 10 cm) packed with Polybuffer anion exchanger 94, equilibrated with 25 mM imidazole hydrochloride (pH 7.4). The column was developed with a linear pH gradient of Polybuffer 74 (pH 4.0). The eluates were immediately neutralized with 0.3 M phosphate buffer (pH 7.4). The active fraction of BLMase was determined as described above.

Isoelectric Focusing. Isoelectric focusing on polyacrylamide slab gels was carried out with a pH 3–10 gradient on ampholine–5% polyacrylamide gel plates (1 mm thick). The gel was focused at 1000 V for 7 h. The slab gel was fixed in 10% trichloroacetic acid and 3.5% 5-sulfosalicylic acid solution for 1 h and stained with silver. The isoelectric pH of purified BLMase was calculated by comparison with the electrophoretic mobility of p*I* markers.

RESULTS

Preparation and Characterization of Anti-BLMase Monoclonal Antibody. We carried out screening of ca. 1000 hybridomas for anti-BLMase antibody and found that hybridoma clone CC413 produced monoclonal antibody specific for BLMase. The subclass of the monoclonal antibody was determined as IgM by Ouchterlony immunodiffusion analysis (data not shown). As illustrated in Figure 1, more BLMase was precipitated with increasing amounts of the antibody, and 7.2×10^{-2} unit of the enzyme was completely precipitated with ca. 3 μg of IgM. However, BLMase was not significantly precipitated with control IgM. Monoclonal antibody against aclarubicin-resistant L5178Y cells, SC438 (Sugimoto et al., 1983), was used as the control IgM. However, the anti-BLMase IgM had little effect on the enzymatic activity (data not shown). BLMase from various rabbit tissues was precipitated with anti-BLMase IgM but not significantly with control IgM (Table IA). The anti-BLMase IgM reacted with the enzyme from livers of various mouse strains but to a lesser degree than that from rabbit liver (Table IB). In mouse tumor cells, anti-BLMase IgM reacted with the enzyme from sarcoma 180 and adenocarcinoma 755 but not significantly with that from MH 134 and Ehrlich carcinoma. The enzyme from L5178Y lymphoma showed an intermediate reactivity (Table IC). The difference in reactivity of the enzyme from various tumors may not be due to different host strains (compare parts B and C in Table I).

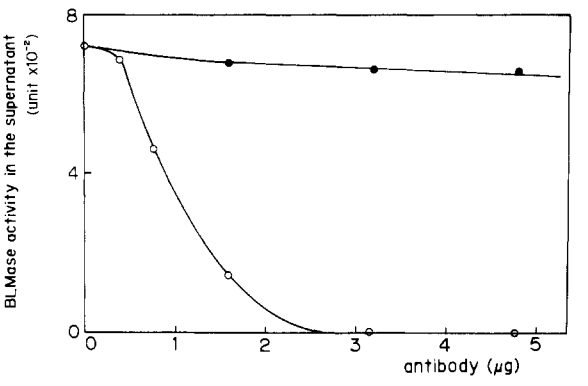

FIGURE 1: Immunoprecipitation of BLMase by monoclonal antibody. The reaction was carried out as described under Methods, except that increasing amounts of anti-BLMase IgM (○) or control IgM (●) were used. The remaining BLMase in the supernatant was assayed.

Table I: Immunoprecipitation of BLMase with Monoclonal Antibodies

source	immunoprecipitation[b] with control IgM	immunoprecipitation[b] with anti-BLMase IgM
(A) Rabbit Tissue		
liver	99[a]	0
lung	99	13
kidney	98	8
spleen	106	20
stomach	99	19
brain	94	10
(B) Mouse Liver		
C3H/He[c]	89	10
ICR	101	11
C57BL/6	91	22
CDF_1	104	23
(C) Mouse Tumor Cell		
MH 134 (C3H/He)[c]	101	99
Ehrlich carcinoma (ICR)	92	86
sarcoma 180 (ICR)	91	19
adenocarcinoma 755 (C57BL/6)	90	24
L5178Y lymphoma (CDF_1)	105	63

[a] The number represents percent enzymatic activity remaining in the supernatant after immunoprecipitation. [b] The immunoprecipitation was performed as described under Methods, except that 10 μg of IgM and the 105000*g* supernatant of tissue or cell homogenate were used. [c] Mouse strain.

Purification of BLMase from Rabbit Liver. BLMase was purified from rabbit liver by immunoaffinity and DEAE-Toyopearl chromatography (Table II). Approximately 1300-fold-purified BLMase was obtained, and the yield was ca. 5%. We eluted the enzyme from the immunoaffinity column at room temperature, using PEP and dithiothreitol.

Table II: Purification of BLMase from Rabbit Liver

purification step	total act. (units)	total protein (mg)	sp act. (units/mg)	yield (%)	purification factor
105000*g* sup	447	145.0	3.08	100	1
immunoaffinity with monoclonal antibody	69.6	0.0315	2210	15.6	718
DEAE-Toyopearl	22.6	0.00582	3880	5.06	1260

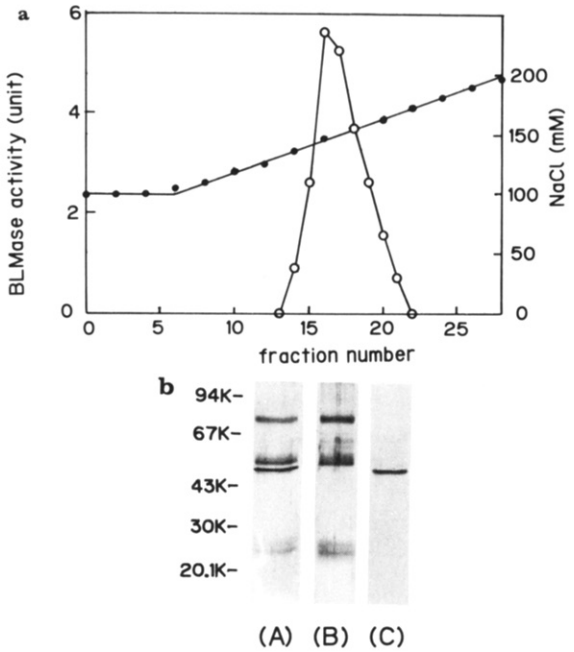

FIGURE 2: Elution profile of BLMase activity from the DEAE-Toyopearl column. The DEAE-Toyopearl column (0.8 × 4 cm) was loaded with the eluate from the immunoaffinity column and eluted with a linear gradient of NaCl (100–200 mM) in 15 mL of the buffer. (a) The BLMase activity (○) in each fraction was determined by the HPLC method, and the NaCl concentration (●) in each fraction was measured by conductance. (b) SDS–PAGE of the eluate from immunoaffinity column and DEAE-Toyopearl column. (A) The eluate from the immunoaffinity column with 10 mM phosphate buffer containing PEP (1.6 mg/mL) and dithiothreitol (2 mM). (B) The flow-through fraction (100 mM NaCl) from DEAE-Toyopearl column. (C) The fraction eluted from DEAE-Toyopearl column with ca. 150 mM NaCl.

The former is the substrate and the latter cleaved IgM. The use of both substances at room temperature resulted in efficient elution. Protein in the eluate of the immunoaffinity column was analyzed by SDS–PAGE (Figure 2b, lane A). The bands of 72K and 23K were presumably produced by the IgM and the bands of 52K and 23K by the IgG. Besides these bands, the band of 48K was also detected. Control experiment showed all these bands except a band of 48K when the eluate from the immunoaffinity column was analyzed without the application of the 105000*g* supernatant from rabbit liver homogenate (data not shown). The purified BLMase was eluted with ca. 150 mM NaCl from the DEAE-Toyopearl column (Figure 2a) and gave a single band of 48K by SDS–PAGE (Figure 2b, lane C). The bands of 72K, 52K, and 23K were detected in the flow-through fraction (100 mM NaCl) (Figure 2b, lane B).

Characterization of BLMase. The purified enzyme lost 50% of its activity when it was stored in the presence of 2 mM dithiothreitol at 4 °C for 3 days. The molecular weight of the enzyme was estimated to be ca. 300K by gel filtration HPLC (data not shown), suggesting a hexameric enzyme. The p*I* value was shown as 5.2 by chromatofocusing and by isoelectric focusing (data not shown). The enzymatic activity of BLMase was studied with PEP and BLM B_2 as substrates. The Lineweaver–Burk plots showed a linear relationship between $1/V$ (unit/mg^{-1}) and $1/S$ (mM^{-1}) and gave a K_m value of 0.79 mM for both substrates and V_{max} values of 6.72 mg min^{-1} mg^{-1} for PEP and 9.24 mg min^{-1} mg^{-1} for BLM B_2. BLM B_2 seemed to be hydrolyzed more rapidly than PEP. The optimum pH of the enzyme was 6.8–7.8 (Figure 3). As presented in Table III, the enzymatic activity was inhibited by E-64, leupeptin, TLCK, NEM, Fe^{2+}, Cu^{2+}, and Zn^{2+}. Little inhibition was observed with bestatin, pepstatin, phosphoramidon, ethylenediaminetetraacetic acid (EDTA), and Mg^{2+}. Intermediate degrees of inhibition were found with antipain, amastatin, and Ca^{2+}. The enzymatic activity was enhanced by dithiothreitol. The results suggest that BLMase is a thiol enzyme.

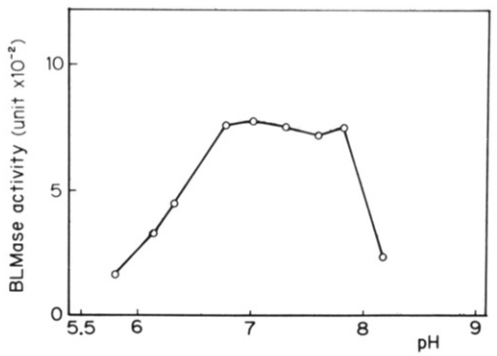

FIGURE 3: Dependency of BLMase on pH. The assay was performed as described under Methods with purified BLMase and different pHs of phosphate buffer.

Table III: Effects of Various Substances on BLMase

additions[a]	concn (mM)	% inhibition
E-64	0.04	100
leupeptin	0.04	54
antipain	0.4	48
amastatin	0.4	28
bestatin	0.4	0
pepstatin	0.4	0
phosphoramidon	0.4	4
TLCK	0.04	100
NEM	0.04	81
dithiothreitol	2.0	−65
EDTA	2.0	7
Fe^{2+}	1.0	100
Cu^{2+}	1.0	100
Zn^{2+}	1.0	100
Ca^{2+}	5.0	39
Mg^{2+}	5.0	0

[a] The reaction mixture (60 μL) contained 7.2 × 10^{-2} unit of purified BLMase and 65 nmol of PEP in 10 mM phosphate buffer (pH 7.4) without dithiothreitol. Tris-HCl buffer (10 mM, pH 7.4) was used in the experiments for the effects of metal ions. The assay was performed as described under Methods.

Discussion

BLMase was so labile that only partially purified BLMase was available as an antigen, but we successfully obtained a monoclonal antibody that reacted with BLMase. Anti-BLMase monoclonal antibody reacts with the enzyme in extracts of various rabbit tissues, mouse livers, sarcoma 180, and adenocarcinoma 755 but not significantly in those of MH 134

and Ehrlich carcinoma. The cross-reactivity with murine livers and tumors may be due to the similar structure of rabbit and mouse enzymes. The failure of reactivity of the enzyme from MH 134 and Ehrlich carcinoma may be due to the existence of BLMase isozyme or contamination by related proteins.

Umezawa et al. (1974) purified BLMase from mouse liver by affinity chromatography using Sepharose 4B–lysinamide and obtained 25-fold-purified enzyme. However, further purification failed, because of the lability of the enzyme. In this respect the immunoaffinity with monoclonal antibody is extremely effective because rapid purification is expected. The two-step rapid purification procedure with immunoaffinity and subsequent DEAE-Toyopearl chromatography enabled us to obtain pure enzyme, which gives a single band in SDS–PAGE. We had first tried to bind anti-BLMase IgM directly to cyanogen bromide activated Sepharose. However, the coupled antibody showed reduced reactivity with the enzyme. Therefore, anti-BLMase monoclonal antibody was adsorbed on protein A–Sepharose coated with anti-mouse IgM.

The purified BLMase is inhibited by E-64, leupeptin, TLCK, and NEM but is enhanced by dithiothreitol. The results suggest that BLMase is a thiol enzyme. Comparison of the results with SDS–PAGE and those with HPLC gel filtration suggests that BLMase is a hexameric enzyme. Since purified BLMase is labile, partially purified enzyme was used for chromatofocusing. The pH of the active fraction eluted from the chromatofocusing column is in accordance with that of a single band shown by isoelectric focusing. The properties of BLMase indicate that BLMase is a previously unrecognized enzyme. The existence of BLMase in various tissues suggests that this enzyme could have a important physiological function.

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500-MHz Proton Homonuclear Overhauser Evidence for Additional Base Pairs in the Common Arm of Eukaryotic Ribosomal 5S RNA: Wheat Germ†

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ABSTRACT: A "common-arm" fragment from wheat germ (*Triticum aestivum*) 5S RNA has been produced by enzymatic cleavage with RNase T1 and sequenced via autoradiography of electrophoresis gels for the end-labeled fragments obtained by further RNase T1 partial digestion. The existence, base pair composition, and base pair sequence of the common arm are demonstrated for the first time by means of proton 500-MHz nuclear magnetic resonance. From Mg^{2+} titration, temperature variation, ring current calculations, sequence comparisons, and proton homonuclear Overhauser enhancement experiments, additional base pairs in the common arm of the eukaryotic 5S RNA secondary structure are detected. Two base pairs, G_{41}·C_{34} and A_{42}·U_{33} in the hairpin loop, could account for the lack of binding between the conserved GAAC segment of 5S RNA and the conserved Watson–Crick-complementary GTΨC segment of tRNAs.

5S RNA is an essential structural component in all ribosomes and is necessary for protein synthesis (Pieler et al., 1984). It is widely believed that at least the eukaryotic 5S RNAs share a common secondary structure [e.g., see Delihas et al. (1984)]. Three universal base-pairing schemes have been adapted to the primary sequence of wheat germ 5S RNA in Figure 1. In particular, each model contains a "common arm" extending from bases corresponding to residues 26–56 in the wheat germ primary sequence. A similar arm is proposed for eukaryotic 5.8S RNA (Lee & Marshall, 1986).

Many early models for 5S RNA did not contain the common-arm helical segment (Boedtker & Kelling, 1967; Brownlee et al., 1972; Du Buy & Weissman, 1971; Monier, 1974). However, once a three-stem model based upon comparative sequence analysis was proposed (Fox & Woese, 1975), all subsequently proposed 5S RNA secondary structures shared a common-arm segment (Delihas et al., 1984; Luoma & Marshall, 1978a,b; Osterberg et al., 1976; Studnicka et al., 1981; de Wachter et al., 1982; Luehrsen & Fox, 1981).

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