Separation of the Protective Enzyme Bleomycin Hydrolase from Rabbit Pulmonary Aminopeptidases†

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ABSTRACT: Bleomycin (BLM) hydrolase inactivates the BLM class of antitumor antibiotics and protects against BLM-induced pulmonary fibrosis. This enzyme is poorly characterized but believed to be an aminopeptidase B. In the present report, both BLM hydrolase and aminopeptidase B from rabbit pulmonary cytosol were retained by arginyl-Sepharose and BLM–Sepharose affinity columns, further suggesting that these two enzymes are similar. When, however, BLM hydrolase was purified over 1800-fold by using our newly developed high-speed liquid chromatography assay for BLM hydrolase coupled with fast protein liquid chromatography, we found that this partially purified BLM hydrolase preparation lacked aminopeptidase B activity. Furthermore, BLM hydrolase was completely separated, by using anion-exchange Mono Q chromatography, from all the aminopeptidases identified in rabbit pulmonary cytosol: one aminopeptidase B, two aminopeptidases N, and one aminopeptidase with both aminopeptidase B and aminopeptidase N activities. Pulmonary BLM hydrolase also had a higher molecular weight than pulmonary aminopeptidase B. In contrast to aminopeptidase B, BLM hydrolase was not activated by NaCl and was much less stable at 4 °C. In addition, bestatin was a potent inhibitor of aminopeptidase B but had little effect on BLM hydrolase, while leupeptin was a potent inhibitor of BLM hydrolase but was less effective against aminopeptidase B. Thus, pulmonary BLM hydrolase and aminopeptidase B have affinity for each other's substrate, but they are clearly distinct enzymes on the basis of charge characteristics, molecular weight, stability, and sensitivity to inhibitors and activators.

The antitumor antibiotic bleomycin (BLM)[1] is widely used to treat human malignancies (Carter, 1985). A unique feature of this drug is the lack of significant bone marrow, hepatic, or renal toxicity (Carter, 1985). The use of BLM is, however, limited by its dose-dependent induction of pulmonary fibrosis (Sikic, 1985).

The metabolic inactivation of BLM is a major factor in determining its biological activity (Umezawa, 1979; Lazo et al., 1987). Incubation of BLM with tissue homogenates from a large number of organs decreases its toxic and antitumor potency (Umezawa et al., 1972). The enzyme responsible for this inactivation has been named BLM hydrolase by Umezawa et al. (1974). It hydrolyzes the carboxamide bond of the β-aminoalaninamide moiety on BLM, resulting in the formation of deamidobleomycin (BLM dA_2), which is 100 times less toxic to tumors (Umezawa, 1979) and does not induce pulmonary fibrosis in animal models (Lazo & Humphreys, 1983). Umezawa et al. (1972, 1974) showed that in vivo BLM inactivation by chemically induced skin tumors from mice correlates inversely with their sensitivity to BLM treatment, suggesting that BLM hydrolase plays an important role in tumor responsiveness to BLM. Lazo and Humphreys (1983) devised an HPLC–fluorometric assay to separate BLM from its inactive metabolite deamido-BLM and demonstrated that species differences in the pulmonary sensitivity to BLM correlated inversely to pulmonary BLM hydrolase activity levels. In vitro and in vivo HPLC data indicated that sensitive species have low BLM hydrolase activity levels in the lungs compared to other organs unaffected by BLM. Thus, BLM hydrolase appears to play a major role in determining pulmonary sensitivity to BLM.

Aminopeptidases are ubiquitous exopeptidases that appear to have an important catabolic function acting in concert with endopeptidases to completely degrade peptides (Barrett, 1977). Aminopeptidases that selectively cleave basic (B), neutral (N), or acidic (A) amino acids from peptides and β-naphthylamide substrates have been isolated from several tissues (McDonald & Schwabe, 1977; Mantle et al., 1985; Hui et al., 1983). Umezawa et al. (1974) suggested that BLM hydrolase is an aminopeptidase B (EC 3.4.11.6) based upon the ability of a partially purified preparation of BLM hydrolase to cleave substrates having lysine or arginine but not leucine on their amino termini and the ability of BLM B_2 to act as a competitive inhibitor of the aminopeptidase B reaction. These studies, however, were conducted with a partially purified (27-fold) BLM hydrolase preparation. Attempts to purify further this biologically relevant enzyme have been hindered by the highly unstable character of BLM hydrolase and the lack of a sensitive and rapid assay to measure BLM metabolism. Previously described bioassays require lengthy incubations and can be affected by numerous endogenous factors (Lazo & Humphrey, 1983; Thomas et al., 1984), while with the previously described HPLC assays metabolite separation and column recycling time are excessively long. We have developed a sensitive high-speed liquid chromatography (HSLC) system and used this new analytical tool coupled with fast protein liquid chromatography

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[1] Abbreviations: BLM A_2, bleomycin A_2; BLM dA_2, deamidobleomycin A_2; Arg, arginine; DTT, dithiothreitol; HSLC, high-speed liquid chromatography; HPLC, high-performance liquid chromatography; FPLC, fast protein liquid chromatography; Tris, tris(hydroxymethyl)aminomethane; SDS, sodium dodecyl sulfate.

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(FPLC) to investigate the relationship between BLM hydrolase and pulmonary aminopeptidases.

Materials and Methods

Materials. Metal-free BLM A_2 was prepared from Blenoxane (Bristol Laboratories, Syracuse, NY) as described by Lazo et al. (1984). BLM dA_2 was enzymatically prepared as described by Lazo and Humphreys (1983). Talisomycin S_{10b} was obtained from Dr. W. T. Bradner (Bristol Laboratories, Syracuse, NY). L-Aminoacyl-β-naphthylamides, L-arginyl-4-methoxy-β-naphthylamide, and Fast Garnet GBC were purchased from Sigma Chemical Co. (St. Louis, MO). Epoxy-activated Sepharose 6B and arginine–Sepharose 4B were purchased from Pharmacia (Piscataway, NJ). Bestatin and leupeptin were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). The lungs were removed from male albino New Zealand rabbits and homogenized in 2 volumes of 10 mM Tris (pH 7.4) buffer at 4 °C as described by Lazo et al. (1984). The homogenate was centrifuged (20800*g*) at 4 °C for 40 min and the supernatant centrifuged (105000*g*) at 4 °C for 60 min. The 105000*g* supernatant fractions were then frozen at −70 °C and thawed as needed.

Arginine–Sepharose 4B Affinity Chromatography. Thirty milligrams of protein from the 105000*g* supernatant of rabbit lung homogenates was applied onto a 1.5 × 8 cm arginine–Sepharose 4B column previously equilibrated with starting buffer (10 mM Na_2HPO_4/0.2 mM DTT, pH 7.2). The column was eluted with 24 mL of starting buffer, followed by a linear gradient of 0–1 M NaCl in starting buffer (220 mL total) at a flow rate of 0.35 mL/min. Four-milliliter fractions were collected, and the absorbance at 280 nm was measured. Aliquots of each fraction (480 μL) were assayed for BLM hydrolase and arginine aminopeptidase (aminopeptidase B) as described below.

Talisomycin–Sepharose 6B Affinity Chromatography. Talisomycin–Sepharose 6B was prepared by covalently coupling talisomycin S_{10b} to epoxy-activated Sepharose 6B (Pharmacia, Piscataway, NJ). This linkage is believed to be between the epoxy groups and the hydroxyls from the sugar moieties on talisomycin. Water-swollen epoxy-activated Sepharose 6B (5 g) was washed with coupling buffer (0.1 M $NaHCO_3$, pH 9) and incubated with 65 mg of talisomycin S_{10b} at 37 °C for 5 h. This was followed by thoroughly washing the gel with coupling buffer, water, and 0.1 M sodium acetate buffer (pH 4). The unreacted sites on the gel were then blocked with 1 M ethanolamine followed by washing the gel with coupling buffer, 0.1 M sodium acetate/0.5 M NaCl buffer (pH 4), and 0.1 M boric acid/0.5 M NaCl buffer (pH 8). Thirty-six milligrams of protein from the 105000*g* supernatant of rabbit lung homogenates was shaken at 37 °C for 30 min with the talisomycin–Sepharose 6B gel. The gel was then washed with 60 mL of starting buffer (10 mM Na_2HPO_4/0.2 mM DTT, pH 7.2) followed by washes with the same buffer containing increasing concentrations of NaCl. The absorbance at 280 nm of each of the 10-mL fractions collected was measured and reported as absorbance per milliliter. Aliquots of each fraction (480 μL) were assayed for BLM hydrolase and aminopeptidase B activities as described below.

Superose 12 Gel Filtration Chromatography. The Superose 12 column (1.0 × 30 cm; Pharmacia, Piscataway, NJ) was connected to the FPLC system (Pharmacia) equipped with two P-500 pumps and an LCC 500 controller. The column was washed with 50 mL of eluting buffer (50 mM Na_2HPO_4/0.2 mM DTT/0.15 M NaCl, pH 7). Five milligrams (250 μL) of protein from the 105000*g* supernatant of rabbit lung homogenates was then loaded onto the column and eluted with the above buffer at a flow rate of 0.4 mL/min for 90 min. One-milliliter fractions were collected, and the absorbance at 280 nm was monitored. Aliquots (100 μL) of each fraction were assayed for BLM hydrolase and aminopeptidase B as described below.

Mono Q Anion-Exchange Chromatography. The Mono Q column (0.5 × 5 cm; Pharmacia) was connected to the FPLC system described above. The column was equilibrated with starting buffer (10 mM Tris, pH 7.4), and 36 mg of protein from the 105000*g* supernatant of rabbit lung homogenates was loaded and eluted at 1 mL/min. One-milliliter fractions were collected, and the absorbance at 280 nm was monitored. BLM hydrolase and aminopeptidase assays were performed on 100 and 50 μL of each fraction, respectively, as described below.

HPLC and HSLC Systems. Cu-complexed BLM A_2 and BLM dA_2 were separated on a Rainin C_8 reverse-phase column (4.6 mm × 150 mm; 5-μm particle size) for HPLC or a Perkin-Elmer C_8 reverse-phase column (4.6 mm × 83 mm, 3-μm particle size) for HSLC. BLM A_2 and BLM dA_2 were detected by fluorescence as described by Lazo and Humphreys (1983). The mobile phase was composed of CH_3OH/CH_3CN/CH_3COOH/H_2O (170:72:8:750 for HPLC and 150:72:8:770 for HSLC). Both mobile phases contained 2 mM heptanesulfonic acid (Eastman Kodak, Rochester, NY) and 25 mM triethylamine (J. T. Baker Chemical Co., Phillipsburg, NJ), and their pH was 5.5. The columns were eluted isocratically at flow rates of 1 mL/min (HPLC) and 2 mL/min (HSLC).

BLM Hydrolase Assay. An aliquot from each fraction obtained after affinity, ion-exchange, and gel filtration chromatography columns was incubated with metal-free BLM A_2 (60 μM) in 10 mM Tris buffer (pH 7.4) at 37 °C. The reaction was stopped by adding 400 μL of methanol and 100 μL of 10 mM $CuSO_4$, centrifuged, and passed through a 0.2-μm ACRO filter. An aliquot was then injected into the HSLC system with a Micromeretics Model 728 autosampler, and the formation of BLM dA_2 was monitored by fluorescence as described above.

Aminopeptidase Assay. Aminopeptidase activities were measured by the well-established and previously described method of Hopsu et al. (1966). Briefly, an aliquot from each fraction obtained after chromatography columns was incubated at 37 °C with L-aminoacyl-β-naphthylamides or L-aminoacyl-4-methoxy-β-naphthylamides at a final concentration of 450 μM in 10 mM Tris buffer (pH 7.4). The reaction was stopped by adding Fast Garnet GBC as described by Hopsu et al. (1966). The color intensity produced by the coupling of Fast Garnet with β-naphthylamine or 4-methoxy-β-naphthylamine, liberated by the enzyme, was monitored by measuring the absorbance at 520 nm.

Inhibition Studies. In order to determine whether BLM hydrolase and aminopeptidase B activities are distinct, we have used bestatin and leupeptin, which are known inhibitors of aminopeptidase B and BLM hydrolase, respectively (Yoshioka et al., 1978; Umezawa et al., 1974). An aliquot of the 105000*g* supernatant (0.65 mg of protein) from rabbit lung homogenates was preincubated with various concentrations of bestatin or leupeptin for 5 min at room temperature. BLM A_2 (60 μM) or L-arginyl-β-naphthylamide (60 μM) was then added and the mixture incubated at 37 °C for 3 h or 15 min, respectively. All reactions were carried out in 10 mM Tris, pH 7.5, buffer. The amount of BLM dA_2 formed was determined by HSLC, and the amount of β-naphthylamine formed was determined by the absorbance at 520 nm as described above.

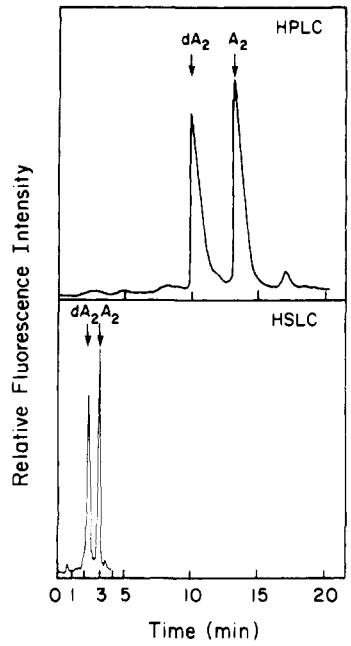

FIGURE 1: Separation of BLM A_2 from its inactive metabolite BLM dA_2 by HPLC and HSLC. A mixture of Cu-complexed BLM A_2 and BLM dA_2 standards was chromatographed on a 4.6 mm × 15 cm HPLC (5-μm particule size) or 4.6 mm × 8 cm HSLC (3-μm particule size) C_8 reverse-phase column as described under Materials and Methods.

Effect of NaCl Concentration on BLM Hydrolase and Aminopeptidase B Activities. Aliquots (0.65 mg of protein) from rabbit lung cytosol were incubated at 37 °C with either BLM A_2 (60 μM) or L-arginyl-β-naphthylamide (60 μM) in the presence of 0, 0.1, 0.2, 0.3, 0.4, or 0.5 M NaCl in 10 mM Tris buffer, pH 7.5. BLM hydrolase and aminopeptidase B assays were then performed as described above.

Stability Studies. Aliquots (0.65 mg of protein) from rabbit lung cytosol were stored at 4 °C in 10 mM Tris (pH 7.5) buffer for 8 days. The samples were then incubated with BLM A_2 (60 μM) or L-arginyl-β-naphthylamide (60 μM) at 37 °C for 3 h or 15 min, respectively. BLM hydrolase and aminopeptidase B were assayed as described above, and their activities were compared to values obtained with frozen (−70 °C) samples assayed under identical conditions.

RESULTS

High-Speed Liquid Chromatography. In order to assay BLM hydrolase activity quickly from a large number of column fractions, we developed a rapid HSLC system for the separation of BLM A_2 from its inactive metabolite BLM dA_2. Figure 1 compares our previously described HPLC (top) and the new HSLC (bottom) fluorescence profiles of a mixture of Cu-complexed BLM A_2 and BLM dA_2. Using both HPLC and HSLC C_8 reverse-phase columns, we were able to resolve BLM A_2 from its metabolite BLM dA_2. The separation, however, was accomplished in 3 min by HSLC as compared to 15 min by HPLC. This 5-fold decrease in separation time did not affect the resolution of the two compounds (R_s = 2.0). Furthermore, the HSLC system, which allowed detection of as low as 300 pg of BLM A_2 or BLM dA_2, was 10 times more sensitive than the HPLC system. Thus, for all subsequent assays of BLM hydrolase, samples were analyzed by the HSLC system linked to an automated injector.

Arginine–Sepharose 4B Affinity Chromatography. To determine whether BLM hydrolase recognizes arginine moieties, we applied the 105000*g* supernatant from rabbit lung homogenates to an arginine–Sepharose 4B affinity column.

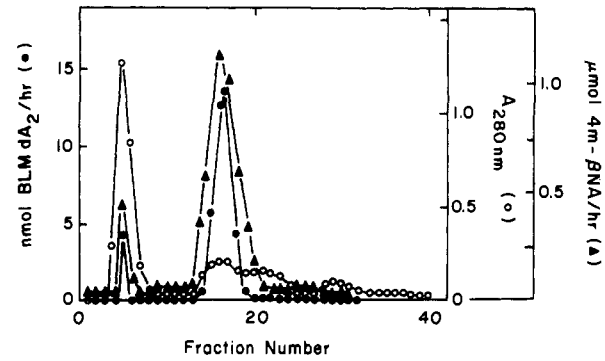

FIGURE 2: Arginine–Sepharose 4B affinity chromatography of rabbit lung cytosol. An aliquot of the 105000*g* supernatant fraction was loaded onto the affinity column and eluted as described under Materials and Methods. The A_{280} (open circles) of each fraction was measured, and BLM hydrolase (closed circles) and aminopeptidase B (closed triangles) activities were assayed as described under Materials and Methods.

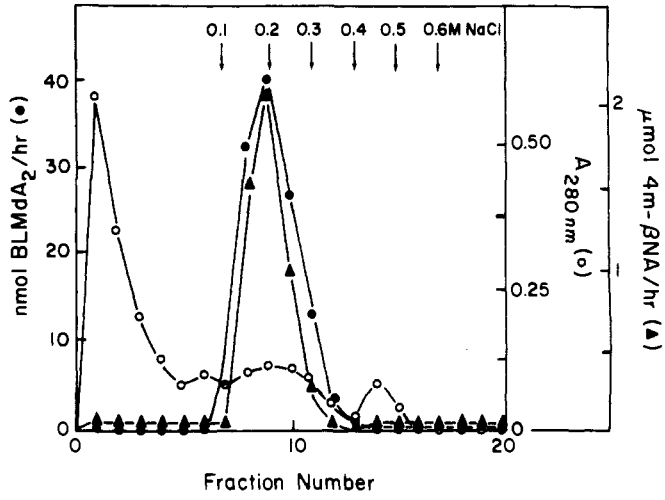

FIGURE 3: Talisomycin S_{10b}–Sepharose 6B affinity chromatography of rabbit lung cytosol. The synthesis of the affinity gel is described under Materials and Methods. An aliquot of the 105000*g* supernatant fraction was incubated with the affinity material, and the gel was washed with increasing NaCl concentrations as described under Materials and Methods. The A_{280} (open circles) of each fraction was measured, and BLM hydrolase (closed circles) and aminopeptidase B (closed triangles) activities were assayed as described under Materials and Methods.

Figure 2 shows that over 80% of the protein (open circles) did not bind to the column but was eluted in the void volume. A small proportion (less than 10%) of both BLM hydrolase (closed circles) and aminopeptidase B (closed triangles) activities also eluted in the void volume of the column. The bulk (over 90%) of the two activities, however, bound to the arginine–Sepharose 4B column and eluted only upon addition of NaCl to the buffer. Thus, BLM hydrolase, like aminopeptidase B, binds to arginine–Sepharose.

Talisomycin–Sepharose 6B Affinity Chromatography. The high affinity of BLM hydrolase for the arginine–Sepharose 4B column suggested that BLM hydrolase and aminopeptidase B may have similar binding domains. Thus, we examined whether pulmonary aminopeptidase B recognizes the BLM structure. A talisomycin–Sepharose 6B affinity material was prepared by reacting talisomycin S_{10b}, a homologue of BLM (Louie et al., 1985), with epoxy-activated Sepharose 6B. An aliquot of the 105000*g* supernatant fraction was added to this gel in a batch fashion, and the gel was washed with increasing amounts of salt. The bulk (over 90%) of the protein did not bind and washed out in the void volume (Figure 3). Both

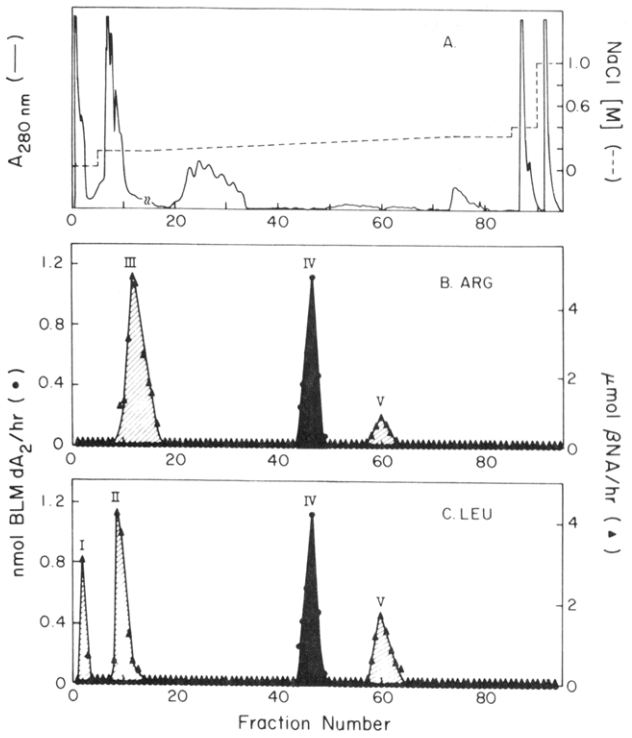

FIGURE 4: Anion-exchange Mono Q chromatography of rabbit lung cytosol. An aliquot of the 105000*g* supernatant was loaded onto the Mono Q column and eluted with various NaCl gradients in 10 mM Tris (pH 7.4) buffer as described under Materials and Methods. Absorbance at 280 nm (solid line) was monitored and is shown with the NaCl gradient profile (dashed line) in panel A. The full scale at 280 nm was 2 absorbance units for the first 15 fractions of the profile and 0.5 absorbance unit for the remaining of the profile. BLM hydrolase (closed circles, blackened area) and aminopeptidase B (panel B) and aminopeptidase N (panel C) (closed triangles, hatched area) were assayed as described under Materials and Methods.

BLM hydrolase and aminopeptidase B activities, however, were completely retained. Upon addition of 0.1 M NaCl, aminopeptidase B and BLM hydrolase coeluted, suggesting further that these two activities may be related.

Mono Q Anion-Exchange Chromatography. Despite similar ligand affinities, further work with anion-exchange chromatography indicated that BLM hydrolase and aminopeptidase B are not the same enzyme. An aliquot of the 105000*g* supernatant fraction was chromatographed on a Mono Q anion-exchange column as described under Materials and Methods. A large proportion of the protein was eluted with the void volume of the column (Figure 4A), but both BLM hydrolase and aminopeptidase B were retained (Figure 4B). BLM hydrolase (Figure 4B, peak IV) was eluted during a shallow 60-mL linear gradient of 0.15–0.30 M NaCl in starting buffer (Figure 4A) and was completely separated from both the major aminopeptidase B (peak III) and the minor aminopeptidase B (peak V). Identical results were obtained when lysyl-β-naphthylamide was used as a substrate for aminopeptidase B. Thus, rabbit pulmonary cytosol contained one major and one minor aminopeptidase B which were clearly distinct from pulmonary BLM hydrolase. Neither of the two aminopeptidase B peaks had any BLM hydrolase activity nor did the BLM hydrolase peak have any aminopeptidase B activity associated with it (Figure 4B).

The BLM hydrolase peak from the Mono Q column had a specific activity of 545 nmol of BLM dA_2 mg^{-1} h^{-1} as compared to a specific activity of 0.3 nmol of BLM dA_2 mg^{-1} h^{-1} of the homogenate. Thus, the BLM hydrolase preparation from the Mono Q column was purified over 1800-fold. This

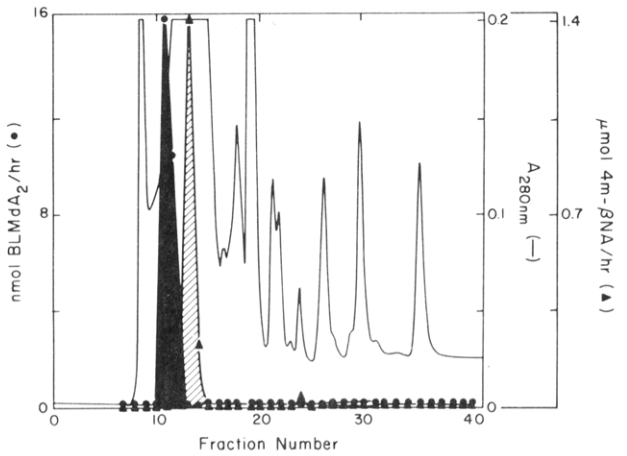

FIGURE 5: Gel permeation Superose 12 chromatography of rabbit lung cytosol. An aliquot of the 105000*g* supernatant fraction was loaded onto the Superose 12 column and eluted as described under Materials and Methods. The absorbance at 280 nm (solid line) was monitored, and BLM hydrolase (closed circles, blackened area) and aminopeptidase B (closed triangles, hatched area) were assayed as described under Materials and Methods.

is a partially purified preparation, which contained several bands on SDS–polyacrylamide gel electrophoresis (data not shown).

We next assayed the Mono Q column fractions for aminopeptidase N activity to determine whether BLM hydrolase could cleave neutral aminoacyl-β-naphthylamides. We identified two major rabbit pulmonary aminopeptidases N (Figure 4C, peaks I and II). Both peaks were resolved from BLM hydrolase (peak IV) as well as the major aminopeptidase B (peak III). Peaks I and II were able to cleave leucine (Figure 4C) as well as alanine and glycine from their corresponding β-naphthylamides. The profiles for alanine and glycine aminopeptidase activities were very similar to that of leucine (Figure 4C) except that glycine aminopeptidase peak I represented a small proportion of the total activity (data not shown). The minor aminopeptidase B peak (Figure 4B, peak V) also had aminopeptidase N activity associated with it (Figure 4C, peak V). As mentioned above, peak V was completely resolved from BLM hydrolase (peak IV) and had no BLM hydrolase activity associated with it. Thus, rabbit pulmonary cytosol contains one aminopeptidase B, two aminopeptidases N, and one aminopeptidase with both aminopeptidase B and aminopeptidase N activities. All four aminopeptidases were distinct from pulmonary BLM hydrolase. We also assayed the Mono Q fractions for cleavage of aspartylacyl-β-naphthylamide and found no aminopeptidase A activity in any of the fractions (data not shown). Thus, rabbit pulmonary cytosol does not contain any detectable aspartic acid–aminopeptidase activity.

Superose 12 Gel Filtration Chromatography. BLM hydrolase and aminopeptidase B have different charge characteristics as demonstrated by Mono Q anion-exchange chromatography. To determine whether these two enzymes also have different molecular weights, we investigated their size properties by gel filtration. Figure 5 shows the FPLC profile of the Superose 12 column separation of rabbit lung cytosolic fractions. BLM hydrolase (closed circles) eluted immediately after the void volume of the column with an apparent molecular mass of 250 000 daltons. In contrast to results from Mono Q chromatography, only one aminopeptidase B peak (closed triangles) was detected, and this peak was separated from the higher molecular weight BLM hydrolase. Thus, these results further demonstrate that BLM hydrolase is distinct

Table I: Inhibition of BLM Hydrolase and Aminopeptidase B by Bestatin and Leupeptin[a]

	% of control[b]					
	bestatin (μM)			leupeptin (μM)		
	2	20	200	2	20	200
BLM hydrolase	115	103	82[c]	96	46	0
aminopeptidase B	55	33	20	96	83	55

[a] Aliquots of the 105000g supernatant from rabbit lung homogenates were preincubated with bestatin or leupeptin for 5 min at room temperature. Each sample was then incubated with either BLM A_2 or Arg-β-naphthylamide as described under Materials and Methods. The values represent the percentage of metabolism found in corresponding samples that were incubated without the inhibitor. [b] Control value for BLM hydrolase was 2 nmol of BLM dA_2 mg^{-1} h^{-1} and for aminopeptidase B 634 nmol of β-naphthylamine mg^{-1} h^{-1}. [c] Each value is an average of two independent determinations.

from pulmonary aminopeptidase B.

Inhibition Studies. Additional evidence distinguishing BLM hydrolase activity from aminopeptidase B activity was provided by the use of inhibitors. Concentrations of bestatin as low as 2 μM decreased the aminopeptidase B activity by 50% in rabbit pulmonary cytosol whereas concentrations up to 20 μM of the same inhibitor had no effect on BLM hydrolase activity in this preparation (Table I). Leupeptin was very effective in inhibiting BLM hydrolase (IC_{50} = 13 μM) but not aminopeptidase B (IC_{50} > 200 μM) (Table I). Thus, BLM hydrolase and aminopeptidase B differ in their sensitivity to bestatin and leupeptin.

Stability Studies and Effect of NaCl Concentration on BLM Hydrolase and Aminopeptidase B. The stabilities of the two enzyme activities were determined by preincubating aliquots of the 105000g supernatants from rabbit lung homogenates at 4 °C followed by assaying for BLM hydrolase and aminopeptidase B as described under Materials and Methods. Prior to incubation at 4 °C, BLM hydrolase had a specific activity of 1.64 nmol of BLM dA_2 mg^{-1} h^{-1} and aminopeptidase B 630 nmol of β-naphthylamine mg^{-1} h^{-1}. BLM hydrolase lost 80% of its activity in 8 days. In contrast, incubation at 4 °C for up to 8 days did not diminish aminopeptidase B activity. Thus, BLM hydrolase is much less stable than aminopeptidase B at 4 °C.

The effect of NaCl on the two enzyme activities was investigated by assaying the 105000g supernatant from rabbit lung homogenates for BLM hydrolase and aminopeptidase B in the presence of various NaCl concentrations as described under Materials and Methods. In the absence of NaCl, BLM hydrolase had a specific activity of 1.31 nmol of BLM dA_2 h^{-1} mg^{-1} and aminopeptidase B 693 nmol of β-naphthylamine mg^{-1} h^{-1}. BLM hydrolase activity was not affected by concentrations of up to 0.5 M NaCl whereas aminopeptidase B activity increased with concentrations of NaCl. The maximum activation of aminopeptidase B activity was seen with 0.2 M NaCl, and this activity was 55% greater than that seen without NaCl. Thus, NaCl did not affect BLM hydrolase activity but significantly increased aminopeptidase B activity.

Discussion

Metabolic inactivation of therapeutic compounds represents an important biochemical mechanism frequently limiting their toxic actions (Jakoby et al., 1982). It has been proposed that BLM hydrolase regulates the antitumor effects and pulmonary toxicity of the complex BLM glycopeptides (Umezawa, 1979; Lazo & Humphreys, 1983). Although BLM hydrolase activity was detected in homogenates from several organs over a decade ago (Umezawa et al., 1972), little is known about the biochemical characteristics of this protective enzyme. Despite the development of direct HPLC assays for BLM hydrolase (Lazo & Humphreys, 1983; Yoshioka et al., 1978; Muraoka, 1979; Akiyama, 1981), the lability of the enzyme and the length of time required for complete separation of deamido-BLM from the parent compound BLM (15 min, Figure 1, top) precluded the practical use of these HPLC assays for the routine analysis of numerous fractions from chromatography columns used in the isolation of BLM hydrolase. Thus, the 3-min HSLC assay described here (Figure 1, bottom) represents an important tool for the purification and characterization of BLM hydrolase. In addition to the speed of the HSLC system, it is also 10 times more sensitive than the HPLC system, allowing analysis of very small amounts of each fraction for BLM hydrolase. Using this analytical method coupled with FPLC, we have purified BLM hydrolase 1800-fold from rabbit lung cytosol; this is a 60-fold improvement over the previously reported purification scheme (Umezawa et al., 1974).

Umezawa et al. (1974, 1979) proposed that BLM hydrolase was an aminopeptidase B because it appeared to hydrolyze substrates with basic amino acids at their amino termini. In addition, BLM B_2 competitively inhibited the hydrolysis of arginine-β-naphthylamide, an artificial substrate commonly used to assay aminopeptidase B (Hopsu et al., 1966; Barrett, 1977). Preliminary studies by Umezawa et al. (1974) also suggested that BLM hydrolase and aminopeptidase B from rat liver might be different. Unfortunately, further characterization of BLM hydrolase was not possible due to its instability, and the relationship between BLM hydrolase and aminopeptidase B was unclear. In the present report, affinity chromatography studies with arginine–Sepharose demonstrated that both BLM hydrolase and aminopeptidase B were selectively retained and, therefore, shared the ability to recognize the arginine moiety. This is in agreement with the report of Umezawa et al. (1974), who showed similar results with a lysine–Sepharose column. BLM hydrolase and aminopeptidase B also shared the ability to recognize the BLM structure by selectively binding to a talisomycin S_{10b}–Sepharose afffinity column. Despite the mutual affinity for BLM- and arginine-containing moieties, BLM hydrolase and aminopeptidase B from rabbit pulmonary cytosol were unambiguously separated by Mono Q anion-exchange and gel filtration chromatography. BLM hydrolase was found to be more anionic (Figure 4B) and of a higher molecular weight than pulmonary aminopeptidase B (Figure 5). Furthermore, we found leupeptin to be a much more effective inhibitor of BLM hydrolase than of aminopeptidase B whereas bestatin, a known aminopeptidase B inhibitor which previously was reported to affect BLM hydrolase activity in crude homogenates (Umezawa et al., 1974), lacked inhibitory actions against BLM hydrolase at concentrations (20 μM) that blocked hydrolysis of the Arg-β-naphthylamide substrate. Aminopeptidase B from various sources is known to be activated by chloride ion (Hopsu et al., 1966; Soderling, 1980). We found that rabbit pulmonary aminopeptidase B was also chloride dependent, but pulmonary BLM hydrolase was not. Finally, BLM hydrolase was very unstable at 4 °C whereas aminopeptidase B was not affected by storage at 4 °C. Thus, BLM hydrolase can be distinguished from aminopeptidase B by charge, size, stability, and sensitivity to inhibitors and activators. Furthermore, several other aminopeptidases were identified in rabbit lungs, and all were also distinct from BLM hydrolase. Besides the aminopeptidase B described above, two aminopeptidases selective for the cleavage of neutral amino acids were also found in rabbit pulmonary cytosol. Interestingly, a fourth amino-

peptidase was identified, and this enzyme was able to cleave basic as well as neutral amino acids from their corresponding β-naphthylamides.

These results demonstrate for the first time that BLM hydrolase is unequivocally distinct from all aminopeptidases identified in rabbit pulmonary cytosol. Furthermore, the HSLC system that has been developed will be of essential value in the further purification and characterization of BLM hydrolase and in exploring its substrate specificity and physiological importance.

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