Human Eosinophil Arylsulfatase B

STRUCTURE AND ACTIVITY OF THE PURIFIED TETRAMERIC LYSOSOMAL HYDROLASE

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ABSTRACT Arylsulfatase B from human eosinophils was purified free of contaminating proteins by gel filtration and sequential affinity chromatography on Affi-Gel Blue and zinc chelate Sepharose. 50 μg of the purified enzyme presented as a single stained band on alkaline disc gel electrophoresis. In both goats and rabbits, the purified enzyme elicited monospecific antisera that yielded single precipitation arcs on Ouchterlony analysis with a human eosinophil extract and the purified enzyme; the immunoprecipitation lines fused in a pattern of identity, providing immunochemical evidence for the homogeneity of the purified enzyme. On sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, a dominant lower molecular weight protein and three other bands with molecular weights approximately two, three, and four times that of the major protein band were resolved. The prominence of the less rapidly migrating protein bands increased relative to the major band if the enzyme was maintained under acidic conditions or was reacted with the cross-linking agent dimethyl suberimidate under alkaline conditions before SDS-polyacrylamide gel electrophoresis, supporting the conclusion that the enzyme consists of four subunits. Two stained bands were present on acid disc gel electrophoresis; they were composed of oligomeric forms of enzyme on analysis by SDS-polyacrylamide gel electrophoresis in a second dimension. A minimum molecular weight of 70,190 was determined from amino acid composition analysis for the tetrameric form of the enzyme. The specific functional activity of the purified arylsulfatase B was concentration and time dependent, compatible with its association or dissociation into subunit forms with differing specific activities. Factors that govern subunit interactions of arylsulfatase B, including local enzyme concentration and pH, provide mechanisms for regulating the enzymatic activity of this lysosomal hydrolase.

INTRODUCTION

Mammalian arylsulfatases include a microsomal enzyme, arylsulfatase C, and two lysosomal enzymes, designated arylsulfatases A and B, which are widely distributed in mammalian tissues (1). The arylsulfatase B enzymes have reported apparent molecular weights in the range of 50,000 to 60,000 and are noncompetitively inhibited by sulfate ions, whereas arylsulfatase A enzymes are of ~100,000 mol wt and are competitively inhibited by sulfate ions (1, 2). The eosinophil expresses the greatest arylsulfatase activity of the various leukocytes (3, 4) and the enzyme has been characterized as being type B (5). Histochemical studies have localized arylsulfatase B to both the large crystalloid-containing granule and to a smaller granule of the eosinophil (6). Although purifications of mammalian arylsulfatase B have been reported from tissues such as human (7, 8) and ox (9) liver, no subunit structure has been recognized previously for this lysosomal enzyme. Human eosinophil arylsulfatase B has been purified to homogeneity, demonstrated to consist of four subunits, and shown to function enzymatically as an associating-dissociating oligomeric enzyme.

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METHODS

Materials. Delipidated bovine serum albumin, dipotassium 2-hydroxy-5 nitrophenyl sulfate (NCS),[1] 4-nitrocatechol (NC), iodoacetamide, Cibacron F3GA dye, glycine, Nonidet P-40 (NP-40) (Sigma Chemical Co., St. Louis, MO); Cibacron Blue F3GA-agarose (Affi-Gel Blue), sodium dodecyl sulfate (SDS), acrylamide, *N,N'*-methylene-*bis* acrylamide, *N,N,N',N'*-tetramethylethylene diamine, urea, dithiothreitol, ammonium persulfate, Coomassie Brilliant Blue stain, Bio-Rad protein assay, bromophenol blue and methyl green dyes, organomercurial agarose (Affi-Gel 501) (Bio-Rad Laboratories, Richmond, CA); Sephadex G-100 gel filtration and electrophoresis molecular weight calibration proteins, 6% Dextran-70 in normal saline (Macrodex), PAA 4/30 4–30% polyacrylamide gradient gel slabs, staphylococcal protein A-Sepharose CL-4B (Pharmacia Fine Chemicals, Piscataway, NJ); acetic acid, sodium acetate, disodium ethylenediamine tetraacetate (EDTA), hydrochloric acid, Tris, potassium ferricyanate, zinc chloride (Fisher Scientific Co., Medford, MA); calcium- and magnesium-free Hanks' balanced salt solution (HBSS) (Microbiological Associates, Walkersville, MD); 10,000-mol wt retention collodion bags (Schleicher and Schuell, Keene, NH); Branson model 350 sonifier (Branson Sonic Power Co., Danbury, CN); immunodiffusion plates (Hyland Laboratories, Cost Mesa, CA); complete and incomplete Freund's adjuvant (Difco Laboratories, Detroit, MI); iminodiacetic-acid Sepharose CL-4B (Pierce Chemical Co., Rockford, IL); dimethyl suberimidate dihydrochloride (Aldrich Chemical Co., Milwaukee, WI); and pH 3–10 and pH 7–9 ampholytes (LKB Instruments, Rockville, MD) were obtained as indicated.

Cell isolation and chromatographic procedures. Blood was obtained from a donor with idiopathic hypereosinophilia (28,000–36,000 leukocytes/mm^3 of blood with 87–94% eosinophils). After citrate dextrose anticoagulation and dextran sedimentation of erythrocytes (10), leukocyte-rich plasma was collected. Leukocytes were washed in calcium- and magnesium-free HBSS by centrifugation at 2,000 *g* for 15 min. A pellet containing 10^9 cells (~90% eosinophils, 5% neutrophils, and 5% mononuclear leukocytes) was frozen at −20°C until it was subjected to three sequential cycles of ultrasonic disruption (model 350 sonifier with microtip at output setting 6), on ice for 2 min in 5 ml of 0.1 M Tris-HCl, pH 7.5, 2 mM EDTA for each cycle. After centrifugation of the sonicates at 2,000 *g* for 10 min, the soluble extracts from each disruption cycle were pooled and analyzed for enzyme activity. Arylsulfatase activity was assessed spectrophotometrically by the formation of NC from NCS (11). Enzyme preparations (10–100 μl) were incubated in 1 ml of 10 mmol NCS 0.5 M sodium acetate-acetic acid, pH 5.7. After 10–30 min, the reactions were terminated by the addition of 1.5 ml of 1 N NaOH and the quantity of NC generated was determined by measuring the OD_{515} (model 240, Gilford Spectrophotometer, Gilford Instrument Laboratories, Oberlin, OH). From the linear portion of a standard curve of OD_{515} plotted against increasing amounts of NC, the micromoles of NC liberated were determined. One unit of arylsulfatase activity is defined as the amount that liberates 1 μmol of NC/h at 37°C in 0.5 M acetate buffer, pH 5.7. For assays used in calculating the activities during purification, 1 mg/ml of bovine serum albumin was present in the reaction mixture. Protein was measured with reagents from Bio-Rad Laboratories by the method of Bradford (12) with bovine gamma globulin as the reference protein. All subsequent chromatographic purification steps were performed at 4°C.

The pooled eosinophil extract was applied to a column (2.6 × 90 cm) of superfine mesh Sephadex G-100 equilibrated and eluted with 0.1 M Tris-HCl, pH 7.5, 2 mM EDTA, at a rate of 35 ml/h. Fractions containing the single peak of arylsulfatase activity eluting at ~46% bed volume were pooled. Reference proteins used as molecular weight markers to calibrate the column included ribonuclease A (13,700 mol wt), chymotrypsinogen A (25,000 mol wt), ovalbumin (43,000 mol wt), and bovine serum albumin (67,000 mol wt) with blue dextran used to determine the void volume.

The pooled gel filtration fractions were applied to a column (1.5 × 28 cm) containing 28 ml of Affi-Gel Blue (100–200 mesh) equilibrated in 0.1 M Tris-HCl, pH 7.5, 2 mM EDTA buffer (buffer A). The column was sequentially eluted at a rate of ~60 ml/h with 50 ml of buffer A, 180 ml of a linear gradient from 0.0 to 0.6 M NaCl in buffer A, 30 ml of 1 M NaCl in buffer A, and 50 ml of 3 M NaCl in buffer A.

Iminodiacetic acid-Sepharose CL-4B (10 ml) in a 1 × 12-cm column was charged with zinc ions by passage of 50 ml of 0.1 M Tris-HCl, pH 7.5 (buffer B) containing 1 mg/ml of $ZnCl_2$. Excess unbound zinc ions in the eluate, demonstrated by the formation of a precipitate with $K_4Fe(CN)_6$, were washed from the column with 50 ml of buffer B. The pooled enzyme sample from the preceding chromatographic step was applied and the column was sequentially eluted at a rate of ~60 ml/h with 30 ml of buffer B; 30 ml of buffer B containing 1 M NaCl; 30 ml of buffer B; 55 ml of 0.1 M sodium acetate-acetic acid buffer, pH 5.0; 20 ml of 0.1 M sodium acetate-acetic acid buffer, pH 5.0, with 0.5 M NaCl; and 40 ml of buffer B with 50 mM EDTA.

Analytical methods. Acid polyacrylamide gel electrophoresis was performed with pH 4.3, 6% polyacrylamide gels and alkaline polyacrylamide gel electrophoresis was performed with pH 8.9, 7.5% polyacrylamide gels, each with 2.5% stacking gels, prepared as detailed (13). Disc gels were electrophoresed at 4°C with a constant current of 1.25 mA/gel until the tracking dye (methyl green or bromophenol blue, respectively) had traversed the stacking gel and then with 2.5 mA/gel until the tracking dye approached the end of the gels. For visualization of protein, gels were fixed in 10% trichloroacetic acid, stained with a solution containing 0.1% Coomassie Brilliant Blue, 45% ethanol, and 10% glacial acetic acid, and destained by diffusion in an aqueous solution of 7% acetic acid and 20% ethanol. To assay for enzymatic activity, 3-mm thick slices from replicate gels were incubated at 37°C in 1 ml of substrate solution (10 mmol NCS, 0.5 M acetate buffer, pH 5.7) for 0.5 to 2.5 h.

Arylsulfatase samples, after acid disc gel electrophoresis, were electrophoresed in the second dimension on SDS-polyacrylamide gradient slab gels. Unfixed acid disc gels were mounted on Pharmacia 4–30% gradient gels previously equilibrated by electrophoresis for 1 h at 70 V, 0.025 M Tris, in pH 8.3, 0.192 M glycine, 0.1% SDS buffer. Electrophoresis in the second dimension was performed in the same buffer at 150 V until the bromophenol blue tracking dye reached the bottom of the gel.

SDS-polyacrylamide disc gel electrophoresis was performed in 7.5 or 10% polyacrylamide gels in pH 7.0, 0.05

[1] *Abbreviations used in this paper*: buffer A, 0.1 M Tris-HCl, pH 7.5, containing 2 mM EDTA; buffer B, 0.1 M Tris-HCl, pH 7.5; EDTA, disodium ethylenediamine tetraacetate; HBSS, Hanks' balanced salt solution; NC, 4-nitrocatechol; NCS, dipotassium 2-hydroxy-5 nitrophenyl sulfate (or nitrocatechol sulfate).

M Na_2HPO_4/NaH_2PO_4 buffer containing 0.1% SDS, by the method of Weber and Osborn (14). Arylsulfatase samples, in pH 5.0, 0.1 M acetate buffer or dialyzed into pH 8.5, 50 mM Tris-HCl buffer, were denatured in 8 M urea with or without reduction in 10 mM dithiothreitol. Reduced samples were alkylated in 100 mM iodoacetamide at 56°C for 15 min. Then SDS was added to a concentration of 1% (wt/vol) and the samples were heated at 100°C for 10 min. Protein standards for molecular weight determination included phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and α-lactalbumin. Stained gels were scanned spectrophotometrically at 600 nm with a Gilford 2520 gel scanner. SDS gels run at polyacrylamide gel concentrations of 4, 6, 8, 10, and 12% were used to assess the validity of molecular weight determinations by examining electrophoretic mobility as a function of acrylamide concentration (15). Protein cross-linking was performed as described by Davies and Stark (16) with dimethyl suberimidate. Arylsulfatase samples at 0.5 to 0.05 mg/ml were incubated overnight at 25°C in pH 8.5, 50 mM Tris-HCl, 2 mM EDTA buffer, containing 5.0 or 0.5 mg/ml dimethyl suberimidate, and then analyzed by SDS-polyacrylamide gel electrophoresis.

Isoelectric focusing was performed in a sucrose density gradient column (model 212, Instrumentation Specialities Co., Lincoln, NB). A 30-ml linear 5–30% sucrose gradient containing 2% (vol/vol) ampholytes (two parts pH 3–10, and three parts pH 7–9), was prefocused at 800 V, at 4°C for 15 h until the current had declined to 1 mA. 1 ml was removed from the mid-portion of the sucrose gradient, mixed with the sample and returned to the mid-portion of the gradient. Isoelectric focusing was resumed for an additional 26 h. The column was harvested by upward displacement, and 0.75-ml fractions were collected. The pH of each fraction was measured and the fractions were dialyzed with 10,000-mol wt cutoff membranes for 48 h against four changes of 40 vol each of 0.05 M sodium acetate acetic acid, pH 5.7, 5 mM EDTA. Isoelectric focusing was also performed as described by O'Farrell (17) in 2.7-mm diam tubes, at 4% polyacrylamide containing 8 M urea, 2% NP-40 and 2% ampholytes (two parts pH 3–10 and three parts of pH 7–9) which were fixed and stained for protein with Coomassie Blue as above.

For amino acid analysis, duplicate 100-μg samples of purified, lyophilized enzyme were hydrolyzed in 6 N HCl at 110°C for 24 h and analyzed using a Beckman 121 MB analyzer. Duplicate analyses yielded identical determinations.

Immunochemical techniques. Rabbits and goats were immunized with 100 μg s.c. of purified arylsulfatase emulsified in complete Freund's adjuvant and boosted subcutaneously with 50–100 μg of enzyme in incomplete Freund's adjuvant at 6 and 10 wk, and then periodically thereafter; immune serum was obtained 1 wk later. Preimmune and immune rabbit sera were applied to a staphylococcal protein A-Sepharose CL-4B column equilibrated in 0.1 M Tris-HCl, pH 7.5, 1 mM EDTA, at 25°C and the bound immunoglobulin G (IgG) was eluted with 100 mM glycine-HCl, pH 2.5 (18).

RESULTS

Purification of human eosinophil arylsulfatase B. A sonicate of 10^9 leukocytes containing 90% eosinophils was applied to a Sephadex G-100 column and yielded a single peak of arylsulfatase activity, at 46% bed volume with a K_{AV} of 0.24, filtering with an apparent molecular weight of 45,000. On application of pooled arylsulfatase to a column containing the sulfonated aromatic dye, Cibacron F3GA (Ciba Geigy Corp., Summit, NJ), the enzyme was completely absorbed. With a gradient from 0 to 0.6 M NaCl, activity eluted in a broad peak beginning at ~10 mS/cm (Fig. 1). The activity eluting between 10 and 40 mS/cm from the Affi-Gel Blue column was applied to a zinc chelate-Sepharose column, and eluted with a decrease in the pH of the buffer from 7.5 to 5.0 (Fig. 2). Arylsulfatase activity was eluted in a discrete peak such that essentially all of the activity was in a single fraction. The activity corresponded to a protein peak in the same fraction. No further activity was recovered when the chelated zinc ligand was removed from the column with the terminal buffer containing 50 mM EDTA. The purification of the enzyme depicted in Figs. 1 and 2 is presented in Table I. The overall yield was 39% with a 126-fold purification. The results from five additional complete purifications with cells obtained on different occasions from the same donor were comparable with a mean yield of 49% (42–70%, range), a mean purification of 105-fold (63- to 140-fold) and a mean specific activity of 140 U/mg (108–174 U/mg).

Elicitation of monospecific antisera with purified eosinophil arylsulfatase. Two rabbits and two goats, immunized with purified eosinophil arylsulfatase, each developed monospecific antiserum, which yielded a single immunoprecipitation line on Ouchterlony analysis with 40 μg of ultrasonically disrupted eosinophil-enriched leukocytes. Each antiserum also gave a single immunoprecipitin line with the purified arylsulfatase; these lines fused in a pattern of immunochemical iden-

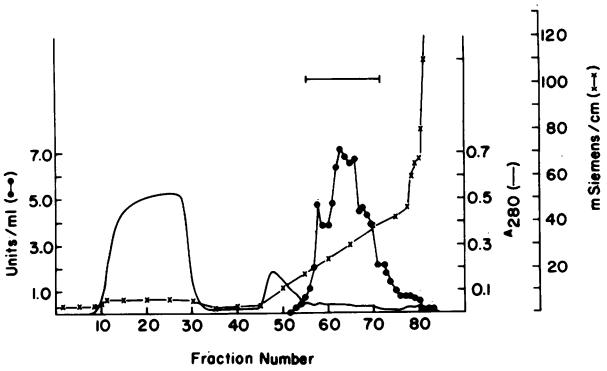

FIGURE 1 Elution of eosinophil arylsulfatase B from a column of Affi-Gel Blue charged with the pooled enzyme activity after Sephadex G-100 gel filtration. The column, equilibrated in buffer A, was washed with buffer A, and eluted sequentially with a gradient from 0 to 0.6 M NaCl in buffer A followed by 1 M NaCl and 3-M NaCl washes in buffer A. 5-ml fractions were collected. Enzyme activity was pooled as indicated (|—|).

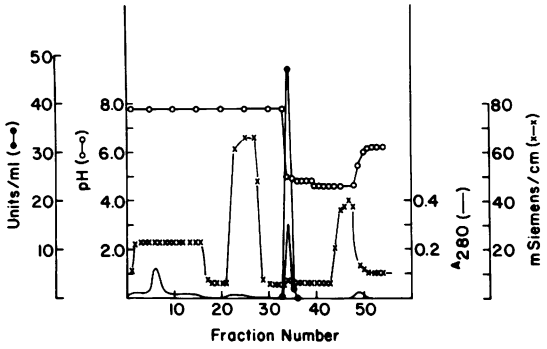

FIGURE 2 Elution of eosinophil arylsulfatase B from a column of zinc-chelate Sepharose equilibrated in buffer B and charged with the pooled enzyme obtained from the Affi-Gel Blue chromatography step. The column was washed with buffer B beginning at fraction 16 and eluted with buffer B containing 1 M NaCl; buffer B; acetate buffer, pH 5.0; acetate buffer, pH 5.0, 1 M NaCl; and finally with buffer B containing 50 mM EDTA. 5-ml fractions were collected.

tity with the immunoprecipitin lines formed against the eosinophil sonicate. The capacity of the purified protein to elicit monospecific sera in four animals representing two different species provides immunochemical evidence for the homogeneity of the purified enzyme.

Subunit structure of purified eosinophil arylsulfatase B. Purified eosinophil arylsulfatase B was subjected to alkaline and acid polyacrylamide gel electrophoreses under non-denaturing conditions. With alkaline conditions, a single band of Coomassie Blue staining protein was detected (Fig. 3A); no enzymatic activity could be eluted from replicated alkaline gels. Under acid conditions, two Coomassie Blue staining bands were present (Fig. 3B). Most of the arylsulfatase B activity assayed from slices of a replicate acid gel coincided with the major protein band.

Electrophoresis under denaturing conditions in SDS of purified arylsulfatase B prepared by dialysis against mildly alkaline buffers (i.e., 50–100 mM Tris-HCl, pH 7.5–8.5) and boiling in 1% SDS for 10 min yielded a predominant protein staining band and three less intensely staining bands with higher apparent molecular weights (Fig. 4A). When purified arylsulfatase B maintained at acidic pH (50–100 mM sodium acetate-acid buffer, pH 5.0) was boiled in 1% SDS for 10 min and subjected to electrophoresis in SDS, the higher molecular weight bands became more prominent (Fig. 4B). Neither treatment of the arylsulfatase B by prior reduction and alkylation with iodoacetamide nor by preparation in 8 M urea altered these patterns on SDS gels. The apparent molecular weights of the three minor protein bands of ~28,500, 40,000, and 60,000 were approximately multiples of the 15,000-mol wt of the principal band (Figs. 4A and B). When undenatured, purified arylsulfatase B, in 50 mM Tris-HCl, pH 8.5, was incubated with the cross-linking reagent dimethyl suberimidate prior to electrophoresis in SDS, there was a shift in protein to the higher molecular weight peaks (Fig. 4C). The finding of four major peaks is compatible with a tetrameric structure for the enzyme.

To analyze the two protein peaks obtained on acid disc gels (Fig. 3B), replicate acid disc gels were subjected to electrophoresis in the second dimension in 0.1% SDS on 4 to 30% polyacrylamide gradient gels. The major protein peak on the acid gel yielded four protein staining bands whose relative migrations were compatible with monomeric, dimeric, trimeric, and tetrameric forms. In contrast, the minor migrating peak on the acid gel yielded a single protein staining band whose electrophoretic migration was identical to that of the monomeric form derived from the major protein peak. Thus, the major peak, composed of multimers, was the predominant source of detectable enzymatic activity.

Molecular weight and amino acid composition. The purified enzyme and six reference proteins were subjected to electrophoresis in gels of different polyacrylamide concentrations. In contrast to the reference proteins, whose electrophoretic migrations yielded lines on a Ferguson plot (15) of $\log_{10}(10 \cdot R_f)$ vs. percent polyacrylamide gel concentrations that had a common intercept at 0% acrylamide (Fig. 5), the monomeric and oligomeric forms of arylsulfatase migrated aberrantly. The free electrophoretic mobility, the intercept

TABLE I
Sequential Purification of Human Eosinophil Arylsulfatase B

Purification step	Protein	Activity	Specific activity	Yield	Purification factor
	mg	*U*	*U/mg*	%	
Sonicate	468	579	1.24	100	—
Sephadex G-100 gel filtration	100	518	5.17	89	4.2
Affi-Gel Blue	4.11	246	59.8	48	48.3
Zinc chelate-Sepharose	1.27	228	156	39	126

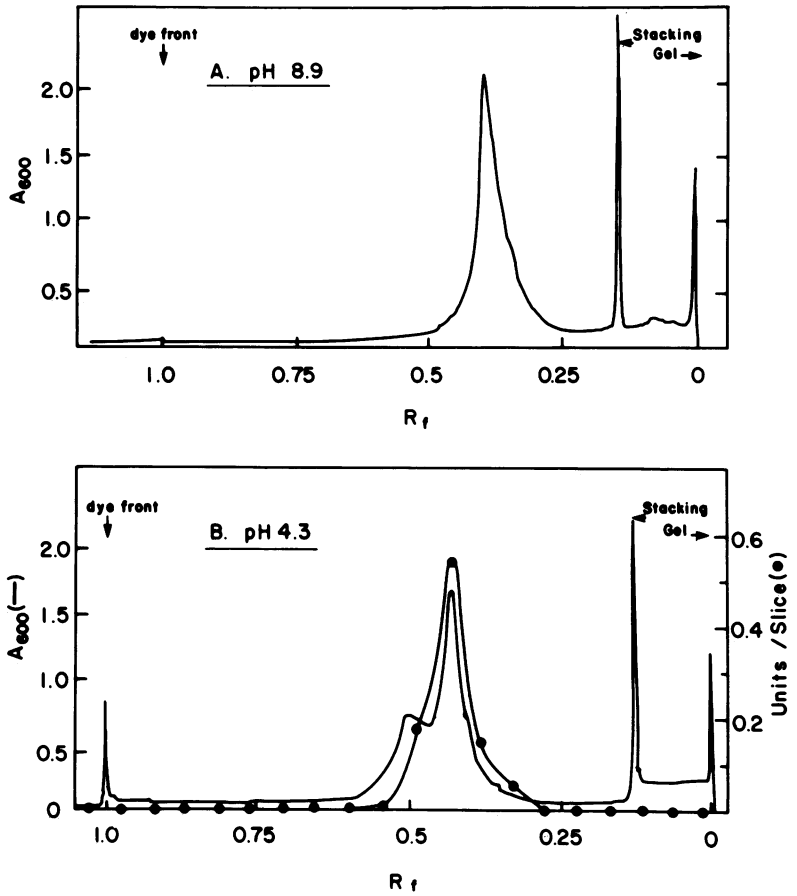

FIGURE 3 Polyacrylamide gel electrophoresis under alkaline (A) and acid (B) conditions of 50 μg of purified eosinophil arylsulfatase B. Gels were stained with Coomassie Blue and scanned at 600 nm.

at 0% acrylamide, was greater for the monomer than for the reference proteins, and rose with increasing polymerization (Fig. 5). As a result of this aberrantly rapid migration in SDS gels, the molecular weights of the monomeric and oligomeric forms of the enzyme were underestimated by comparison to those of reference proteins.

The average amino acid composition of arylsulfatase B from two 24 h acid hydrolysates is shown in Table II. The number of residues of each amino acid per 1,000 residues is presented. The number of moles of each amino acid per mole of enzyme has been calculated with the assumption that each of the four subunits contains one methionine residue. With this assumption, the molecular weight of the tetrameric enzyme would be 70,190, based on the molecular weights of the determined amino acids. Because of limited amounts of enzyme, separate analyses for determination of cysteine and tryptophan and longer duration hydrolyses were not performed.

On isoelectric focusing in sucrose of 200 μg of arylsulfatase B, a single zone of enzymatic activity was found at pH 8.76. Focusing in polyacrylamide gels containing urea and nonionic detergent yielded a single protein band focusing between pH 7.30 and 7.65.

Enzymatic behavior of purified arylsulfatase B as an associating-dissociating enzyme. When 43-μg samples of purified enzyme were assayed in 1 ml of 10 mM NCS for 1, 2, 5, and 15 min, the mean rate of 14.7 nmol of product generated/min was constant, but declined to 12.7 nmol/min over a 30-min assay. Similar results were found with other preparations of enzyme and were not explicable by substrate depletion or product inhibition. This decrease in the rate of hydrolysis of substrate with time suggested the possibility that arylsulfatase might dissociate with loss of enzymatic activity.

When arylsulfatase B was diluted before assay, the specific activities of the diluted preparations diminished progressively with dilution (Fig. 6A). Compati-

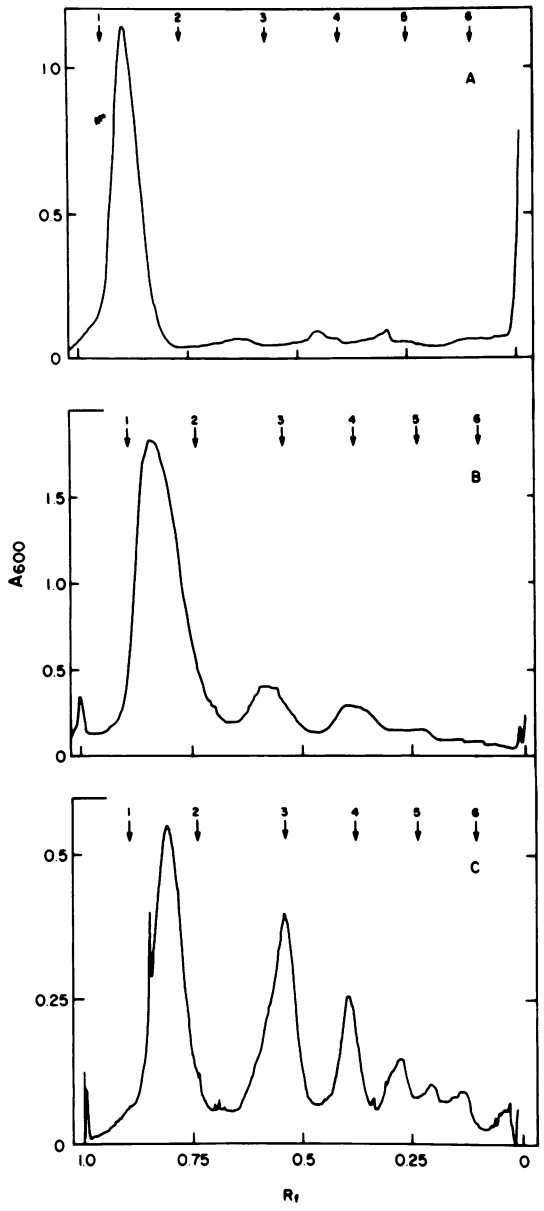

FIGURE 4 Electrophoresis in 0.1% SDS with 7.5% polyacrylamide gels of eosinophil arylsulfatase B. In A, 45 μg of purified enzyme were dialyzed against 50 mM Tris-HCl, pH 8.5, before electrophoresis. In B, 55 μg of purified enzyme were in 50 mM sodium acetate-acetic acid, pH 5.0, before electrophoresis. In C, 50 μg of purified arylsulfatase B, dialyzed at a concentration of 500 μg/ml against 50 mM Tris-HCl, pH 8.5, were treated with 500 μg/ml of the cross-linking reagent dimethyl suberimidate at 25°C overnight and then boiled in 1% SDS for 10 min before electrophoresis. The migrations of reference proteins in replicate gels (1. α-lactalbumin, 14,400 mol wt; 2. soybean trypsin inhibitor, 20,100 mol wt; 3. carbonic anhydrase, 30,000 mol wt; 4. ovalbumin, 43,000 mol wt; 5. bovine serum albumin, 67,000 mol wt; 6. phosphorylase b, 94,000 mol wt) are indicated. Gels were stained with Coomassie Blue and scanned at 600 nm.

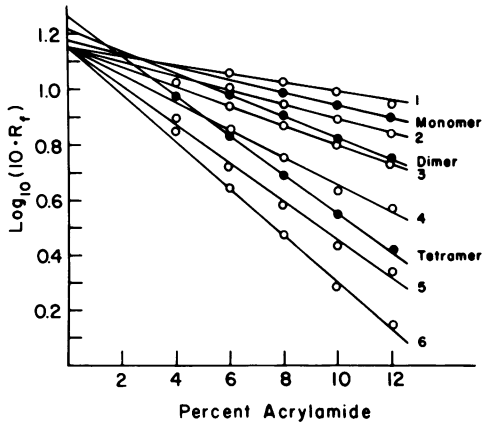

FIGURE 5 Ferguson plot of the electrophoretic migration of purified eosinophil arylsulfatase B and six reference proteins in 0.1% SDS with polyacrylamide gels of different acrylamide concentrations. R_f was determined from the distance of migration of bromophenol blue. The migrations of the monomeric, dimeric, trimeric (not shown), and tetrameric forms of eosinophil arylsulfatase are aberrantly fast relative to those of the six reference proteins (1. α-lactalbumin; 2. soybean trypsin inhibitor; 3. carbonic anhydrase; 4. ovalbumin; 5. bovine serum albumin; 6. phosphorylase b).

ble with the dissociation of the arylsulfatase as shown in Fig. 6B, dilution of purified enzyme was associated with an immediate decrease in specific activity and a further decline over the following hour. If dilution was made into buffer containing bovine serum albumin, no loss in specific activity accompanied dilution (Fig. 6A). In an additional experiment, concentrations of albumin in the diluent and assay mixture from 1 mg/ml to 62.5 μg/ml prevented the 56% loss of arylsulfatase activity occurring when a 20-μg portion of purified enzyme was diluted 1:4 in 0.5 M sodium acetate-acetic acid, pH 5.7. A concentration of albumin of 31.25 μg/ml was partially protective, limiting loss of activity to 20%, whereas 16 μg/ml was without effect.

To confirm that loss of enzymatic activity occurring with dilution was ascribable to dissociation of subunits and not to irreversible denaturation of diluted protein, 1.5 ml of purified arylsulfatase B was diluted to 6 ml with pH 5.7, 0.5 M sodium acetate-acetic acid buffer. 4 ml was neutralized and this diluted enzyme reconcentrated to 0.5 ml by adsorption to zinc-chelate Sepharose and elution with pH 5.0, 0.1 M sodium acetate-acetic acid buffer containing 100 mM EDTA. In 1-ml reaction mixtures, 1.5 μg of enzyme from the initial and diluted samples, and 2.5 μg from the reconcentrated sample were assayed. The specific activity of the undiluted starting material was 112 U/mg and declined to 48.9 U/mg after 3 h of dilution; neither value was affected by addition of EDTA to replicate

TABLE II
Amino Acid Composition of Human Eosinophil Arylsulfatase B

Amino acid	Amino acid residues/ 1,000 residues	Moles amino acid/ mole arylsulfatase B*
Asp	103.1	68
Thr	53.4	35
Ser	59.0	39
Glu	70.4	46
Pro	54.1	35
Gly	82.3	54
Ala	119.3	78
Val	85.3	56
Ileu	8.1	5
Leu	122.2	80
Tyr	17.0	11
Phe	51.6	34
His	60.5	40
Lys	81.0	53
Arg	25.4	17
Met	6.1	4

* Based on the tetrameric form of the enzyme.

samples. In contrast, reconcentration of the diluted sample after 3 h increased the specific activity to 178 U/mg.

DISCUSSION

Arylsulfatase B (5) from human eosinophil leukocytes (3, 19, 20) has been purified to homogeneity from the limited amounts of starting material available in the blood from a donor with a 90% eosinophilia. After an initial gel filtration step, eosinophil arylsulfatase B was bound to Affi-Gel Blue, as reported for arylsulfatase B from human urine, rat liver and rat and sheep brain (21), and was eluted with a linear salt gradient between 10 and 40 mS/cm (Fig. 1). The immobilized ligand on Affi-Gel Blue is an aromatic sulfonated blue dye, structurally analogous to the synthetic substrates of the enzyme. A final chromatographic step relied on the affinity of the arylsulfatase B for solid phase-chelated zinc ions and elution was effected by a decrease in pH to 5.0 (Fig. 2). An alternative final step using copper chelate chromatography required a further decrement in pH to about 3.5 for quantitative elution of the purified enzyme (data not shown). The requirement of such acidic conditions for elution from these metal-chelate resins implicates binding by histidine and/or cysteine residues in the enzyme (22), and the failure of the enzyme to bind to sulfhydryl-reactive organomercurial agarose (data not shown) suggests that the histidine residues of eosinophil arylsulfatase are principally responsible for the interaction with these metallic ions. The purification sequence (Table I) gave a 39% yield of enzyme with a specific activity of 156 U/mg. Five additional purifications yielded comparable results.

The purified arylsulfatase B was homogeneous by both physicochemical and immunochemical criteria. Analytic alkaline disc gel electrophoresis of 50 μg of enzyme demonstrated a single stained band of protein (Fig. 3A) but enzymatic activity was not recoverable from replicate alkaline gels. Immunization of both rabbits and goats with the purified protein elicited antisera that were monospecific in their immunoprecipitation reactions with the starting material, sonicated eosinophil-enriched leukocytes. Each antiserum also yielded a single immunoprecipitin line with purified enzyme that fused in a pattern of immunochemical identity with the precipitin line to sonicated eosinophils, thereby providing immunochemical evidence for the homogeneity of the purified enzyme.

SDS-polyacrylamide gels of purified arylsulfatase B dialyzed into mildly alkaline buffers and boiled in 1% SDS revealed a major peak of stained protein with several minor bands of higher molecular weights (Fig.

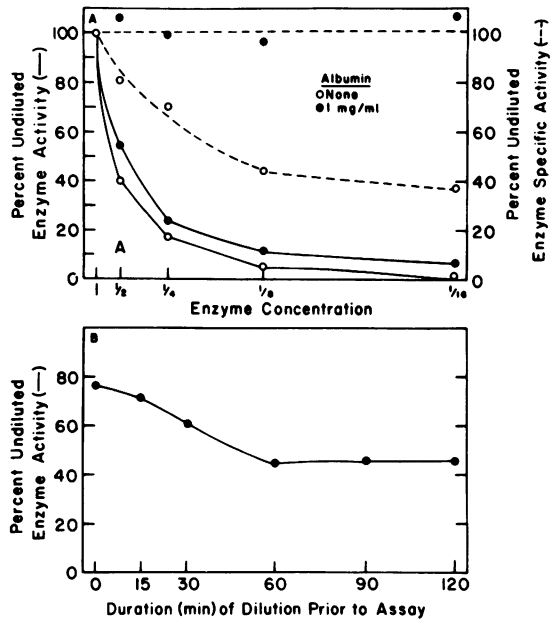

FIGURE 6 Effect of dilution and time of dilution on eosinophil arylsulfatase B. A. Purified eosinophil arylsulfatase B (10 μg) in 0.5 M sodium acetate-acetic acid buffer, pH 5.7, was diluted in the same buffer with (●) or without (○) 1 mg/ml bovine serum albumin for 5 min before assay of enzymatic activity for 15 min at 37°C in 1 ml of 10 mM NCS in the diluent. Net (——) and specific activities (- - -) are expressed relative to the activities of the undiluted enzyme. B. Purified eosinophil arylsulfatase B (7 μg) was diluted 1:20 in 0.5% sodium acetate-acetic acid, pH 5.7, at 25°C for varying durations before assay of enzymatic activity for 15 min at 37°C in 1 ml of 10 mM NCS in the same buffer. Activities are expressed relative to the activity of an amount of undiluted enzyme equal to the initial 1:20 dilution.

4A). That these higher molecular weight protein bands represented oligomeric forms of the enzyme was suggested by the increased intensity of these bands when the purified enzyme was maintained at an acidic pH before electrophoresis (Fig. 4B) or was incubated with the cross-linking agent dimethyl suberimidate at alkaline conditions (Fig. 4C). The finding of the same four protein peaks with chemical cross-linking over a range of 50 to 500 μg protein/ml favors a tetrameric structure for the enzyme. Because reduction and alkylation of arylsulfatase B does not alter the relative amounts of subunits present on SDS-polyacrylamide gel electrophoresis, their interactions are not due to disulfide bonding. Replicate analytical acid disc gel electrophoresis (Fig. 3B) demonstrated arylsulfatase activity to be coincident with the major of two Coomassie Blue staining protein bands. On analysis by SDS electrophoresis in the second dimension, this major protein yielded bands with relative migrations compatible with monomeric, dimeric, trimeric, and tetrameric forms of the enzyme, while the minor peak from acid gel electrophoresis yielded a single protein migrating as the monomeric form of the enzyme. Because alkaline conditions appear to favor dissociation of enzyme into its subunits, the single band on alkaline disc gels (Fig. 3A) may represent monomer, thereby accounting for the failure to elute measurable enzymatic activity from a replicate gel. Thus, all detectable protein on SDS gels and on acid and alkaline disc gels of purified arylsulfatase B appears to represent monomeric and oligomeric forms of the enzyme, and no contaminating proteins were recognized.

Eosinophil arylsulfatase B is a glycoprotein, as evidenced by its binding and saccharide specific elution from columns of immobilized concanavalin A, wheat germ agglutinin, and ricinus communis lectins (data not shown). Since the molecular weights of some glycoproteins are not necessarily accurately assessed by SDS-PAGE, the validity of the molecular weights of the arylsulfatase subunits estimated by SDS-PAGE were examined by Ferguson plots (Fig. 5). The monomeric and polymeric forms of arylsulfatase B were found to migrate with a free electrophoretic mobility (23) (Fig. 5) that increased progressively with the increasing polymerization of the subunits. This aberrantly rapid migration would lead to underestimates of the true molecular weights of the arylsulfatase oligomers and is contrary to the usual overestimation of molecular weights of glycoproteins on SDS-polyacrylamide gel electrophoresis (24). Aberrantly rapid mobility (25) has been recorded previously with proteins binding quantities of SDS in excess of 1.4 g SDS/g of protein (26), for ferridoxins due to the inherent charge of acidic residues (27), and for a few proteins without explanation (28) as is the situation with eosinophil arylsulfatase B. Apparent molecular weights by SDS-PAGE, as in Fig. 4, of ~15,000 for the monomer, 28,000 for the dimer, 42,000 for the trimer, and 60,000 for the tetramer, therefore, are underestimates of the molecular weights.

The number of residues per mole of tetrameric enzyme was calculated (Table II) with the assumption that each subunit contains a single methionine. If all of the four subunits were identical, the molecular weight would be 70,190 for the tetramer and 17,548 for the monomer. These estimates represent minimal molecular weights since cysteine and tryptophan were not assessed and no corrections were made for losses in serine and threonine residues with hydrolysis. Similarly, sugar residues were not included. Because the Ferguson plot analysis of the SDS gels indicated that the tetrameric molecular weight would be in excess of 60,000 (Fig. 4), these estimates of molecular weight by amino acid composition are in accord. The previous molecular weight estimate of eosinophil arylsulfatase B of 45,000 by gel filtration is similar to values found by gel filtration for arylsulfatase B from ox liver (45,000–47,000) (9) and human liver (47,000–54,000) (7, 8). Equilibrium sedimentation assessments of molecular weights for these enzymes were in the range of 55,300 to 61,000 (7–9). Recognition of the subunit interactions of arylsulfatase B would provide an explanation for these molecular weight determinations since rapid equilibrium between dimeric and tetrameric species on equilibrium sedimentation or gel filtration might account for molecular weight determinations intermediate between those of tetrameric and dimeric molecular species (29). Human placental arylsulfatase B has been resolved into fractions with reported molecular weights of 71,500, 60,000, and 48,000 (30). Smaller molecular weight forms of human liver arylsulfatase B, an enzyme whose cold lability is a feature of some oligomerizing enzymes (31), tentatively have been recognized after cold treatment (32). Thus, a polymeric structure may be present in arylsulfatase B enzymes of other tissues similar to the tetrameric structure of human eosinophil arylsulfatase B.

The enzymatic behavior of eosinophil arylsulfatase is compatible with an associating-dissociating enzyme. The decrease in enzymatic activity with duration of assay, which is not attributable to substrate depletion or product inhibition, and the decrease in specific activity with dilution of enzyme concentration (Fig. 6) are compatible with dissociation of the enzyme into subunit forms having lesser specific activity than the more polymeric form(s) (31–33). The addition of exogenous bovine serum albumin did not augment enzyme activity, but prevented the decreases in specific activity that occurred upon dilution of the enzyme (31). That the loss of activity with dilution was not

attributable to denaturation of the diluted protein was established by reconcentrating diluted enzyme to a specific activity of 178 U/mg, which was 1.5-fold the original undiluted enzyme and 3.5-fold that of the diluted enzyme. These findings are compatible with concentration-dependent reassociation of subunits into polymeric forms with greater enzymatic activity.

The dependence of enzymatic activity on the state of association of subunits contributes to some of the variability found with the six purifications of the enzyme. For instance, the results of the above concentration experiment would increase the yield of this preparation from 42 to 63%, and the purification factor from 85- to 126-fold. Since the specific activity of eosinophil arylsulfatase B is dependent on the concentration of the enzyme, comparisons with published specific activities of other purified forms of the enzyme require consideration of protein concentrations. The specific activity for NCS of 156 U/mg, at an assay concentration of 127 μg/ml (Table I), compares with the values of 244.2 for human liver (7) at 350 μg/ml, whereas 5,598 U/mg (8) were reported at an unstated concentration for human liver arylsulfatase B and 5,820 for ox brain arylsulfatase B at 9,100 μg/ml (32).

The factors recognized as governing subunit interactions of arylsulfatase B, an eosinophil lysosomal hydrolase (6), provide mechanisms for regulating its enzymatic function. Thus, the local concentration of enzyme and acidic pH of lysosomes would favor subunit association and augmented specific activity, whereas the dilution of the enzyme and neutral pH encountered with release of the enzyme into the extracellular milieu would favor dissociation of subunits and a decrease in functional activity.

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