Purification and Kinetic and Physical Characterization of Bovine Ascorbate-2-sulfate Sulfohydrolase†

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ABSTRACT: Ascorbate-2-sulfate sulfohydrolase (ascorbate sulfatase) has been purified 70 000-fold to homogeneity from bovine liver. The purification procedure consists of pH fractionation, ammonium sulfate fractionation, hydroxylapatite column chromatography, Sephadex A-50 column chromatography, and preparative ultracentrifugation. Gel electrophoresis and sieving columns show that the aggregation state of the enzyme is pH dependent. Arylsulfatase and ascorbate sulfatase activities of the homogeneous enzyme show the same banding pattern on gel electrophoresis. Gel electrophoresis in the presence of sodium dodecyl sulfate and 8 M urea shows that the enzyme is comprised of two protein species of 59 000 and 52 000 molecular weight. Both of these bands stain positively for glycoprotein. The enzyme also binds to concanavalin A-Sepharose. The most powerful inhibitor of ascorbate-2-sulfate sulfohydrolase is ascorbate 2-phosphate, followed by inorganic phosphate and the nucleotide triphosphates. Ascorbate 2-sulfate hydrolysis is competitively inhibited by ascorbate 2-phosphate, Na_2HPO_4, and *p*-nitrocatechol sulfate with K_i values of 0.5 μM, 3.3 μM, and 0.1 mM. The K_m for ascorbate 2-sulfate changes from 13 mM to 2.9 mM upon freezing and thawing the enzyme and then to 0.5 mM upon removal of an inactive protein. Partially purified ascorbate-2-sulfate sulfohydrolase is activated by $(NH_4)_2SO_4$, while the highly purified enzyme is competitively inhibited by sulfate with a K_i of 0.04 mM. The pH optimum of partially purified ascorbate-2-sulfate sulfohydrolase is 5.4; pure enzyme, 4.8. The K_m and V_{max} values for isoascorbate 2-sulfate hydrolysis are lower than values for ascorbate 2-sulfate hydrolysis. Nitrocatechol sulfate hydrolysis by ascorbate-2-sulfate sulfohydrolase is competitively inhibited by ascorbate 2-phosphate and ascorbate 2-sulfate with K_i values of 1.8 μM and 3.8 mM, respectively. The apparent K_m for nitrocatechol sulfate hydrolysis is 0.5 mM. Both ascorbate 2-sulfate and nitrocatechol sulfate hydrolyses are substrate inhibited. It is suggested that the enzyme may have multiple unequivalent binding sites for these substrates.

Ascorbate 2-sulfate was discovered in brine shrimp cysts in 1969 (Mead and Finamore, 1969). Soon after its discovery improved procedures were developed for synthesizing ascorbate 2-sulfate salts and for quantitating it from biological tissues (March, 1972; Tolbert et al., 1975). Ascorbate 2-sulfate appears to be a ubiquitous substance in animals found in the urine or tissue of every species tested, including man (Mead and Finamore, 1969; March, 1972; Halver et al., 1975; Baker et al., 1971, 1975). The wide distribution of ascorbate 2-sulfate in animals suggests that this compound may play a role in ascorbic acid biochemistry. Ascorbate 2-sulfate is as effective as ascorbic acid in relieving scurvy in trout (Halver et al., 1975). Ascorbate 2-sulfate appears to be converted to ascorbic acid in vivo and ascorbic acid is converted to ascorbate 2-sulfate in primates (Baker et al., 1975). Therefore the enzymatic hydrolysis of ascorbate 2-sulfate to ascorbic acid should be a nutritionally important step in certain biological systems. Experiments were undertaken to isolate and characterize the enzyme activity which carries out the hydrolysis of ascorbate 2-sulfate to ascorbate.

Preliminary results have shown that a crude bovine liver arylsulfatase A (EC 3.1.6.1) preparation hydrolyzes ascorbate 2-sulfate to ascorbate (Bullen, 1972). This enzyme activity was named ascorbate-2-sulfate sulfohydrolase (ascorbate sulfatase). It has been shown that ascorbate sulfatase activity is present in many tissues from several species of animals (Knight, 1974). More recently, the partial copurification and characterization of ascorbate sulfatase and arylsulfatase from the liver of a marine gastropod, *Charonia lampas,* have been reported (Hatanaka et al., 1975a). The two enzymes responded differently to certain inhibitors (Hatanaka et al., 1975b,c). Ascorbate 2-sulfate is also a substrate for pure arylsulfatase A (Roy, 1975). Partial purification from bovine liver, using ammonium sulfate reverse phase column chromatography, has shown that arylsulfatase A and ascorbate sulfatase activities copurify (Carlson, 1974). However, the ratio of their specific activities changes with purification and the activities of this enzyme preparation respond differently to 1 mM ammonium sulfate. Ascorbate sulfatase activity for partly purified enzyme is activated 5-fold, while arylsulfatase activity is inhibited (Carlson, 1974). Ascorbate sulfatase purified 40 000-fold has been shown to be powerfully inhibited by phosphate and ascorbate 2-phosphate (Carlson et al., 1976). These results raised some questions: Is there a specific ascorbate sulfatase which can be separated from arylsulfatase? What are its properties? To answer these questions bovine ascorbate sulfatase was purified to homogeneity using procedures which are specific for isolating an ascorbate 2-sulfate hydrolyzing enzyme rather than procedures based on purifying arylsulfatase A. Physical and kinetic characteristics of this enzyme were determined.

Materials and Methods

Materials. Dipotassium L-ascorbate 2-sulfate was prepared in our laboratory by previously described methods (Tolbert et al., 1975). Trisodium L-ascorbate 2-phosphate was prepared by methods generously supplied by Dr. Paul Seib of Kansas State University, Manhattan, Kansas. Nucleotides were purchased from Calbiochem and Sigma Chemical Co. D-Glucose 6-phosphate, D-fructose 6-phosphate, glucose 6-sul-

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fate, and chondroitin sulfate were purchased from Sigma Chemical Co.

Ascorbate Sulfatase Assay. The assay consists of following the reduction of 2,6-dichloroindophenol (DCIP)[1] by the ascorbic acid produced from the enzymic hydrolysis of ascorbate 2-sulfate. On reduction DCIP changes from a blue to a colorless molecule. The decrease in absorbance is followed at 516 nm, the isosbestic point. The molar extinction coefficient of the potassium salt of DCIP at 516 nm is 9300. Two and one-half milliliters of a 0.15 mM DCIP solution in 0.1 M acetate buffer (pH 4.8) was added to a 4-mL cuvette. To the cuvette was added 0.5 mL of 40 mM dipotassium ascorbate 2-sulfate. The decrease in absorbance with time was measured before adding the enzyme since there is a slight acid-catalyzed ascorbate 2-sulfate hydrolysis at pH 4.8. Up to 0.2 mL of the enzyme fraction was added and the decrease in absorbance at 516 nm was followed using a recording spectrophotometer. No decrease in absorbance was detected in the presence of enzyme without ascorbate 2-sulfate. Enzyme fractions boiled prior to assay show no measurable activity.

The ascorbate sulfatase assay procedure in crude homogenates requires some modification since there is a low concentration of enzyme, a great deal of protein precipitation and nonspecific reduction of the DCIP. Two and one-half milliliters of the same DCIP solution were added to 0.05 or 0.10 mL of crude dialyzed homogenate. This solution was allowed to stand for 10 to 15 min and was then centrifuged. The supernatant was poured into a 3-mL cuvette. Substrate, 0.5 mL, was added and the decrease in absorbance at 516 nm was followed. The control reactions without the addition of substrate and the nonenzyme hydrolysis of ascorbate 2-sulfate were subtracted from the experimental assay. During purification all assays were done using enzyme samples which had been dialyzed against cold 5 mM Tris base, pH 7.0.

For kinetic studies it was necessary to maintain a constant ionic strength and therefore the assay was modified as follows. One and one-half milliliter of 0.15 mM 2,6-dichloroindophenol in 0.05 M KAc (pH 4.8) was placed in a 3-mL cuvette. To this was added an appropriate amount of 100 mM dipotassium ascorbate 2-sulfate, 0.5 M KAc (pH 4.8), and water to give a total volume of 2 mL and a final ionic strength of 0.1. The rate of reaction at each substrate concentration was measured several times. Velocities were calculated during the first 6 to 10 min of hydrolysis. The rate of ascorbate 2-sulfate hydrolysis is calculated from a line drawn tangent to the initial progress curve. In most cases the hydrolysis rate did not change appreciably during the first 6 to 10 min. The assay is continuous and linear with enzyme concentration up to reaction rates giving 7.0 nmol ascorbic acid/min. One unit of activity is defined as that amount of enzyme which will hydrolyze 1 μmol of ascorbate 2-sulfate per min. Typical changes in absorbance would be from a change of 0.02 absorbance unit/5 min to about 0.2 absorbance unit/5 min.

Arylsulfatase Assay. The procedure is similar to a previously described procedure (Nichol and Roy, 1964). Up to 0.2 mL of the enzyme fraction was added to 5 mL of a solution of 3.0 mM *p*-nitrocatechol sulfate in 0.1 M acetate buffer at pH 4.9. This solution was incubated at 37 °C for 1 h. One-half milliliter of this solution was placed in 5.0 mL of 0.2 N NaOH and the absorbance at 510 nm was measured. The molar extinction coefficient of the nitrocatechol anion is 12 500. A control was run without enzyme, and no detectable amount of the nitrocatechol anion was observed. This assay is point by point and is not linear with enzyme concentration. The enzyme concentration of each assay was adjusted so that the 510-nm absorbance was maintained at about 0.12.

For kinetic studies it was necessary to modify the assay procedure in order to maintain a constant ionic strength and to obtain reaction rates during the first 10 min of hydrolysis. Solutions of 50 mM dipotassium nitrocatechol sulfate and 0.5 M acetate buffer made pH 4.8 were prepared. Using these solutions and distilled water, the desired nitrocatechol sulfate concentrations were prepared by dilution in test tubes and maintained at a final ionic strength of 0.1. The total volume at each substrate concentration was 1 mL. Fifty microliters of the enzyme preparation was added and the reaction mixture was incubated for 10 min at 37 °C. Three milliliters of 0.2 N NaOH was then added and the absorbance at 510 nm was measured. Several determinations were made at each substrate concentration. When determining the pH optimum, the pH was adjusted using acetic acid or KOH. The substrate concentration during the pH optimum determination was 3 mM. Nitrocatechol sulfate hydrolysis progress curves show that the rate of hydrolysis decreases significantly during the 10-min incubation time. Thus a K_m for nitrocatechol sulfate was also determined by using initial velocities calculated from progress curves according to the method of Stinshoff (1972). The ionic strength, pH, and temperature of the assay were as described above.

Calculation of the K_m and K_i Values. K_m and K_i values were calculated from the $1/s$ intercept of Lineweaver-Burk plots. Each K_m and K_i value was computed from a linear regression fit of the data points. For each K_m value, three to four determinations were made and the average value and standard deviation from the average were computed. For K_i values, three to four determinations were made at each of two inhibitor concentrations and the average value and standard deviation from the average computed.

Protein Determination. Protein concentrations were determined by the method of Schacterle and Pollack using bovine serum albumin as the standard (Schacterle and Pollack, 1973).

Ascorbate Sulfatase Purification Procedure. Twenty kilograms of fresh bovine liver was obtained, stored frozen, and processed within 2 weeks. All steps in the purification procedure are done at about 5 °C.

Step 1: pH Fractionation. One kilogram of liver was homogenized in 1800 mL of cold distilled water. The homogenate was adjusted to pH 5.9 using 2 M acetic acid and centrifuged at 13 000*g* for 45 min. The supernatant was adjusted to pH 4.7 using 2 M acetic acid and centrifuged at 13 000*g* for 15 to 20 min. The sediment was suspended in 500 mL of cold distilled water and adjusted to pH 7.0 with 0.2 N NaOH. Four kilograms of liver was processed and the combined solutions were stirred for 30 min after the last solution was added. Time for processing 4 kg of liver to this point was about 2 h.

Step 2: Ammonium Sulfate Fractionation. The above solution was made 20% ammonium sulfate (114 g/L), centrifuged at 13 000*g* for 30 min and the precipitate was discarded. The ammonium sulfate concentration of the supernatant was increased to 55% (225 g/L) and allowed to stand overnight. The solution was centrifuged at 13 000*g* for 20 min and the supernatant was discarded. The sediment was collected and stored in the freezer. Twenty kilograms of liver was processed through this step before going on. The enzyme is stable indefinitely in ammonium sulfate solution, or suspension.

Step 3: Hydroxylapatite Column Chromatography. The precipitate from 20 kg of liver was dissolved in 1 L of distilled

[1] Abbreviations used: DCIP, 2,6-dichloroindophenol; Tris, tris(hydroxymethyl)aminomethane; AcO, acetate; EDTA, ethylenediaminetetraacetic acid.

water and dialyzed overnight against 55 L of 5 mM Tris base adjusted to pH 7.0 with HCl. After dialysis the solution was centrifuged at 20 000*g* for 1 h to remove any sediment. Two hundred grams of Bio-Rad HTP hydroxylapatite was prepared according to Bio-Rad instructions in 0.1 M acetate buffer (pH 5.0). A 3.5 × 57 cm column was prepared and washed with about 1.5 L of 0.1 M acetate buffer (pH 5.0). One-third of the enzyme preparation was applied to this column. The column was washed with 0.1 M acetate buffer (pH 5.0) until the effluent showed negligible absorbance at 280 nm. This effluent had no measurable ascorbate sulfatase activity. The enzyme activity was eluted from the column with 0.3 M ammonium sulfate in 0.1 M acetate buffer, pH 5.0. The column was stripped of protein with 0.4 M phosphate adjusted to pH 5.4 with acetic acid. Elution fractions, 50 mL each, were dialyzed against cold 5 mM Tris (pH 7.0) and assayed. Protein concentration was monitored by following the 280-nm absorbance. After each column run, the hydroxylapatite was removed from the column, washed with several volumes of 0.4 M phosphate, and then the column was repacked.

Step 4: Sephadex A-50 Column Chromatography. The ascorbate sulfatase activity from the hydroxylapatite columns was combined and concentrated by ultrafiltration from a volume of 1.8 L to 108 mL. The concentrated solution was made 0.1 M acetate (pH 5.0) using 1.0 M acetate buffer (pH 5.0). The enzyme solution was applied to a 2.5 × 55 cm Sephadex A-50 column which had been equilibrated with 0.1 M acetate buffer (pH 5.0). The enzyme was eluted with 1 L of a NaCl gradient, 0 to 0.4 M NaCl, prepared in 0.1 M acetate buffer (pH 5.0).

Step 5: A Second Hydroxylapatite Column. A small hydroxylapatite column with a bed volume of 10 mL was prepared as described above. The enzyme solution was dialyzed against 25 L of cold 5 mM Tris (pH 7.0) and applied to the column. The column was washed with 50 mL of 0.1 M acetate buffer (pH 5.0). The enzyme was eluted with 40 mL of 0 to 0.4 M ammonium sulfate gradient in 0.1 M acetate buffer (pH 5.0). Tenfold dilutions of each fraction were assayed for enzyme activity. Figure 1 shows the elution profile of a typical hydroxylapatite column. The ascorbate sulfatase activity was pooled and dialyzed against cold 5 mM Tris (pH 7.0). Gel electrophoresis of the protein at this point showed that there was a heavy protein species present in addition to the enzyme band.

Step 6: Preparative Ultracentrifugation. The enzyme solution was concentrated to 3 mL by ultrafiltration and layered on two 5–20% sucrose gradients prepared in 0.375 M Tris buffer (pH 8.8). The tubes were centrifuged at 24 000*g* for 40 h and punctured, and 1-mL fractions were collected. Gel electrophoresis of the fractions showed that the heavy protein separated from the enzyme.

Some preliminary data reported here were obtained using enzyme which had been partially purified using the procedure of Nichol and Roy through the acetone cut (Nichol and Roy, 1964), or by ammonium sulfate reverse phase solubility chromatography (Carlson, 1974). Ammonium sulfate reverse-phase solubility chromatography has been used in the purification of arylsulfatase A from human urine (Stevens et al., 1975).

Gel Electrophoresis Procedures: General Procedures. Gel electrophoresis was carried out using a Tris-glycine system (Ornstein, 1964; Laemmli, 1970) and an Ortec 4100 pulsed power supply. All the gels were slab gels 1 mm thick × 20 cm long.

Staining Procedures. The gels were stained for protein using Coomassie Brilliant Blue R, purchased from Sigma Chemical Co. (Fairbanks et al., 1971). The gels were stained for glycoprotein using Alcian Blue, purchased from Kodak (Wardi and Michos, 1972). Gels were stained for arylsulfatase activity by placing the gel in a solution of 3 mM nitrocatechol sulfate, which was 0.1 M acetate (pH 4.9), for 30 min at 37 °C. The gel was removed from the nitrocatechol sulfate solution and placed in 0.2 N NaOH. A bright red band, due to the nitrocatechol anion, appears where the enzyme is located and quickly diffuses. Gels were stained for ascorbate sulfatase activity by placing them in a 10 mM solution of dipotassium ascorbate 2-sulfate, which was 0.1 M acetate (pH 4.8), for 30 to 45 min at room temperature. Then the gel was placed in 0.5 M $AgNO_3$ in the dark. A metallic silver band appears at the location of the enzyme and does not diffuse. The gel can be placed in distilled water to remove excess $AgNO_3$.

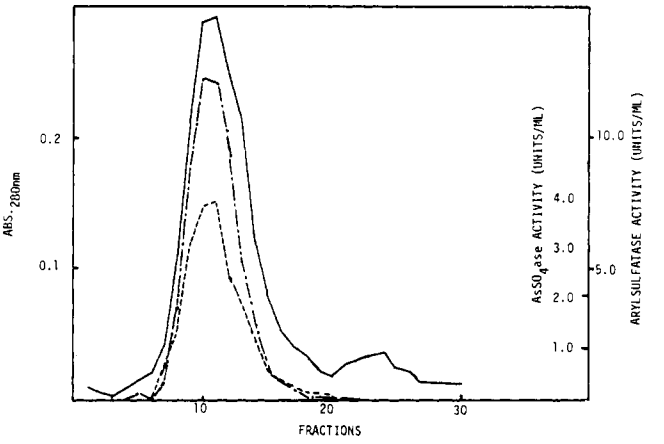

FIGURE 1: The elution pattern of the second hydroxylapatite column. Each fraction has a volume of 3 mL. (—) The 280-nm absorbance; (- - -) ascorbate sulfatase; (- · · · -) arylsulfatase.

Results

The results of the purification procedure are summarized in Table I. The liver homogenate has a large amount of arylsulfatase activity relative to the ascorbate sulfatase activity. This is reflected in the low ratio of ascorbate sulfatase to arylsulfatase specific activities. After the pH fractionation, there is a large increase in this ratio. These data suggest that pH fractionation enriches sulfatase enzymes which hydrolyze ascorbate 2-sulfate. The supernatant from the pH 4.7 precipitation step contains arylsulfatase activity but has no measurable ascorbate sulfatase activity. Homogeneous bovine ascorbate sulfatase has arylsulfatase activity. Table I also shows an increase in yield of both activities after ammonium sulfate fractionation. The reason for the effect is not known, but was observed repeatedly. The 70 000-fold purification necessary to obtain homogeneous ascorbate sulfatase shows that levels of this enzyme in liver are quite low.

Gel electrophoresis of ascorbate sulfatase shows that both ascorbate sulfatase and arylsulfatase activities have identical protein and activity banding patterns. This pattern consists of a major sharp band located between the bovine serum albumin dimer and monomer bands followed by a light streaking pattern. This streaking pattern was seen repeatedly. It was noted that, when the enzyme preparation was exposed to pH 8.3 or pH 8.8 conditions for 2 to 3 days, this banding pattern shifted so that the streaking portion of the pattern became more dominant. This streaking pattern can be seen in the first dimension of the two-dimensional gel shown in Figure 2. This suggested that perhaps there was pH-dependent aggregation of the enzyme. Subsequent gels have shown that the lower

TABLE I: Ascorbate Sulfatase Purification.

	Units (μmol/min)			Spec Act.		Purification		
	Aryl-sulfatase	Ascorbate sulfatase	Protein (mg)	Aryl-sulfatase	Ascorbate sulfatase	Aryl-sulfatase	Ascorbate sulfatase	Ratio[a]
Crude liver homogenate	4400[b]	525[b]	2.3×10^{6}[b]	0.0019	0.00023			0.12
Step 1: pH fractionation	690	480	2.7×10^{5}	0.0026	0.0018	1.4	7.8	0.69
Step 2: ammonium sulfate fractionation	1330	745	4.0×10^{4}	0.034	0.019	18	83	0.56
Step 3: hydroxylapatite column chromatography	306	400	100	3.1	4.0	1630	17 400	1.3
Step 4: Sephadex A-50 column chromatography	230	390	55	4.2	7.1	2200	31 000	1.7
Step 5: hydroxylapatite column chromatography	150	150	15	7.1–10.0[c]	8.2–10.0[c]	3700–5300[c]	36 000–45 000[c]	1.0
Step 6: preparative ultracentrifugation	31	40	3	11–13[c]	13–16[c]	5800–6800[c]	57 000–70 000[c]	1.2

[a] The ratio is the ascorbate sulfatase to arylsulfatase specific activity ratio. [b] These values are average values determined from several 1-kg crude liver homogenates. The values are then extrapolated to the values for 20 kg of liver. [c] The two values are representative of two 20-kg liver preparations.

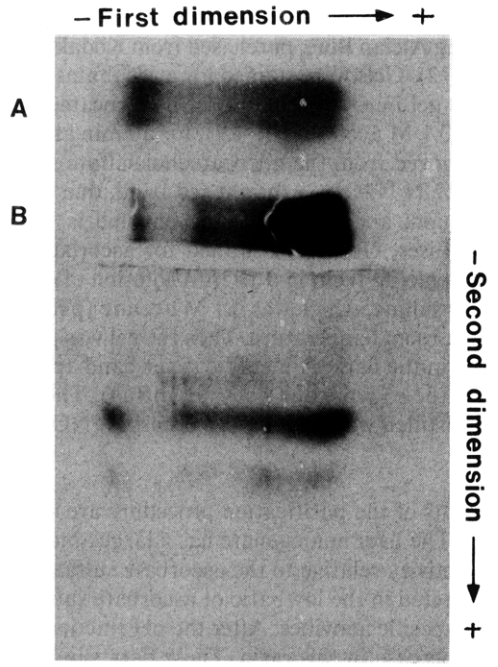

FIGURE 2: Two-dimensional gel electrophoresis of homogeneous bovine ascorbate sulfatase. The conditions for the first dimension are as follows. The stacking gel was 5% acrylamide in 0.375 M Tris buffer which is pH 8.8. The running gel was 10% acrylamide at pH 8.8. One hundred volts at 100 PPS was applied to the gel for 18.5 h. The initial current was 20 mA and the final current was 15 mA. Section A was stained for ascorbate sulfatase activity. Section B was stained for protein. A third section was electrophoresed in the second dimension. Each section contains 50 μg of ascorbate sulfatase. The second dimension was sodium dodecyl sulfate/8 M urea gel in Tris–glycine. The third section of the first dimension was placed in a solution of 0.125 M Tris (pH 6.8), 2% β-mercaptoethanol, 8 M urea, and 2% sodium dodecyl sulfate at 37 °C for 2 h. This section was placed on top of a running gel of 10% acrylamide prepared in 0.1% sodium dodecyl sulfate, 8 M urea, and 0.325 M Tris-HCl (pH 8.8). A 10% acrylamide capping gel prepared in 0.1% sodium dodecyl sulfate, 8 M urea, and 0.125 M Tris-HCl (pH 6.8) was poured on top of the 10% running gel. One hundred volts at 100 PPS was applied to the gel for 18 h. The initial current was 20 mA and the final current was 10 mA. The gel was stained for protein.

streaking band becomes the dominant band when the pH of the stacking gel is 8.8 and that the upper band is dominant when the stacking gel is pH 6.8. Only the upper band is present when gel electrophoresis is done in which the gel is pH 7.2 throughout. These gels are prepared and electrophoresed in 0.15 M Tris (pH 7.2). These results indicate that there is a shift in the aggregation state of the enzyme which is dependent upon pH. A sizing column, using Bio-Gel P-300 in 25 mM Tris (pH 8.0) and impure enzyme, gives a molecular weight for ascorbate sulfatase of 138 000. Another sizing column, using Agarose A-1.5m in 0.1 M acetate (pH 5.4) buffer and impure enzyme, gives a molecular weight of about 300 000 for ascorbate sulfatase. Both sizing column and gel electrophoresis experiments indicate that ascorbate sulfatase changes aggregation states depending upon pH. The kinetics responsible for the shift in aggregation state was not investigated further.

Gels of ascorbate sulfatase, run in the Tris–glycine system in the presence of 0.1% sodium dodecyl sulfate and 8 M urea, show the presence of two protein bands. These two protein bands have relative mobilities corresponding to molecular weights of 59 000 and 52 000. The 59 000 molecular weight band is more highly stained than the 52 000 molecular weight band. Both bands stain positively for glycoproteins. To determine whether one of these two protein bands could be an impurity, a two-dimensional gel was run. The first dimension was run under nondenaturing conditions where the pH of the stacking gel and running gel was 8.8. The second dimension was run under denaturing conditions. This gel, pictured in Figure 2, shows that the two bands seen under denaturing conditions are everywhere associated with the enzyme activity and the protein banding patterns observed in the first dimension. These results indicate that the ascorbate sulfatase activity is homogeneous even though it consists of two closely associated protein species.

The pH optimum of homogeneous ascorbate sulfatase is 4.8 at ionic strengths of 0.1 and 0.2. The pH optimum of ascorbate sulfatase partially purified through the acetone cut using the procedure of Nichol and Roy is 5.4 (Nichol and Roy, 1964). The pH optimum of the arylsulfatase activity of homogeneous bovine ascorbate sulfatase is pH 5.6 to pH 5.7.

Table II shows the effect of several compounds on ascorbate sulfatase activity. There appears to be a slight activation by $Mg(AcO)_2$, $Mn(AcO)_2$, and $ZnCl_2$. There is a slight inhibition by NaCl. Ascorbate 2-phosphate is the most powerful inhibitor followed by Na_2HPO_4 and then by the nucleotide triphosphates. It is possible that the nucleotide triphosphate inhibition is due, in part, to the presence of some inorganic phosphate. However, ascorbate 2-phosphate contains less than 0.1% inorganic phosphate. Glucose 6-sulfate and chondroitin sulfate are very poor inhibitors which suggests that they probably are poor substrates. EDTA does not affect ascorbate sulfatase indicating that divalent metal ions are not necessary for en-

TABLE II: The Effect of Various Compounds on Ascorbate Sulfatase Activity.

Reagent (1 mM)	% inhibition (% activation)
Na_2HPO_4	99
GTP	98
CTP	94
ATP	88
ADP	68
UMP	18
CMP	6
D-Glucose-6-PO_4	65
D-Fructose-6-PO_4	92
Ascorbate-2-PO_4	100
Na_2SO_4	77
Na_2AsO_3	63
Glucose-6-SO_4	0
Chondroitin-SO_4 (1 mg/mL)	6
Galactose	0
NaCl	4
LiCl	0
NH_4AcO	0
$Mg(AcO)_2$	(8)
$Mn(AcO)_2$	(13)
$ZnCl_2$	(6)
EDTA	0

zyme activity. Similar effects have been reported for the inhibition by sugar phosphates and by nucleotides of ascorbate sulfatase partially purified from the marine gastropod *Charonia lampas* (Hatanaka et al., 1975b). Bovine ascorbate sulfatase does not hydrolyze ascorbate 2-phosphate; however, *E. coli* alkaline phosphatase does hydrolyze ascorbate 2-phosphate (unpublished data). Phosphate inhibition of other sulfatase activities has not been studied in any detail.

The kinetics of ascorbate sulfatase depend upon ionic strength. At an ionic strength of 0.2 there is apparent substrate inhibition beginning at 4 to 5 mM ascorbate 2-sulfate. At ionic strength 0.1 substrate inhibition is not observed. At an ionic strength of 0.1 the arylsulfatase activity of pure ascorbate sulfatase is also substrate inhibited. This can be seen in Figure 3 where the Lineweaver–Burk plot results in an upward curving line near the $1/v$ intercept.

The kinetic properties of ascorbate sulfatase are summarized in Table III. The third and fourth columns of Table III compare the hydrolysis of ascorbate 2-sulfate with isoascorbate 2-sulfate. The enzyme preparation and the assay conditions are given in Table III. Ascorbate 2-phosphate competitively inhibits the hydrolysis of isoascorbate 2-sulfate and ascorbate 2-sulfate with a K_i of about 0.4 μM. The type of inhibition by inorganic phosphate is not clearly competitive or noncompetitive for either substrate using this enzyme preparation; Na_2HPO_4 inhibition of the pure enzyme is competitive with a K_i of 3.3 μM as recorded in the first column of Table III. The K_m for isoascorbate 2-sulfate hydrolysis is about 13-fold lower than the K_m for ascorbate 2-sulfate. Not given in Table III are data which show that the V_{max} for isoascorbate 2-sulfate hydrolysis is 16-fold lower than the V_{max} for ascorbate 2-sulfate hydrolysis. The K_m for ascorbate 2-sulfate after freezing and thawing this enzyme preparation is 2.9 mM. Ascorbate 2-sulfate hydrolysis is competitively inhibited by inorganic sulfate with a K_i of 0.04 mM.

The first and second columns of Table III compare ascorbate 2-sulfate hydrolysis with nitrocatechol sulfate hydrolysis. These studies were done using the homogeneous enzyme. The assay conditions are given above and stated briefly in Table III. Inhibition is competitive for all cases except for phosphate inhibition of the arylsulfatase activity. The Lineweaver–Burk plot for phosphate inhibition of the arylsulfatase activity resulted in upward curving lines, as shown in Figure 3. K_m and K_i values given in Table III are significantly higher for arylsulfatase activity than for the ascorbate sulfatase activity. The K_m for nitrocatechol sulfate calculated from initial velocities (Stinshoff, 1972) is 0.32 mM ± 0.02 mM which is not significantly different from the K_m of 0.5 mM ± 0.3 mM (see Table III) which was calculated from 10-min velocities.

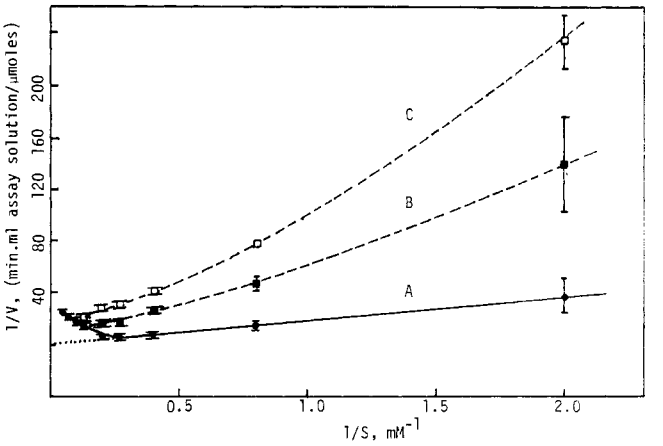

FIGURE 3: A Lineweaver–Burk plot of the arylsulfatase activity of ascorbate sulfatase and its inhibition by phosphate. The protein concentration of each assay is 0.37 μg/mL. The temperature is 37 °C and the ionic strength is 0.1. (A) Without phosphate; (B) with 25 μM Na_2HPO_4; and (C) with 50 μM Na_2HPO_4.

Discussion

The major purification step of ascorbate sulfatase is hydroxylapatite column chromatography. It was discovered earlier that ascorbate sulfatase is powerfully inhibited by inorganic phosphate (Carlson et al., 1976). Several methods of purification were attempted based on this inhibition. Hydroxylapatite was chosen since it is an inorganic phosphate material. The enzyme bound to the hydroxylapatite and could be removed with low phosphate concentrations (10 mM) or with 0.3 M ammonium sulfate. Under these conditions very little of the protein is removed from the column but most of the enzyme activity is removed. Ammonium sulfate was chosen to elute the enzyme because it is a less powerful inhibitor than inorganic phosphate and therefore facilitates the assaying of the fractions. The second hydroxylapatite column (step 5) results in very little purification, gives a poor yield, and is probably an unnecessary step.

pH fractionation enriches sulfatase enzymes which hydrolyze both ascorbate 2-sulfate and nitrocatechol sulfate. The literature supports the idea that pH fractionation may remove arylsulfatase activity which does not hydrolyze ascorbate 2-sulfate. Ascorbate 2-sulfate is a very poor substrate for arylsulfatase B, while arylsulfatase A readily hydrolyzes this molecule (Roy, 1975). At pH 4.7 bovine arylsulfatase A precipitates while arylsulfatase B remains in solution (Nichol and Roy, 1964; Allen and Roy, 1968). Thus it is possible that sulfatase enzymes which hydrolyze ascorbate 2-sulfate are separated from sulfatase enzymes which do not hydrolyze ascorbate 2-sulfate during the pH fractionation.

A purification procedure based on concanavalin A-Sepharose affinity chromatography was attempted (unpublished data). Arylsulfatase from sheep brain has been purified using this method (Bishayee et al., 1973; Bishayee and Bachhawat, 1974). Ascorbate sulfatase binds to concanavalin A-Sepharose and could not be removed using a variety of eluents and con-

TABLE III: Kinetic Properties of Ascorbate-2-sulfate Sulfohydrolase.

Substrate or inhibitor	Ascorbate sulfatase act.,[a] ascorbate 2-sulfate hydrolysis	Arylsulfatase act.,[a] nitrocatechol sulfate hydrolysis	Ascorbate 2-sulfate[b] hydrolysis	Isoascorbate 2-sulfate[b] hydrolysis
NCS	$K_i = 0.09 \pm 0.01$ mM	$K_m = 0.50 \pm 0.3$ mM		
As-2-SO_4	$K_m = 1.2 \pm 0.2$ mM[c]	$K_i = 3.8 \pm 1.8$ mM	$K_m = 13 \pm 3$ mM[d]	
As-2-PO_4	$K_i = 0.5 \pm 0.2$ μM	$K_i = 1.8 \pm 0.5$ μM	$K_i = 0.4 \pm 0.4$ μM	$K_i = 0.4 \pm 0.1$ μM
Na_2HPO_4	$K_i = 3.3 \pm 0.9$ μM	$K_i = ?$[e]	$[Na_2HPO_4]$ at 50% inhibition is 8 μM[f]	$[Na_2HPO_4]$ at 50% inhibition is 8 μM[f]
Iso-As-2-SO_4				$K_m = 1.1 \pm 0.1$ mM
Na_2SO_4			$K_i = 0.04 \pm 0.01$ mM	

[a] These studies were done using pure enzyme. The conditions were: temperature 37 °C; ionic strength 0.1; pH 4.8. [b] These studies were done using enzyme purified through the second hydroxylapatite column. The conditions were: temperature 25 °C; ionic strength 0.1; and pH 4.8. [c] The K_m of the pure enzyme at a temperature of 25 °C, ionic strength 0.1, and pH 4.8 is 0.5 ± 0.1 mM. [d] The K_m of the enzyme at this stage of purification is 2.9 ± 1.1 mM after freezing and thawing. [e] The Lineweaver–Burk plot of phosphate inhibition of the arylsulfatase activity results in upward curving lines, and the K_i cannot be determined by this type of plot. [f] Inhibition is 50% at ascorbate 2-sulfate concentration of 4 to 6 mM. Inhibition is 50% at an isoascorbate 2-sulfate concentration of 0.9 mM.

ditions. It is not known whether the column destroyed the enzyme activity; however, concanavalin A, in concentrations up to 0.6 mg/mL, does not inhibit ascorbate sulfatase activity. The binding of the enzyme suggests that it is a glycoprotein.

Initially, it was hoped that there would be an ascorbate sulfatase enzyme which would prove to be distinct from arylsulfatase, and which could be separated from arylsulfatase activity during purification. However, arylsulfatase and ascorbate sulfatase activities copurify to homogeneity. Several enzymic properties of ascorbate sulfatase change during purification. These include a change from activation to inhibition of the enzyme by sulfate, changes in K_m values, and changes in the pH optimum. Therefore there is good reason to believe that the in vivo enzyme is different from the homogeneous enzyme. Arylsulfatase A, prepared by Nichol and Roy (1964), is probably also a modified enzyme and differences between ascorbate sulfatase and arylsulfatase A could rise from the fact that the homogeneous preparations are different. Whether ascorbate sulfatase and arylsulfatase A are the same enzyme or have a common origin is unknown. Similarities between the two enzymes are discussed below. The data do not rule out the possibility that there is an ascorbate sulfatase enzyme which does not have arylsulfatase activity. Such an enzyme would be difficult to purify because it would be masked by the enzyme purified here which has both ascorbate sulfatase and arylsulfatase activities.

Gel electrophoresis and sizing columns show that ascorbate sulfatase changes aggregation states depending upon pH. These gels also show that both ascorbate sulfatase and arylsulfatase activities are associated with the same protein species. pH dependent changes in aggregation states have been described for bovine arylsulfatase A. Ultracentrifugation studies show that arylsulfatase A changes from a molecular weight of 100 000–120 000 at pH 7.5 to a molecular weight of about 400 000 at pH 5.5 (Nichol and Roy, 1964, 1965).

Gels run under denaturing conditions, i.e., sodium dodecyl sulfate and 8 M urea, and two-dimensional gel electrophoresis shows that ascorbate sulfatase is pure and that it consists of two distinct protein species. These protein species consist of a dark staining Coomassie blue band with a molecular weight of 59 000 and a light staining Coomassie blue band with a molecular weight of 52 000. The difference in the intensity of staining is not understood. If the intensity of staining is proportional to the amount of each protein species, then the two proteins are not present in a reasonable ratio for the enzyme to be a simple two subunit enzyme. These data suggest that bovine ascorbate sulfatase is a complex enzyme physically. Ascorbate sulfatase has similar physical properties to arylsulfatase A.

Differences between the kinetic characteristics of nitrocatechol sulfate hydrolysis with those of ascorbate 2-sulfate hydrolysis shown in Table III suggest that the enzymic mechanism of ascorbate sulfatase is more complex than a simple one-site model mechanism. A simple one-site model would dictate that the K_m for ascorbate 2-sulfate equal the K_i value for ascorbate 2-sulfate inhibition of the arylsulfatase activity. They are significantly different. The K_i values for ascorbate 2-phosphate inhibition of both arylsulfatase and ascorbate sulfatase activities should be equal, but are not. The K_m for nitrocatechol sulfate should equal the K_i for nitrocatechol sulfate inhibition of the ascorbate sulfatase activity. Again, they are not equal. One explanation of these data could be that the enzyme has multiple unequivalent binding sites for both nitrocatechol sulfate and ascorbate 2-sulfate. Other data presented in this paper also suggest that the enzyme has multiple binding sites. The nonlinear Lineweaver–Burk plot for phosphate inhibition of the arylsulfatase activity is suggestive of multiple binding sites. Substrate inhibition by both ascorbate 2-sulfate and nitrocatechol sulfate suggests multiple binding sites for these molecules. Thus, ascorbate sulfatase is a complex enzyme kinetically as well as physically. Further work is necessary to understand the kinetic and physical complexity of this enzyme. Attention should be focused on the relationship of the two protein species which comprise ascorbate sulfatase and to the fact that this enzyme is a glycoprotein.

It is known that ascorbate 2-sulfate is converted to ascorbic acid in several biological systems (Halver et al., 1975; Baker et al., 1975; Knight, 1974). The most obvious role for ascorbate sulfatase would be to carry out this conversion. However, from the data presented in this paper, it is not so obvious that ascorbate sulfatase in its present state would hydrolyze ascorbate 2-sulfate in vivo. The best estimates of urine and tissue homogenate levels of ascorbate 2-sulfate in the rat (March, 1972) are much lower than the K_m value. In addition, physiological concentrations of phosphate are much higher than the K_i value for phosphate of 3.3 μM. These data make it unlikely that purified ascorbate sulfatase could hydrolyze ascorbate 2-sulfate in vivo. It is possible, however, that microenvironmental concentrations of these molecules in vivo could be such that hydrolysis would take place. It is also possible that an ascorbate sulfatase exists in the trout which has quite different properties since in this species ascorbate 2-sulfate is an effective vitamin

substitute for ascorbic acid (Halver et al., 1975). Ascorbate sulfatase is a glycoprotein and as such it could be membrane associated where phosphate concentrations are low and ascorbate 2-sulfate hydrolysis could take place. The rate of ascorbate 2-sulfate hydrolysis in tissues is not known. Thus, the physiological significance of the data presented in this paper concerning the in vivo role of ascorbate sulfatase is uncertain.

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