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Ascorbic acid sulfate sulfohydrolase (C_2 sulfatase): The modulator of cellular levels of L-ascorbic acid in rainbow trout

(aquaculture/fish nutrition/vitamin C/arylsulfatase)

LITA V. BENITEZ* AND JOHN E. HALVER†

*Aquaculture Department, Southeast Asian Fisheries Development Center, P.O. Box 256, Iloilo City, Philippines; and †School of Fisheries, University of Washington, Seattle, Washington 98195

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ABSTRACT **The enzyme L-ascorbic acid 2-sulfate sulfohydrolase (C_2 sulfatase) was purified from rainbow trout liver. The enzyme catalyzes the hydrolysis of L-ascorbic acid 2-sulfate and has a pH optimum at 6.0. It has a molecular weight of about 117,500 at pH 5.0 and is inhibited by a number of sulfhydryl blocking agents including L-ascorbic acid. C_2 sulfatase activity was observed in most metabolic organs of rainbow trout. These findings suggest that the physiologic role of the enzyme is to maintain adequate cellular concentrations of L-ascorbic acid in the fish. The activity of the enzyme is controlled by L-ascorbic acid through feedback inhibition. Comparison of kinetic constants and inhibition patterns suggests that C_2 sulfatase is structurally identical to human arylsulfatase A. However, unlike C_2 sulfatase, human arylsulfatase A may not be involved in ascorbate metabolism. Its physiologic substrate is reported to be cerebroside-3-sulfate, not L-ascorbic acid 2-sulfate. A scheme is proposed to account for the functional divergence of these two structurally identical enzymes.**

L-Ascorbic acid (C_1) plays a critical role in all living organisms. Trout, salmon, and a number of other fish species (1–6) have a dietary requirement for C_1. As in mammalian systems, C_1 appears to be involved in collagen synthesis. Fish reared on C_1-deficient diets develop signs traceable to impaired collagen biosynthesis; i.e., lordosis, scoliosis, vertebral dislocation, deformation of support cartilage, and delayed wound repair (4–6).

C_1 is the most chemically unstable component of fish feeds. L-Ascorbic acid 2-sulfate (C_2), a more stable derivative of C_1 was discovered in brine shrimp cyst (*Artemia salina*) (7, 8). It was also detected as a metabolite in human urine (9) and in the liver, spleen, adrenal glands, and bile of rats (10, 11). C_2 promptly arrested signs of fish scurvy in rainbow trout reared on a scorbutic diet (12). In a group of rainbow trout fed for 1 yr an artificial diet containing C_2 as sole source of ascorbate, normal growth, diet efficiency, and absence of scurvy indicated that C_2 sulfate was an adequate source of C_1.

Enzymatic hydrolysis of C_2 to C_1 by a sulfohydrolase would be a critical step in the utilization of C_2 as a vitamin source. This activity has been detected in fish tissues (12, 13), in the liver of the gastropod *Charonia lampas* (14), and in extracts of mammalian tissues (15, 16). The ability of rainbow trout to utilize C_2 as sole dietary source of C makes this fish a model test organism for the study of the sulfatase.

This paper deals with the purification and properties of C_2 sulfohydrolase (C_2 sulfatase) from rainbow trout liver. Experimental evidence suggests that the enzyme modulates cellular levels of C_1 in the fish.

MATERIALS AND METHODS

Materials. Ultrapure grade C_1 was from Hoffmann–La Roche. The dipotassium salt of C_2 was generously donated by Paul A. Seib (Kansas State University). Dipotassium 4-nitrocatechol sulfate, crystalline 4-nitrocatechol, crystallized and lyophilized bovine serum albumin, apoferritin, myoglobin, dithioerythritol, sodium iodoacetate, and *p*-chloromercuriphenylsulfonic acid were from Sigma. Sephadex resins were from Pharmacia. All other reagents were of analytical grade.

Fish Samples. Rainbow trout (*Salmo gairdneri*) (mean weight, 334 g) were sampled randomly from grow-out tanks of the Seward Park Hatchery (University of Washington). The fish were maintained on commercial feed pellets (WFA trout fish food, stage 6-finisher 1; 3/16-in pellets) prior to sampling. Immediately after sampling, the livers from 100 fishes were dissected out, the gallbladders were excised, and the livers were pooled. These were kept at −20°C and processed by 12 hr.

Enzyme Purification. All steps in the purification of the enzyme were performed in the cold (0–4°C).

1. About 317 g of frozen pooled livers was minced and then homogenized in a Waring Blendor with about 1 liter of 0.2 M OAc/0.1 M EDTA, pH 5.0. The homogenate was then centrifuged at 15,000 rpm for 30 min. The supernatant was collected and the pellet was extracted twice by resuspension in buffer and centrifugation to obtain the supernatant.

2. The crude extract was precipitated with ammonium sulfate at 25% saturation. The mixture was allowed to equilibrate overnight with occasional stirring and then was centrifuged at 15,000 rpm for 30 min. The pellet was discarded and the supernatant was further precipitated with ammonium sulfate at 60% saturation. The pellet was collected by centrifugation and dissolved in a minimum volume of 20 mM OAc/10 mM EDTA, pH 5.0.

3. The active fraction from step 2 was loaded on a Sephadex G-25 column (4.5 × 55 cm) which had been equilibrated with 20 mM OAc/10 mM EDTA, pH 5.0. The column was eluted with the same buffer, and 4.5-ml fractions were collected at a flow rate of 20 ml/hr. The most active protein fractions (42–51) were pooled and precipitated with ammonium sulfate. The precipitate was dissolved in a minimal volume of the eluting buffer.

4. The active fraction from step 3 was loaded on a Sephadex G-100 column (4.5 × 54 cm) which was equilibrated and eluted with 20 mM OAc/10 mM EDTA, pH 5.0. Fractions (4.3 ml) were collected at an elution rate of about 21 ml/hr. The fractions containing the enzyme (45–50) were pooled, precipitated with ammonium sulfate, and centrifuged.

5. The product from step 4 was applied to a Sephadex G-200 column (2.5 × 48 cm) which had been equilibrated with 20 mM OAc/10 mM EDTA, pH 5.0. The column was eluted with the same buffer at a flow rate of 21 ml/hr, and 3.6-ml fractions were collected. Fractions 34–38 were pooled and precipitated with ammonium sulfate. The precipitate was dissolved in eluting buffer and dialyzed exhaustively against the same buffer.

6. The dialysate was loaded on a column of SP Sephadex C-25 (2.4 × 24 cm) which was equilibrated with buffer A (20

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Abbreviations: C_1, L-ascorbic acid; C_2, L-ascorbic acid 2-sulfate.

mM OAc/10 mM EDTA, pH 5.0, containing 20 mM NaCl), and 63 3.0-ml fractions were collected. The column was then eluted with buffer B (20 mM OAc/10 mM EDTA, pH 5.0, containing 200 mM NaCl) until most of the proteins were desorbed. The active enzyme was recovered in fractions 5–11. The enzyme solution was stored at −20°C without further treatment.

Molecular Weight Determination. The molecular weight of the enzyme was determined by gel filtration on Sephadex G-200 by the method of Andrews (17). A column of Sephadex G-200 (2.5 × 51 cm) was equilibrated with 20 mM OAc/20 mM EDTA, pH 5.0, and calibrated with apoferritin (460,000), bovine serum albumin (68,000), and myoglobin (17,200) as standard molecular weight markers. The enzyme and markers were loaded and then eluted from the column with 20 mM OAc/10 mM EDTA, pH 5.0, at 18 ml/hr; 2.8-ml fractions were collected. The protein concentration of each fraction was estimated from the absorbances at 225 and 280 nm. Fractions were assayed for C_2 sulfatase activity to determine the precise elution volume of the active enzyme.

Enzyme Assays. The activity of C_2 sulfatase was determined by a modification of the procedure of Stevens *et al.* (18). The assay mixture contained 10 mM C_2, 0.17 mM 2,6 dichloroindophenol, 20 mM Tris, and 10 mM EDTA in a total volume of 3.0 ml at pH 6.0. The mixture was preincubated for 15 min and reaction was initiated by the addition of 50–100 μl of enzyme solution. After 1 hr at room temperature, the absorbance at 516 nm was determined. A control mixture containing all components except the enzyme was run simultaneously. Difference in the absorbances of the two mixtures was an index of enzyme activity. Enzyme activity was expressed as μmol of ascorbate released per hr per mg of protein.

The enzyme was assayed with K_2 4-nitrocatechol sulfate as substrate by a modification of Baum *et al.* (19). The assay mixture contained 2.5 mM 4-nitrocatechol sulfate and 10–50 μl of enzyme in 20 mM Tris/10 mM EDTA, pH 6.0, in a total volume of 2.0 ml. After 1 hr at room temperature, the reaction was stopped by addition of 1.0 ml of 2 M NaOH. Absorbance of the resulting solution was determined at 515 nm.

The protein concentration of fractions obtained by gel filtration and chromatography were estimated from absorbance at 280 nm (20). The protein concentration of the crude extracts and of the pooled fractions were determined by the method of Lowry *et al.* (21) with bovine serum albumin as standard. All assays described in this section were done at room temperature.

RESULTS

Purification of C_2 Sulfatase. Typical elution profiles in the purification steps are shown in Fig. 1. A significant amount of protein was bound tightly to the resin in the first two gel filtration steps (Sephadexes G-25 and G-100). The protein did not have any C_2 sulfatase activity and its adsorption on the resins represented an effective purification mechanism. The fractions containing the active enzyme catalyzed the hydrolysis of both C_2 and K_2 4-nitrocatechol sulfate. The latter assay method was used during the purification process because it required the use of less enzyme. However, the peak fractions and pooled enzyme fractions were also assayed by the dichloroindophenol/C_2 method.

The purification of the enzyme is summarized in Table 1. The final product gave a 477-fold increase in specific activity and a yield of 18%. Variations in the value for enzyme activity of the crude extract and of protein fractions from early stages of purification may be due to the presence of endogenous inhibitors, the active binding of dichloroindophenol to some proteins, or the competitive reduction of dichloroindophenol by other enzymes. Some dehydrogenases in liver extracts can both bind and reduce dichloroindophenol under reaction conditions similar to those of the C_2 sulfatase assay (22).

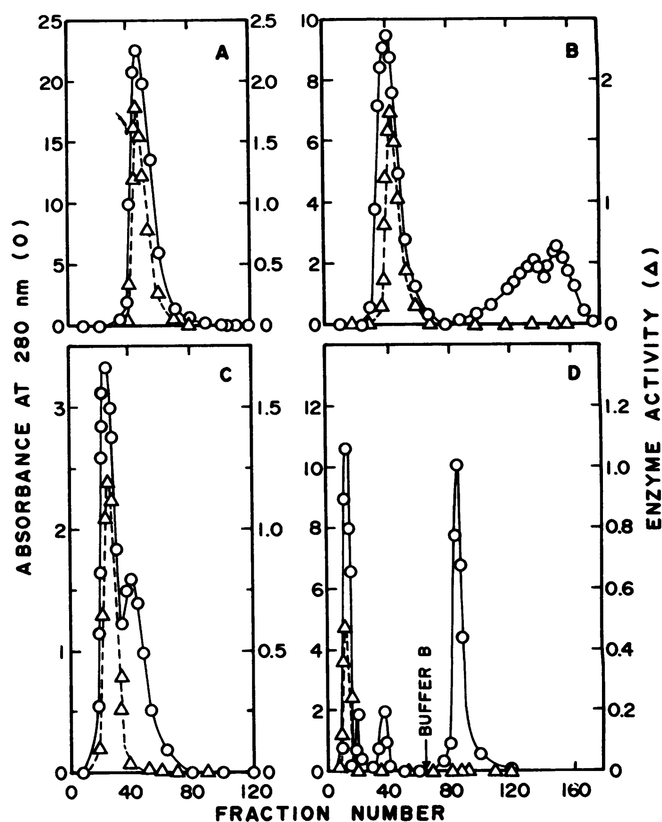

FIG. 1. Elution profiles of C_2 sulfatase from various Sephadex resins during purification. (*A*) Gel filtration on Sephadex G-25; (*B*) gel filtration on Sephadex G-100; (*C*) gel filtration on Sephadex G-200; and (*D*) ion-exchange chromatography on SP Sephadex C-25.

Properties of C_2 Sulfatase. The molecular weight of the enzyme was determined by gel filtration on Sephadex G-200 (Fig. 2). The enzyme had an apparent molecular weight of 117,500 at pH 5.0 in 20 mM OAc/10 mM EDTA.

The pH activity profile of C_2 sulfatase is shown in Fig. 3. Hydrolysis of both substrates was maximal at pH 6.0. The enzyme was active only at acidic pH values. At pH 8.0, <10% of the enzyme activity was detectable. The data in Fig. 3 were calculated on the basis of 1-hr incubation; however, the pH optimum remained constant regardless of time of incubation.

C_2 sulfatase was inhibited by compounds known to be sulfhydryl blocking agents (Fig. 4). Of compounds tested, sulfite was the most potent; complete inhibition was obtained at a concentration of 1.61 mM. The inhibitors of C_2 sulfatase listed in order of inhibitory action are: sulfite, *p*-chloromercuriphenylsulfonic

Table 1. Purification of C_2 sulfatase from rainbow trout liver

Stage	Total protein, mg	Total activity, μmol of ascorbate/hr	Specific activity,* μmol ascorbate/hr/mg protein	Purification, fold	Recovery, %
1. Crude extract	24,347	202	0.0083	1	100
2. $(NH_4)_2SO_4$	640	189	0.2960	36	93
3. Sephadex G-25	255	173	0.6800	82	86
4. Sephadex G-100	56	130	2.3135	279	64
5. Sephadex G-200	18	67	3.7400	451	33
6. Sephadex C-25	9	36	3.9600	477	18

* Values represent means of at least three determinations.

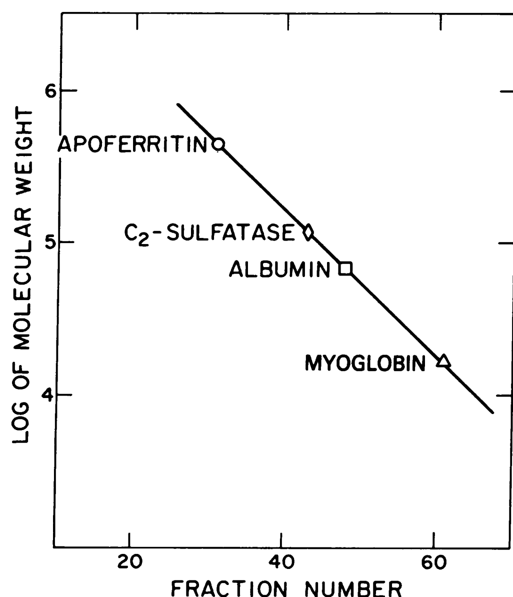

FIG. 2. Determination of molecular weight of C_2 sulfatase by gel filtration on Sephadex G-200.

acid, C_1, sulfate, iodoacetate, and dithioerythritol. Iodoacetate is one of the more reactive sulfhydryl reagents and has been used extensively for carboxymethylation of enzymes with active-site sulfhydryl groups. However, with C_2 sulfatase, iodoacetate was less effective than the organomercurial. The close structural similarity between the organomercurial and the substrates C_2 and nitrocatechol sulfate might account for its strong inhibitory action on the C_2 sulfatase reaction. Dithioerythritol is not considered to be a sulfhydryl-blocking agent. Its mild inhibitory action on C_2 sulfatase might be traced to its ability to reduce disulfide bonds which in turn would affect the conformational integrity of the enzyme.

Because some of the inhibitors tested interfered with the dichloroindophenol/C_2 assay method, all inhibition studies shown in Fig. 4 were conducted with nitrocatechol sulfate as substrate. However, for inhibitors that did not interfere with the dichloroindophenol/C_2 assay, levels of inactivation were comparable with those obtained with nitrocatechol.

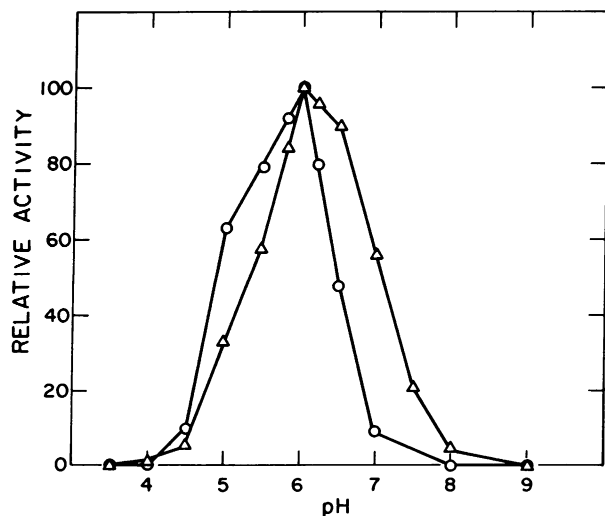

FIG. 3. pH activity profile. The enzyme concentrations were 9.61 μg/ml and 5.76 μg/ml and the substrate concentrations were 1 μmol/ml and 2.5 μmol/ml for the assay methods using C_2 (○) and K_2 4-nitrocatechol sulfate (△) as substrate, respectively.

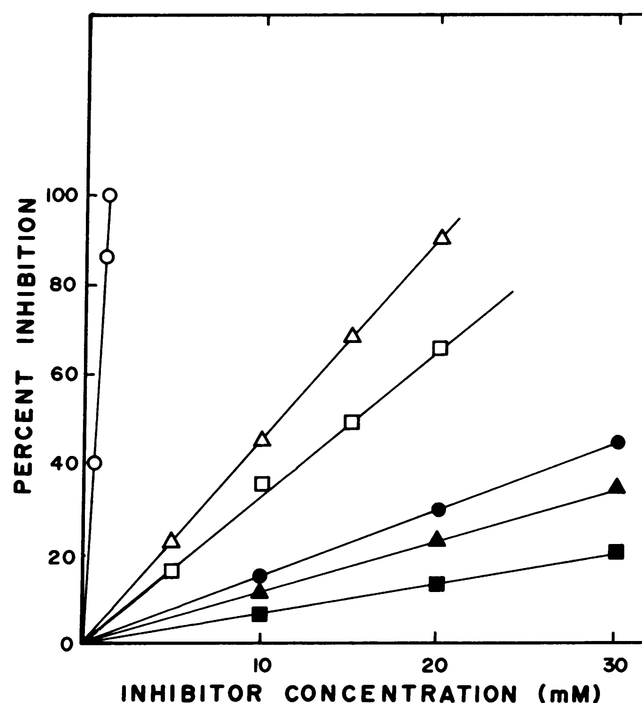

FIG. 4. The response of C_2 sulfatase to various inhibitors: ○, sulfite; △, *p*-chloromercuriphenylsulfonic acid; □, C_1; ●, sulfate; ▲, iodoacetate; ■, dithioerythritol. The nitrocatechol sulfate assay method was used for all inhibitors tested. The enzyme (5.76 μg/ml) in a total volume of 1.0 ml of 20 mM OAc/10 mM EDTA, pH 6.0, was incubated with the inhibitor at the indicated concentrations for 30 min at room temperature. The reaction was initiated by the addition of 5 μmol of substrate in 1.0 ml of the same buffer. The complete reaction mixture was incubated for 1 hr at room temperature and then 1.0 ml of 2 M NaOH was added. The activity of the reaction mixture that contained no inhibitor was taken to be 100%.

DISCUSSION

C_2 sulfatase purified from rainbow trout liver was found to have arylsulfatase A activity and a kinetic pattern common to many previously purified arylsulfatases. This kinetic pattern is characterized by a rapid initial reaction followed by a slower linear rate. A similar pattern has been observed for a number of hydrolases and has been interpreted (23) as a sequential reaction involving two steps:

$$E + S \rightleftharpoons ES\,I \rightarrow P_1 + ES\,II \rightarrow P_2 + E \quad [1]$$

in which E represents the enzyme and S is the substrate. Two enzyme–substrate complexes, ES I and ES II, and two products, P_1 and P_2, are formed sequentially. Fig. 5 shows a proposed reaction mechanism for C_2 sulfatase which is patterned after Eq. 1. This mechanism is consistent with the kinetic properties and inhibition pattern of C_2 sulfatase and shows: (*a*) involvement of a sulfhydryl group in the active site of the enzyme; (*b*) acid-base catalysis possibly involving a histidyl residue positioned near the active site; (*c*) sequential formation of two enzyme–substrate complexes, the second being more firmly bound than the first; and (*d*) sequential formation of two products, C_1 (L-ascorbic acid) and then sulfate.

Under normal physiologic conditions, the sulfhydryl groups in cysteine residues of enzymes are generally the most reactive of all amino acid side chains. They may be alkylated, oxidized, arylated, or acylated readily and form complexes with heavy metal ions (24). The inactivation of C_2 sulfatase by sulfhydryl-specific reagents in the present study suggests the involvement of a sulfhydryl group in the active site of the enzyme.

Acid-base catalysis may be important in the desulfation of enzyme–substrate complex II, the rate-determining step in the

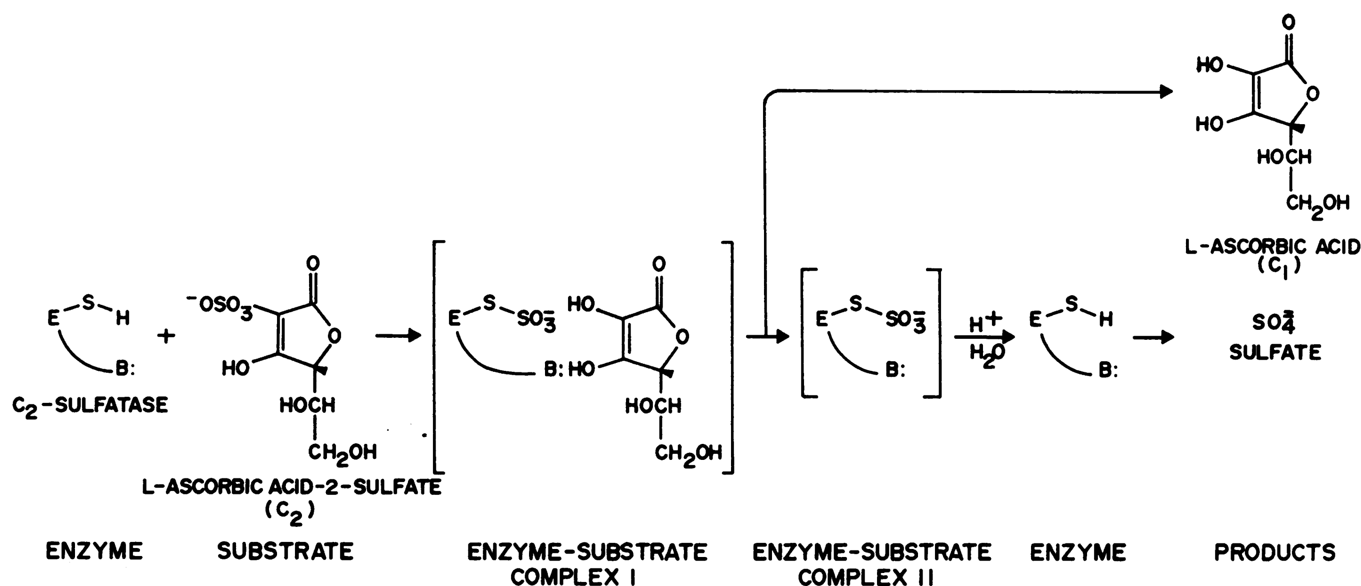

FIG. 5. Proposed reaction mechanism of C_2 sulfatase.

reaction sequence. The imidazole group of the histidine residue can act as a general acid-base catalyst in many enzyme-catalyzed reactions and appears to be pertinent in the C_2 sulfatase reaction as well. An essential histidyl group has been implicated in the active site of ox liver arylsulfatase A, an enzyme that was subsequently found to have C_2 sulfatase activity (25, 26). Hydrolysis of an organic sulfate ester catalyzed by an intramolecular imidazole group has been proposed as a model system for aryl sulfate sulfohydrolase (27). A synthetic polyethyleneimine polymer containing an imidazole group proved to be a highly efficient catalyst for the hydrolysis of nitrocatechol sulfate (28).

The present study suggests that, in rainbow trout, the major physiologic function of C_2 sulfatase is to modulate and to maintain adequate cellular levels of C_1 consistent with the physiologic requirement of the fish. Rainbow trout and other fishes can survive and grow normally with C_2 as the sole dietary source of ascorbate (12, 13, 29, 30). The ubiquitous distribution of C_2 in tissues of fish (12, ‡) suggests that this sulfated derivative may be the storage form of C in these animals. The distribution of C_2 in fish tissues parallels the distribution of C_2 sulfatase in spleen, liver, brain, kidney, skin, and male and female gonads.

A critical step in the utilization of the stored C_2 involves the hydrolysis of C_2 to C_1 catalyzed by C_2 sulfatase, the activity of which is controlled by C_1 through feedback inhibition. C_2 sulfatase activity is inhibited by relatively low concentrations of C_1. The formation of the weakly bound enzyme–substrate complex I (Fig. 5) rationalizes the mechanism of inhibition of C_2 sulfatase activity by C_1 and represents an effective mechanism for the control of cellular concentrations of ascorbate in the fish. High dietary levels of C_1 suppress C_2 sulfatase activity and allow conservation of stored C_2. Rapid utilization of cellular C_1 causes derepression of C_2 sulfatase which in turn increases the rate of hydrolysis of C_2 and results in the replenishment of cellular C_1.

C_2 sulfatase appears to be identical structurally to arylsulfatase A of human tissues and other mammalian sources (Table 2). Results of this study on co-purification suggest that C_2 sulfatase and arylsulfatase A are identical (46, 47).

Arylsulfatase activity was first demonstrated in extracts of a marine mollusc (48) and later in other aquatic organisms (13, 38, 41, 49). Widespread distribution of arylsulfatases was established as the enzyme was detected and purified from a number of organisms living in different environments varying in physiologic complexity from microorganisms to man.

Although C_2 sulfatase and human arylsulfatase A are identical structurally, these enzymes have different physiologic functions. Trout C_2 sulfatase appears to be involved in the maintenance of cellular levels of C_1 through hydrolysis of C_2. In contrast, human arylsulfatase A is not involved in ascorbate metabolism, although *in vitro* it catalyzes the hydrolysis of C_2. C_2 was discovered in the urine of humans in whom scurvy had been induced (9). In subhuman primates, intravenously injected C_2 was effectively removed from the blood through the kidneys (50–52).

Mammalian tissues contain arylsulfatase A and other arylsulfatases which have no defined function and were known

Table 2. Similarities between C_2 sulfatase and arylsulfatase A

Feature	C_2 sulfatase*	Arylsulfatase A†
Molecular weight: comparable	117,500 (by gel filtration)	107,000 (31) 109,600 (32) 110,000 (33) 120,000–135,000 (34) 130,000 (35)
pH optimum: acidic	pH 6.0	pH 4.0–4.5 (18) pH 4.6–5.3 (34) pH 4.8 (36, 37) pH 5.5 (38) pH 5.5–6.0 (39)
Substrate specificity	Hydrolyzes C_2 Hydrolyzes nitrocatechol sulfate	Hydrolyzes C_2 (18, 36, 37, 40) Hydrolyzes nitrocatechol sulfate (19, 38, 41–43)
K_m with C_2 as substrate	3.06 mM at pH 6.0	2.5–3.0 mM at pH 4.0 (40)
K_m with nitrocatechol sulfate as substrate	0.64 mM at pH 6.0	0.49 mM at pH 5.6 (42)
Response to inhibitors similar; inhibitors include sulfite, sulfate, and ascorbate (42–45)		

* From present study.

† Data from references given in parentheses.

‡ Halver, J. E., Tucker, B., Benitez, L. V. & Smith, R. R. (1981) World Conference on Aquaculture, Venice, Italy, p. 39 (abstr.).

to hydrolyze only nonphysiologic substrates such as *p*-acetylphenylsulfate, *p*-nitrophenylsulfate, nitrocatechol sulfate, and 4-methylumbilliferylsulfate (53). The physiologic substrate of arylsulfatase A is cerebroside-3-sulfate, and in metachromatic leukodystrophy a cellular deficiency in arylsulfatase A (54–57) is characterized by accumulation of cerebroside-3-sulfate which leads to degeneration of myelin and death (56–61).

The gene coding for C_2 sulfatase may be the ancestral gene from which human arylsulfatase A evolved. The selection pressure for evolution and divergence of the gene is the obligatory requirement for C_1 in the cells. Fish and other aquatic animals derive C_1 mainly from the sulfated derivative, C_2. Aquatic organisms acquired the ability to convert C_2 to C_1 through the synthesis of C_2 sulfatase. C_2 is stable in water and is also resistant to oxidative degradation (10, 62, 63). It has a selective advantage as the primary source and precursor of C_1 in the aquatic environment. The transition from aquatic to terrestrial environment involved the scarcity of C_2 and the preponderance of C_1 in the diet of terrestrial animals.

Arylsulfatase A, B, and C have been identified as the three major isozymes in human tissues (64–67). Metabolic disorders resulting in accumulation of sulfate esters have been correlated with hereditary deficiency of any one or all of the three isozymes (53). The structural and functional relationships among the three isozymes are not yet known. However, it has been shown that human arylsulfatase B does not seem to have any significant C_2 sulfatase activity (40).

The dietary requirement for ascorbate in fish species should be reevaluated with C_2 as sole ascorbate source. C_2 should be provided at a concentration sufficient to saturate storage pools and to induce full activity of the C_2 sulfatase enzyme in major metabolic organs.

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