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ASCORBIC ACID-2-SULFATE SULFHOHYDROLASE ACTIVITY OF HUMAN ARYLSULFATASE A

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Summary

Pure human arylsulfatase A (EC 3.1.6.1) was found to hydrolyze ascorbic acid 2-sulfate to ascorbic acid and inorganic sulfate at rates from 200 to 2000 μmol/mg per h depending on the method of assay. This rate was lower than that observed with the synthetic substrate 4-nitrocatechol sulfate, but higher than that seen with the physiological substrate cerebroside sulfate. Extracts of cultured fibroblasts from normal subjects were also shown to hydrolyze ascorbic acid 2-sulfate; extracts of fibroblasts from patients with metachromatic leukodystrophy, known to be deficient in arylsulfatase A, did not. Similarly, hydrolysis of ascorbic acid 2-sulfate was not observed when a partially purified preparation of human arylsulfatase B was tested under a variety of conditions. Thus, in the human, arylsulfatase A appears to be the major, if not the only, ascorbic acid-2-sulfate sulfohydrolase.

Introduction

Ascorbic acid 2-sulfate appears to be a widely distributed metabolite of ascorbic acid. It was initially discovered in cysts of the brine shrimp *Artemia salina* where is was first believed to be the 3-sulfate [1,2]. It was then identified in human urine where it constituted up to 25% of the excreted ascorbic acid metabolites [3]. Subsequently, it has been found in rat liver, spleen, adrenal gland and urine [4]. In the liver it represented about 20% of the total ascorbate. While its biological significance has not been established, ascorbic acid 2-sulfate has been suggested as a storage form for ascorbic acid, as a transport de-

Abbreviation: DCIP, 2,6-dichlorophenolindophenol.

rivative, or as a direct sulfate donor for sulfate ester biosynthesis.

Reports in abstract form from Tolbert's group [5,6] have indicated that arylsulfatase A (EC 3.1.6.1) fractions from beef liver exhibit ascorbic acid-2-sulfate sulfohydrolase activity. The established physiological substrates for this enzyme are sulfated galactolipids [7—9] which are found only in a limited number of tissues while arylsulfatase A occurs ubiquitously. The wide distribution of ascorbic acid 2-sulfate parallels that of arylsulfatase A. We have recently purified arylsulfatase A from human urine [10], and it appeared propitious to test the ability of the pure enzyme to hydrolyze ascorbic acid 2-sulfate.

Human arylsulfatase A hydrolyzed ascorbic acid 2-sulfate at rates comparable to those for other substrates, while partially purified arylsulfatase B was inactive. Fibroblast extracts derived from patients with metachromatic leukodystrophy, unlike extracts of normal fibroblasts, did not hydrolyze this vitamer, suggesting that arylsulfatase A is the principal if not the only enzyme capable of hydrolyzing this substrate.

Experimental

Ascorbic acid 2-sulfate (dipotassium salt) and ascorbic acid 2-[^{35}S]sulfate (barium salt) were supplied by the Department of Chemical Research, Hoffmann-La Roche Inc. 2,6-Dichlorophenolindophenol (disodium salt) (DCIP) and ascorbic acid, U.S.P., were obtained from Calbiochem. The liquid scintillation cocktail consisted of 50 mg POPOP and 4 gm PPO per l of an equivolume mixture of toluene and 2-methoxyethanol [11]. Counting was done in a Packard TriCarb spectrometer. Human arylsulfatase A was isolated from urine and was essentially pure [10]. Dilute enzyme solutions were stabilized by bovine serum albumin (1 mg/ml) and showed 800—4000 4-nitrocatechol sulfate units/mg enzyme. Activity measurements toward 4-nitrocatechol sulfate, 4-methylumbelliferyl sulfate and cerebroside sulfate were carried out as previously described [10].

Fibroblast cultures were initiated from skin biopsies by the explant technique and maintained in the usual manner [12]. Extracts were prepared by suspending cells in an equal volume of 0.2 M Tris · HCl, pH 7.4 and subjecting them to six cycles of freezing and thawing. After removal of cell debris by centrifugation for 2 min in the Brinkman Microfuge (14 000 × *g*), the supernatant fluids were dialized overnight against 1000 volumes of 25 mM Tris · HCl, pH 7.4. Arylsulfatase A activity was determined by the procedure of Baum et al. [13] as employed in this laboratory [10]. A unit of activity is defined as 1 μmol of 4-nitrocatechol sulfate hydrolyzed per h. Protein was determined by the procedure of Lowry et al. [14].

Four methods were developed for monitoring the hydrolysis of ascorbic acid 2-sulfate based on: continuous decrease in ultraviolet absorbance of the substrate; continuous reduction of DCIP by ascorbic acid; reduction of DCIP by accumulated ascorbic acid; and determination of radioactivity of accumulated sulfate (Stevens et al., unpublished). The present studies were conducted largely with the latter two methods.

In the reduction of DCIP by accumulated ascorbic acid, the reaction mixture consisted of 20 μl of 20 mM ascorbic acid 2-sulfate in 0.1 M acetate, pH 4.0, and 20 μl of enzyme solution. Following incubation at 37°C for 15 min the

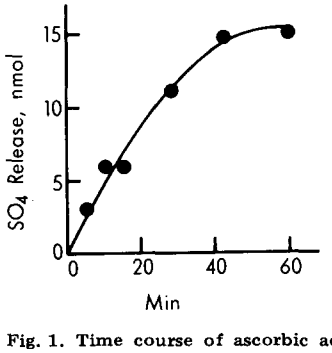
reaction was stopped with 0.4 ml of 0.15 mM DCIP in 0.5 M sodium phosphate, pH 8.0 (freshly prepared by dilution of a 10 mM DCIP stock solution). The absorbance at 600 nm was determined within 30 s after the addition of the DCIP reagent. In the control, enzyme was replaced by bovine serum albumin (1 mg/ml). "No substrate" and zero time controls were also evaluated but were not significantly different from the albumin control.

In the determination of radioactivity of accumulated sulfate, the reaction mixture consisted of 50 µl of 2 mM ascorbic acid 2-[^{35}S]sulfate (approx. 5000 cpm) in 0.2 mM sodium acetate, pH 4.5, and 100 µl of either pure enzyme or fibroblast extract preparation. Following incubation at 37°C for 30 min the reaction was stopped with 0.1 ml of 50 mM Na_2SO_4 in 0.2 M Tris · HCl, pH 8.0; then 10—20 mg of Celite, 9 ml of water, and 0.08 ml of 30 mM $BaCl_2$ were added. The Celite-aggregated $BaSO_4$ was centrifuged, washed, and counted. In the control the extract was replaced by bovine serum albumin, 1 mg/ml in 25 mM Tris · HCl, pH 7.4.

Results

Human arylsulfatase A hydrolyzed ascorbic acid 2-sulfate at rates in the range of 200—2000 µmol/mg per h depending on specific assay protocol utilized and the history of the enzyme sample. Under optimal conditions, this activity was about one-half that obtained with 4-nitrocatechol sulfate and 10-fold greater than with 4-methylumbelliferyl sulfate or cerebroside sulfate.

The pH optimum for the reaction was between 4.0 and 4.5 and was independent of the method of assay. The rate of reaction was proportional to enzyme concentration and the initial rate was linear. However, the reaction slowed and stopped at around 40 min, well before complete hydrolysis occurred, as shown in Fig. 1 for the radioactivity method. The reaction ceased between 20 and 60 min in the other methods (Stevens et al., unpublished).



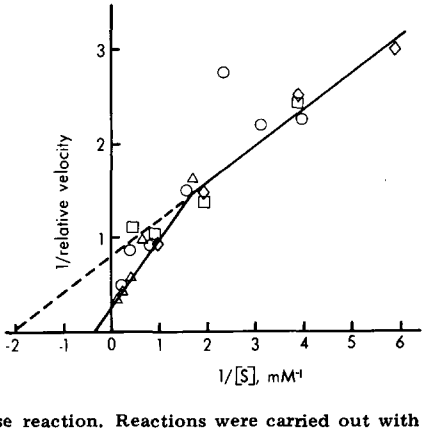


Fig. 1. Time course of ascorbic acid-2-sulfate sulfohydrolase reaction. Reactions were carried out with 100 ng of arylsulfatase A by the radioactivity assay.

Fig. 2. Lineweaver-Burk plot of ascornic acid-2-sulfate sulfohydrolase activity. Assay method: ○, radioactivity assay; □ and ◇, decrease of ultraviolet absorbance of substrate (Stevens et al., unpublished), two independent determinations; and △, reduction of DCIP by ascorbic acid.

When examined by the accumulated ascorbic acid DCIP method the ascorbic acid 2-sulfate hydrolysis reaction yielded a linear Lineweaver-Burk plot between 1 and 10 mM substrate with a K_m of 2.5—3.0 mM. Methods which operate at lower substrate concentrations also yielded linear kinetic plots, but K_m values were less than 1 mM. When results from the four assay methods were combined, as in Fig. 2, there was curvature of the double reciprocal plot at lower substrate concentrations. Data from low (< 1 mM) substrate levels extrapolates to a K_m value of about 0.5 mM.

Ascorbic acid 2-sulfate hydrolysis could not be demonstrated with crude tissue extracts despite the presence of adequate arylsulfatase A activity. Preliminary tests suggested that this might be due to an inhibitor, the destruction of ascorbate, or hemoglobin interference with the dye reduction. Therefore, extracts of cultured human fibroblasts, which are free of blood elements, were tested with the radioactive sulfate method for investigating ascorbic acid 2-sulfate hydrolysis in crude systems. Hydrolysis could be observed with dialyzed fibroblast extracts, but it was less than that expected from the arylsulfatase A activity. Substrate dependence, pH optimum, and the time course of the reaction were similar to those for the pure enzyme. When the protein dependence of the fibroblast extracts was examined, it was found that activity failed to increase with protein above about 0.2 mg per assay (cf. Figs. 3 and 4). The radioactive sulfate released in the linear range did not exceed the zero time blank by more than 2—3-fold, so the confidence level of individual determinations was not high. Therefore, dialyzed fibroblast extracts were subjected to several purification steps in an attempt to separate the enzyme from material interfering with the assay. Chromatography on DEAE-cellulose and then on concanavalin A-Sepharose was only partially effective in relieving this apparent inhibition. Further purification was not pursued since the objective was to be able to assay activity in crude fibroblast extracts.

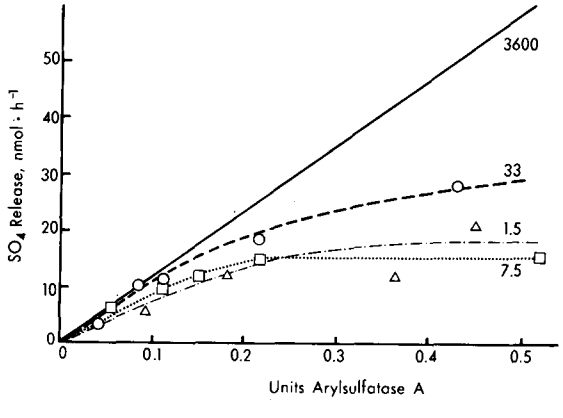


Fig. 3. Effect of purification and enzyme concentration on ascorbic acid-2-sulfate sulfohydrolase activity of fibroblast extracts. Dialyzed fibroblast extract untreated, △; then chromatographed on DEAE-cellulose, □; then treated with concanavalin A-Sepharose, ○. The solid line represents the reaction with pure arylsulfatase A. The specific activities toward 4-nitrocatechol sulfate as units/mg protein are indicated by the numbers opposite the plots. The radioactivity assay was employed.

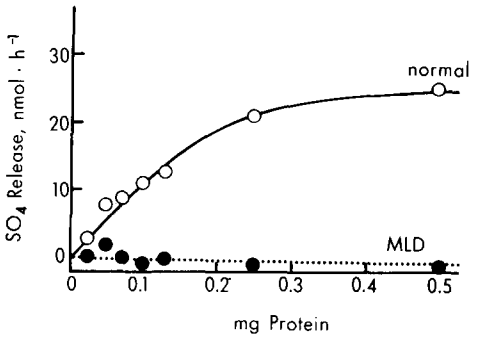


Fig. 4. Ascorbic acid-2-sulfate sulfohydrolase activity of normal and metachromatic leukodystrophy (MLD) fibroblast extracts measured by the radioactivity assay.

Repeated trials with dialyzed extracts were carried out at several different protein levels and the results were reasonably reproducible. The data collected from dialyzed extracts were compared to similar data from pure urinary arylsulfatase A and enzyme preparations from partially purified fibroblast extracts (Fig. 3). Ascorbic acid-2-sulfate sulfohydrolase activity levels of extracts were obtained by extrapolation to zero enzyme concentration. With this approach extracts of fibroblasts from a patient with late infantile metachromatic leukodystrophy were compared with extracts of normal cells (Fig. 4). The extracts of normal cells showed ascorbic acid-2-sulfate sulfohydrolase activity nearly commensurate with its arylsulfatase A activity while no hydrolysis could be detected with extracts of metachromatic leukodystrophy cells at any level. The arylsulfatase A activity of the latter was at the limits of detection, less than 5% of that in the normal control. When the deficient extracts were supplemented with pure arylsulfatase A to normal levels the ascorbic acid 2-sulfate hydrolysis was identical to that in control cells showing that the lack of activity was not due to an excessive level of inhibitor in these cells.

Arylsulfatase B, partially purified from human placenta [15], was incubated with ascorbic acid 2-sulfate. It did not produce any detectable level of hydrolysis, even at a 500-fold excess of 4-nitrocatechol sulfate units. Adjusting either the pH or the substrate concentration or both to those for optimal hydrolysis of 4-nitrocatechol sulfate were of no avail. The conditions for hydrolysis of UDP-*N*-acetylgalactosamine 4-sulfate, a recently described physiological substrate for arylsulfatase B, are quite different from those for synthetic substrates [15]. Ascorbic acid 2-[^{35}S]sulfate hydrolysis by arylsulfatase B under nucleotide cleavage conditions were also ineffective. The minor "B-like" sulfatase components [16] retained by DEAE-cellulose and eluted before arylsulfatase A by salt gradients likewise failed to show any ascorbic acid-2-sulfate sulfohydrolase activity.

Discussion

The present results using pure human arylsulfatase A, confirm the tenet that arylsulfatase A catalyzes the hydrolysis of ascorbic acid 2-sulfate. A similar observation has been made with pure ox liver enzyme by Roy [17] who kindly

provided a prepublication copy of his studies. The possibility that arylsulfatase A and ascorbic acid-2-sulfate sulfohydrolase are identical was originally suggested by Tolbert's group [5,6] from observations on co-purification.

Ascorbic acid-2-sulfate sulfohydrolase activity has also been observed in liver extracts of a marine gastropod by Hatanaka et al. [18]. They have purified the enzyme and found that it co-purified with arylsulfatase [19]. There is apparently only one arylsulfatase in the gastropod liver and it is not certain whether it corresponds to the mammalian A or B enzyme, or whether it is functionally unrelated. Nevertheless, its ascorbic acid-2-sulfate sulfohydrolase activity is of teleological interest.

Human arylsulfatase A hydrolyzed ascorbic acid 2-sulfate almost is effectively as it hydrolyzed 4-nitrocatechol sulfate and more effectively than 4-methylumbelliferyl sulfate, cerebroside sulfate, or testicular sulfoglycerogalactolipid. It thus appears unlikely that this is an adventitious side reaction, but rather an operative metabolic activity. The affinity of the enzyme for ascorbic acid 2-sulfate as deduced from an apparent K_m value around 1—2 mM is consistent with a physiological role for this reaction.

The cessation of the ascorbic acid-2-sulfate sulfohydrolase activity after the initial reaction is reminiscent of the anomalous kinetic behavior of arylsulfatase A with 4-nitrocatechol sulfate [21]. The latter has been shown to be due to a reversible modification of the enzyme by this substrate to an inactive form. Ox liver arylsulfatase A also exhibited similar behavior with ascorbic acid 2-sulfate, and Roy [17] has provided evidence that substrate modification may occur.

With pure human arylsulfatase A we have shown that varying either the enzyme or substrate concentration does not appreciably affect the time dependence of the inactivation; however, the possible inactivation by the product, ascorbic acid, could not be disregarded. Ascorbic acid, particularly in the presence of Cu^{2+}, inactivates arylsulfatase A [21]; yet, including 0.1 mM EDTA in reaction mixtures or in dialysis media of cell extracts did not alter the time-dependent inactivation. Conducting the reaction in the presence of DCIP, which continuously oxidized the ascorbic acid as it was formed, was also ineffectual. Our findings appear to preclude inactivation by ascorbic acid per se.

The specificity of ascorbic acid 2-sulfate hydrolysis for arylsulfatase A was somewhat unexpected. We had presumed that ascorbic acid 2-sulfate and 4-nitrocatechol sulfate were isosteric analogs since they are both esters of resonance-stabilized cyclic enediols. Consequently, we had predicted that ascorbic acid 2-sulfate, like 4-nitrocatechol sulfate, would be hydrolyzed equally well by arylsulfatase A and arylsulfatase B. Roy [17] found that ox brain arylsulfatase B had only 1% of the activity of ox liver arylsulfatase A. Similarly, we were unable to show any activity with human placental arylsulfatase B. Both studies indicate that there must be finite but subtle differences at the substrate binding site of arylsulfatase A and arylsulfatase B. On the one hand, both enzymes react with synthetic substrates like 4-nitrocatechol sulfate and 4-methylumbelliferyl sulfate [16]; yet in stark contrast, they exhibit a high degree of specificity toward natural substrates. The structures of substrates acted on by both arylsulfatases are presented in Fig. 5 in a manner emphasizing the similarities in electron distribution around the putative reaction center, the ester sulfate. It is paradoxical that ascorbic acid 2-sulfate and 4-nitrocatechol sulfate are

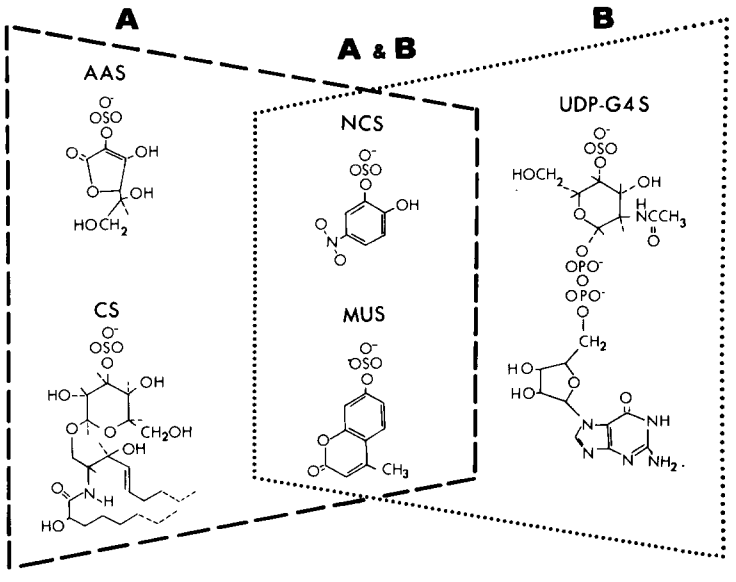


Fig. 5. Structures of substrates hydrolyzed by arylsulfatase A and arylsulfatase B. AAS, ascorbic acid 2-sulfate; CS, cerebroside sulfate; NCS, 4-nitrocatechol sulfate; MUS, 4-methylumbelliferyl sulfate. UDP-G4S, uridine diphospho-*N*-acetylgalactosamine 4-sulfate.

acceptable at the site which recognizes galactose 3-sulfate (arylsulfatase A), while 4-nitrocatechol sulfate but not ascorbic acid 2-sulfate is acceptable at the site which recognizes *N*-acetylgalactosamine 4-sulfate (arylsulfatase B). The basis for the stereochemical specificities of the active site of sulfatases remain unclear at present.

The present results and those of Roy [17] suggest that arylsulfatase A may be the major mammalian enzyme for the hydrolysis of ascorbic acid 2-sulfate. However, the biological relevance of the reaction is difficult to assess, largely due to the uncertainty of the function of ascorbic acid 2-sulfate. If it is a storage form of ascorbic acid, as some have suggested, its hydrolysis would serve to maintain cellular levels of the vitamin. Such a role for arylsulfatase A is commensurate with its ubiquitous occurrence, as opposed to its rather limited role as a sulfogalactolipid sulfohydrolase. The failure of ascorbic acid 2-sulfate to serve as an anti-scorbutic agent in guinea pigs [22] and monkeys [23] has dampened enthusiasm for such a role, however, the latter failing may simply reflect an inability of the compound to cross cell membranes, as appears to be the case with cultured cells (Fluharty, A.L. and Churlik, C.A., unpublished results).

There have been reports which suggest that ascorbic acid 2-sulfate may function as a sulfate donor. Verlangieri and Mumma [24] reported that incorporation of label into cholesterol [^{35}S]sulfate by rats was more efficient when the label was administered as ascorbic-acid 2-[^{35}S]sulfate rather than inorganic [^{35}S]sulfate. Hatanaka et al. [25] found that radioactivity from ascorbic acid 2-[^{35}S]sulfate was incorporated to almost the same extent as that from inorganic [^{35}S]sulfate by embryonic chick cartilage epiphyses into chondroitin sulfates. However, the direct involvement of ascorbic acid 2-sulfate as sulfate do-

nor in such systems has been questioned by Shapiro and Poon [26] who found that incorporated radioactivity was mainly derived from a decomposition product, possibly inorganic sulfate. If ascorbic acid 2-sulfate does in fact function in biological sulfation reactions, then the sulfohydrolase activity of arylsulfatase A would be relegated to an ancillary "house-keeping" role.

We have found a profound deficiency of ascorbic acid-2-sulfate sulfohydrolase activity in cultured human fibroblasts derived from patients with metachromatic leukodystrophy. This deficiency is commensurate with the lack of arylsulfatase A activity in these fibroblasts. This disease state is characterized by an over accumulation of cerebroside sulfate in peripheral and central nervous tissue and associated myelin degeneration. Pathological features do not implicate abnormal ascorbic acid metabolism and no metachromatic leukodystrophy patients have ever been diagnosed as scorbutic. However, now that this enzyme deficiency has been reported in fibroblasts, investigation into enzyme deficiency in tissues and any possible pathology must be considered.

Ascorbic acid is particularly rich in neural tissue: in brain, its concentration is 2—3 times higher than that of glucose. Just how much occurs as the sulfate ester is uncertain, since brain was not included in the study of the distribution of ascorbic acid 2-sulfate in rat tissues [4]. If the ratio of ascorbic acid to ascorbic acid 2-sulfate found in rat liver is typical of other tissues, the amount occurring in brain would be highly significant. Ascorbic acid has been implicated in a variety of hydroxylation reactions. Such reactions are important in neural tissue for the synthesis of adrenergic and cholinergic transmitter substances, fatty acid hydroxylation and fatty acid chain elongation. Despite the absence of overt ascorbic acid deficiency in patients with metachromatic leukodystrophy, the deficiency of arylsulfatase A could cause local perturbations which might affect synthetic reactions. Such subtle compromises could contribute to the severe neuropathy in metachromatic leukodystrophy which is not fully accountable by over accumulation of cerebroside sulfate alone.

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