ASCORBATE-2-PHOSPHATE INHIBITION OF ASCORBATE-2-SULFATE SULFOHYDROLASE FROM BOVINE LIVER

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Summary. The hydrolysis of ascorbate-2-sulfate by the enzyme, ascorbate-2-sulfate sulfohydrolase, purified from bovine liver has been shown to be powerfully inhibited by ascorbate-2-phosphate. The inhibition by ascorbate phosphate is competitive with a K_I of 0.3 μM. Na_2HPO_4 also inhibits by an apparent non-competitive process. The Na_2HPO_4 concentration at 50% inhibition is 7.7 μM. A possible control role for ascorbate phosphate in ascorbate biochemistry is suggested.

Ascorbate-2-sulfate is a naturally occurring metabolite of ascorbic acid which was first found in brine shrimp cysts and since then in the urine of man, monkeys, rats, and guinea pigs (1, 2, 3, 4, 5). It is probably a storage form of ascorbate in trout, and in fact relieves scurvy in trout (2).

The enzymic hydrolysis of ascorbate sulfate was first observed by Bullen using a crude arylsulfatase A preparation from bovine liver (6). Since then, ascorbate sulfate sulfohydrolase has been partially purified and characterized from bovine liver (7, 8) and from a marine gastropod (9, 10, 11). In both of these cases, the ascorbate sulfate sufohydrolase activity co-purified with arylsulfatase A activity measured using p-nitrocatechol sulfate as a substrate. Ascorbate sulfate is a good substrate for purified arylsulfatase A (12). Both ascorbate sulfate sulfohydrolase and arylsulfatase activities are inhibited by ascorbate phosphate and by Na_2HPO_4. However, arylsulfatase is inhibited to a lesser degree than ascorbate sulfate sulfohydrolase (unpublished data). In the case of the marine gastropod, ascorbate

sulfate sulfohydrolase is strongly inhibited by inorganic phosphate and by several nucleotides (11).

We report here a very powerful inhibition of a highly purified ascorbate sulfate sulfohydrolase from bovine liver by ascorbate phosphate and Na_2HPO_4. The inhibition by ascorbate phosphate is stronger than the inhibition by Na_2HPO_4. The possible role of ascorbate phosphate in ascorbate biochemistry is discussed.

MATERIALS AND METHODS

Ascorbate-2-sulfate and Ascorbate-2-phosphate. Ascorbate-2-sulfate and ascorbate-2-phosphate are prepared by direct sulfation or phosphorylation using $(CH_3)_3N{\cdot}SO_3$ (5) and $POCl_3$ respectively.

Ascorbate-2-sulfate Sulfohydrolase. The enzyme used in these studies has been purified 40,000 fold from bovine liver by procedures which are in preparation for publication. Arylsulfatase A activity co-purifies with ascorbate sulfate sulfohydrolase throughout these procedures.

Enzyme Assay. The enzyme assay consists of following the reduction of 2,6-dichloroindophenol by the ascorbate which is produced during the enzymic hydrolysis. The decrease in absorbance is followed at 516 nm which is the isobestic point of 2,6-dichloroindophenol. The pH of the assay reaction is pH 4.8 which is also the optimum. Since ascorbate sulfate is slowly acid hydrolyzed at this pH, a non-enzymic hydrolysis is used as a control. In the kinetic studies, 1.5 ml of 0.15 mM 2,6-dichloroindophenol in 0.05 M KAc pH 4.8 are placed in a 3 ml cuvette. To this is added an appropriate amount of 70 mM dipotassium ascorbate sulfate, 0.5 M NaAc or KAc, and water to give a total volume of 2 ml and an ionic strength of 0.1. Trisodium ascorbate phosphate and Na_2HPO_4 are added so that their final concentrations are from 0.2 to 0.4 μM and from 7.7 to 14.8 μM respectively. To the cuvette is added 0.05 ml of a 40/1 dilution of purified enzyme solution which had an initial protein concentration of 0.26 mg/ml (determined by the Lowry method using BSA as a standard). For each substrate concentration the non-enzymic hydrolysis rate is subtracted from the hydrolysis rate with the enzyme to give the true rate of enzymic hydrolysis. The rate of reaction at each substrate concentration is measured three to four times.

RESULTS AND DISCUSSION

Figure 1 (Top) shows that the Km for ascorbate sulfate is 12 mM, and that the inhibition by ascorbate phosphate is competitive and very powerful with a K_I of 0.3 μM. Figure 1 (Bottom) shows the inhibition by Na_2HPO_4. The intersection point is to the left of the 1/v axis which indicates non-competitive inhibition. However, the area of intersection is close to the zero line and a small systematic error could give competitive inhibition. Thus the type of inhibition by Na_2HPO_4 is questionable. The reaction is inhibited 50% by 7.7 μM Na_2HPO_4.

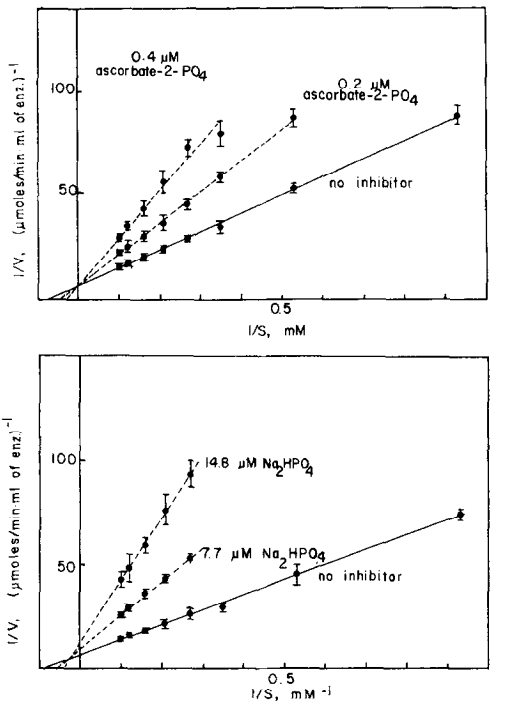

Figure 1. Top: A Lineweaver-Burke plot showing the inhibition of ascorbate-2-sulfate sulfohydrolase by ascorbate-2-phosphate. Bottom: A Lineweaver-Burke plot showing the inhibition of ascorbate-2-sulfate sulfohydrolase by Na_2HPO_4. The dashed lines are with the indicated inhibitor concentration and the solid lines are without inhibitor.

These results are summarized on Table I. The inhibition by ascorbate phosphate is approximately 25 fold more powerful than the inhibition by Na_2HPO_4. The K_I for ascorbate phosphate is 40,000 fold lower than the Km. The powerful inhibition of ascorbate sulfate sulfohydrolase by ascorbate phosphate with a K_I of 0.3 μM shows that ascorbate phosphate is bound very tightly to the enzyme. The fact that this binding is 25 fold more powerful than it is for Na_2HPO_4 and that the type of inhibition by ascorbate phosphate is different from Na_2HPO_4 suggests that the binding of ascorbate phosphate is quite specific.

Ascorbate phosphate is an interesting molecule. It is stable for weeks in neutral aqueous solution. It can be used to cure scurvy in the guinea pig (13). It is subject to rapid hydrolysis by alkaline phosphatase (unpub-

TABLE I

Inhibition of Ascorbate-2-Sulfate Sulfohydrolase by Na_2HPO_4 and by Ascorbate-2-Phosphate.

Inhibitor	K_I or Inhibitor Concentration at 50% Inhibition $[I]_{50\%}$	Type of Inhibition
None	Km = 12 mM	---
Ascorbate-2-phosphate	K_I = 0.3 μM	Competitive
Na_2HPO_4	$[I]_{50\%}$ = 7.7 μM	Non-competitive[a]

[a]The type of inhibition by Na_2HPO_4 is questionable. This is explained in the text.

lished data) and probably many other phosphatases since it is unstable in biological fluids. Ascorbate phosphate should be a moderately high energy compound, yet it would have a short life time in biological tissue. These properties, coupled with the very tight binding of ascorbate phosphate reported above, lead us to suggest that ascorbate phosphate may function as an active form of ascorbic acid under biological conditions.

Ascorbate phosphate could serve as a control compound for the ascorbate sulfatase function described in this report. An example of such a control might be present in the trout. Ascorbate sulfate prevents scurvy in trout and salmon (2) by a process in which ascorbate is derived from ascorbate sulfate. Ascorbate phosphate is a likely candidate for control of the regeneration if the enzyme is similar to that described in this paper. The enzyme described in this paper, ascorbate sulfate sulfohydrolase, seems to be part of the arylsulfatase A enzymes (EC 3.1.6.1) and its major biological roles are uncertain. Details of the properties of this enzyme will be published later.

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