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THE pH DEPENDENCE OF THE INHIBITION OF ASCORBATE OXIDASE BY ANIONS

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SUMMARY

Double-reciprocal plots of azide inhibition, with respect to ascorbate, of ascorbate oxidase indicate mixed-type inhibition at pH values above 6. This is in contrast to the simple competitive inhibition previously observed at pH 5.6. Linear replots of the slopes and intercepts of the double-reciprocal plots yield two inhibition constants. Both constants are pH dependent. Similar inhibition patterns are obtained with fluoride and thiocyanate. These results suggest the presence of two inhibitor binding sites, one of which is competitive with respect to ascorbate and the other uncompetitive.

INTRODUCTION

Ascorbate oxidase (E.C. 1.10.3.3.) belongs to the group of copper-containing enzymes referred to as the "blue" oxidases. The other members of this group are laccase and ceruloplasmin. These enzymes catalyze the four electron reduction of molecular oxygen to water (1). All of the "blue" oxidases possess types 1, 2, and 3 copper, as defined by Malkin and Malmstrom (2), and catalyze one-electron oxidation of the reducing substrate (3). The mechanism of the reaction is not understood at present but at least some and perhaps all of the copper ions undergo oxidation and reduction during the catalytic cycle (4,5). The "blue" oxidases are inhibited by a variety of anions capable of coordinating to the active site copper, but the number of anion binding sites remains unclear (6,7).

Inhibition of ascorbate oxidase by azide and fluoride at pH 5.6 was found to be competitive with respect to ascorbate (8). We have studied the inhibition by azide over the pH range 5.6-8.0 and we find that the pattern of inhibition at the higher pH values is more complicated than previously reported at pH 5.6. The same is true of inhibition by fluoride. We have also

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found that thiocyanate inhibits ascorbate oxidase in a manner similar to azide and fluoride. These results suggest two anion binding sites in ascorbate oxidase.

MATERIALS AND METHODS

Ascorbate oxidase was purified from zucchini squash (Cucurbita pepo medullosa) as previously described (9). The copper content was 0.31% by weight. The initial rate of ascorbate oxidation was measured using an oxygen electrode. All measurements were conducted in phosphate-citrate buffer at 25.0°C (8). The concentration of the buffer varied with the pH in order to maintain a constant ionic strength of 1.0. Inhibition constants measured in acetate or phosphate buffers did not differ, within experimental error, from those determined in phosphate-citrate buffer.

The buffer contained 0.05% bovine serum albumin to stabilize the enzyme. Solutions of ascorbic acid contained 0.1% metaphosphoric acid (10). Concentrated solutions of inhibitors were prepared in deionized water and small aliquots added to the reaction mixture just before the start of the reaction, which was initiated by adding the substrate.

All reagents were of the highest purity available and were used without further purification. Distilled, deionized water was used in preparing all solutions.

RESULTS AND DISCUSSION

Figure 1 shows a double reciprocal plot for azide inhibition at pH 7.17 in air-saturated buffer. Mixed-type inhibition is clearly evident. Similar plots were obtained at all pH values above 6. Two inhibition constants, referred to as K_{slope} and $K_{intercept}$, were obtained from linear replots of the slopes and y-intercepts respectively of the double reciprocal plots vs. azide concentration. At pH 5.60 azide was found to show simple competitive inhibition, in agreement with earlier work (8). The kinetic data can be described by the equation

$$\frac{1}{v} = \frac{K_M}{V_{max}}\left(1 + [I]K_{slope}\right)\frac{1}{[ascorbate]} + \frac{1}{V_{max}}\left(1 + [I]K_{intercept}\right)$$

where v is the initial rate of the reaction, [I] is the inhibitor concentration, K_{slope} and $K_{intercept}$ are the two inhibition constants and K_M and V_{max}, which are functions of the fixed oxygen concentration, have the usual meaning.

Figure 2 shows a plot of K_{slope} and $K_{intercept}$ vs. pH. K_{slope} shows a very substantial pH dependence. $K_{intercept}$ could only be measured at the higher

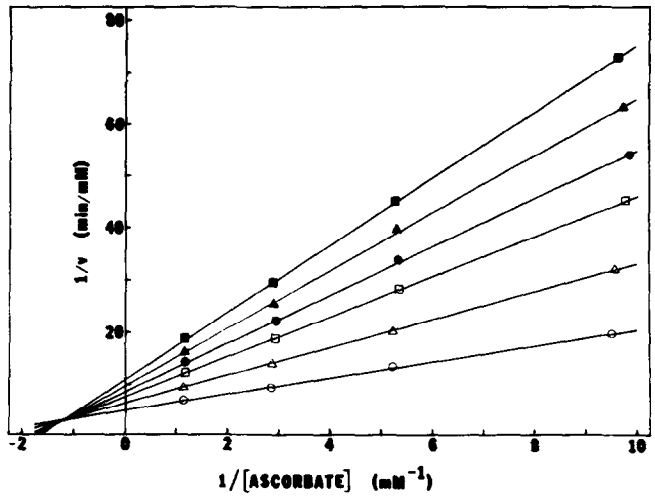

Figure 1. Inhibition of ascorbate oxidase with respect to ascorbate at pH 7.17 and 25°C with 0 (○), 0.820 (△), 1.67 (□), 2.41 (●), 3.33 (▲), and 4.21 mM (■) azide. Each data point is the average of at least two determinations and the lines were obtained by the least squares method.

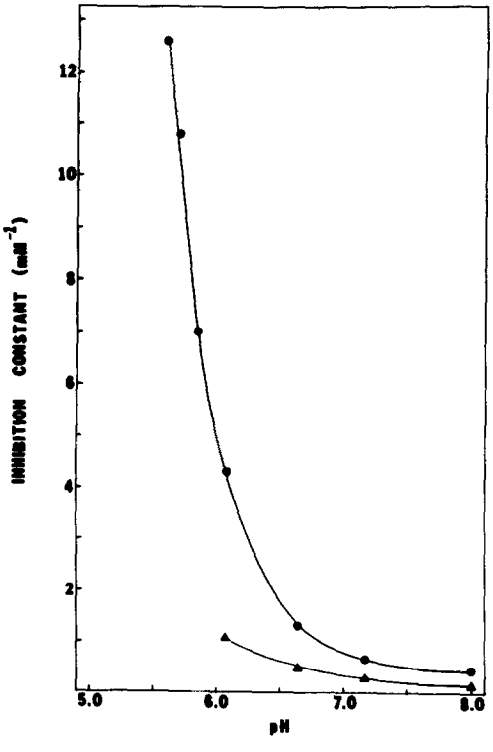

Figure 2. The pH dependence of K_{slope} (●) and $K_{intercept}$ (▲) for azide inhibition of ascorbate oxidase.

pH values and it also decreases somewhat with pH. K_{slope} is more strongly pH dependent than $K_{intercept}$, at least at the acid end of the pH range investigated. Table I gives the inhibition constants for fluoride and thiocyanate. Fluoride, like azide, shows mixed-type inhibition at the higher pH values. Thiocyanate is a relatively weak inhibitor and gives a mixed-type inhibition pattern at all pH values investigated. The K_M for ascorbate is independent of pH over the range 5.6-8.0, in agreement with an earlier report (11). The K_M for oxygen is also independent of pH over this range (12).

The interaction of anions with oxidized ascorbate oxidase has been followed by changes in the visible absorption spectrum of the protein (8). Azide produces an increase in absorbance at 430 nm and a decrease in the 330 nm band of type 3 copper. Fluoride produces almost no change in the absorption spectrum but an analysis of azide binding in the presence of fluoride indicated partial competitive binding of the two anions. Either ion can bind in the presence of the other, but with a lower binding constant. Both type 2 and type 3 copper have been suggested as anion binding sites in the "blue" oxidases (7). ESR data indicate that fluoride and azide bind to type 2 copper of laccase (13,14). There is no evidence that anions bind to type 1 copper. Spectroscopically determined binding constants for azide and other

TABLE I

INHIBITION OF ASCORBATE OXIDASE BY THIOCYANATE AND FLUORIDE

pH	$K_{slope}(M^{-1})$	$K_{intercept}(M^{-1})$
	THIOCYANATE	
5.77	183	37
6.57	122	33
7.51	49	4
	FLUORIDE	
5.60	1640*	0*
7.00	395	39

*Reference 8.

anions are generally lower than kinetically determined binding constants for all of the "blue" oxidases (6,15).

Simple mixed-type inhibition suggests the possibility of two inhibitor binding sites. The following scheme can account for the kinetic data (16).

$$\begin{array}{ccccccc} E & + & S & \rightleftarrows & ES & \rightarrow\rightarrow & \text{PRODUCTS} \\ + & & & & + & & \\ I & & & & I & & \\ \updownarrow K_{slope} & & & & \updownarrow K_{intercept} & & \\ EI & & & & IES & & \end{array}$$

The inhibitor (I) can combine with two forms of the enzyme, E and ES, where S stands for ascorbate. E could be the fully oxidized enzyme but it need not be since it is likely that two one-electron transfers from ascorbate precede oxygen binding (8). The complex of I with E is competitive with respect to ascorbate while the complex of I with ES is uncompetitive. Thus, I would give mixed-type inhibition. The complexes EI and IES likely reflect azide binding to two distinct sites on the protein, as suggested by the differences in pH dependence of K_{slope} and $K_{intercept}$. Simultaneous binding of two azide ions is inconsistent with the linear replots of slopes and intercepts, since it would introduce an $[I]^2$ term in the kinetic expression.

There are two possible means of accounting for the difference in the data at pH 5.6 and that at pH > 6. The first is that the complex IES cannot form at pH 5.6. The second is that at pH 5.6 $K_{slope} >> K_{intercept}$ so that, with the range of inhibitor concentrations giving measurable reaction rates, the quantity $1 + [I]K_{intercept}$ is approximately one and the y-intercepts of the lines with and without inhibitor present are the same within experimental error. At higher pH values, K_{slope} and $K_{intercept}$ are more nearly equal, so mixed-type inhibition is observed.

The great difference between the kinetic and spectroscopic binding constants would suggest that anion inhibition involves a partially reduced form of the enzyme. The first step in the catalytic cycle involves reduction of type 1 copper (5), which may produce a conformational change in the protein

that facilitates anion binding. The sites of anion binding could be the same as in the fully oxidized enzyme. In the proposed scheme, the anion binding site that is competitive with ascorbate would be the type 2 copper, in agreement with earlier suggestions (8), and the uncompetitive site would be the type 3 copper.

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