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A KINETIC STUDY OF THE EFFECTS OF HYDROGEN PEROXIDE AND pH ON ASCORBATE OXIDASE

Ronald E. Strothkamp and Charles R. Dawson

Department of Chemistry, Columbia University, New York, N.Y. 10027

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SUMMARY

Addition of hydrogen peroxide to ascorbate oxidase results in formation of a complex which has been analyzed kinetically. Since reduction of molecular oxygen to water by this enzyme occurs in more than one step, the peroxide complex may be a mimic for a catalytic intermediate. The properties of the complex are similar to those reported for a peroxide complex with laccase. Changes in the activity of ascorbate oxidase as a function of pH indicate the presence of a group in the enzyme with an apparent pK of 7.8 which must be protonated in order for the enzyme to function. The ascorbate K_m is known to be insensitive to pH in this region and the present work indicates the same to be true for the oxygen K_m. It would appear that the rate determining step in the catalytic mechanism involves protonation of an intermediate.

INTRODUCTION

Ascorbate oxidase (EC 1.10.3.3, l-ascorbate:O_2 oxidoreductase), laccase, and ceruloplasmin comprise a class of enzymes known as "blue" copper oxidases, all of which possess many similar properties, and a comprehensive review on these enzymes has recently appeared (1). The reduction of the "blue" oxidases occurs via several one-electron transfers from a suitable substrate (2,3). The reduced enzyme can then reduce oxygen to water with the addition of four electrons, possibly via two, two-electron transfers (4). Assuming the reduction of molecular oxygen occurs in two steps, a peroxide-like intermediate must be formed. Evidence for such a species has been observed for laccase and ceruloplasmin (5,6). Also, addition of hydrogen peroxide to resting laccase results in the formation of a complex which may be related to a catalytic intermediate (7,8). Based on these results, it was felt that a study of the effect of hydrogen peroxide on ascorbate oxidase was in order. If all of the "blue" oxidases function in a similar manner, their reaction with

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hydrogen peroxide should be comparable. It was also felt that a close look at the pH dependence of the activity of ascorbate oxidase might reveal additional information concerning the mechanism of oxygen reduction. The results of both of these studies will be presented here.

MATERIALS AND METHODS

Ascorbate oxidase was obtained from green zucchini squash (Cucurbita pepo medullosa) as previously described (9), and was homogeneous by polyacrylamide gel electrophoresis at pH 9.5 performed according to the procedure of Davis (10). All reagents were of the highest purity available and all aqueous solutions were prepared with deionized water just prior to use. Protein concentration was determined according to the Lowry procedure (11), using bovine serum albumin as standard. Copper determinations were performed according to Stark and Dawson (12). Enzymatic activity was determined at 25° C in a 1.5 ml reaction volume using a Clark oxygen electrode on a Gilson Model KM Oxygraph, under concentration and buffer conditions previously described (13).

The formation of a hydrogen peroxide complex with ascorbate oxidase was followed by observing absorbance changes with a Cary 17 recording spectrophotometer, utilizing a thermostated cell holder maintained at 25 ±0.3° C. Initial experiments with full visible region scans showed the wavelength of maximum absorbance to be 350 nm. The buffer used throughout was 0.01 M phosphate pH 7.6. Hydrogen peroxide solutions were prepared by suitable deionized water dilutions of Merck 30% Superoxol. The actual peroxide concentration of these solutions was then determined by titration with a standardized permanganate solution as previously described (14). All peroxide solutions were prepared fresh daily.

The dependence of K_m and V_{max} on pH was determined using the Gilson Oxygraph and Clark oxygen electrode. The 1.5 ml reaction cell was thermostated at 25 ±0.2° C. The standard buffer used in the enzyme assay was 0.20 M phosphate-citrate pH 5.6. Portions of this buffer were titrated with phosphoric acid or sodium hydroxide to pH values between 5.1 and 8.3. All buffers contained 0.05% bovine serum albumin for greater enzyme stability. Ascorbic acid solutions contained 0.1% meta-phosphoric acid to ensure stability (13). A 1.5 ml volume of the appropriate buffer and 20 ul of diluted enzyme solution were placed in the oxygraph cell and equilibrated. Oxygen variation was accomplished by passing oxygen or nitrogen gas over the reaction mixture in the oxygraph cell until the desired oxygen concentration was achieved. This was followed by immediate initiation of the reaction with a fixed amount of ascorbic acid solution.

RESULTS AND DISCUSSION

Addition of hydrogen peroxide to ascorbate oxidase produces a broad absorption band centered at 350 nm with a $\Delta\varepsilon$ of 900 $M^{-1}cm^{-1}$ in phosphate buffer at pH 7.6. This $\Delta\varepsilon$ was calculated from a difference spectrum of treated minus untreated enzyme. The absorption band is fully formed within several minutes and decays over several hours. The spectrum of the complexed enzyme could be produced with near stoichiometric quantities of hydrogen peroxide. Additions

of catalase to the system to scavenge free hydrogen peroxide failed to produce any increase in the rate of decomposition of the enzyme-peroxide complex. After complete decay of the spectrum, the specific activity of the enzyme was unchanged from its initial value.

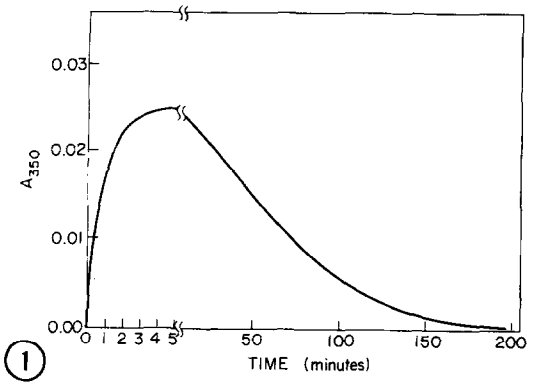
The rate of formation of the enzyme-peroxide complex was followed at 350 nm as a function of initial peroxide concentration. A plot of absorbance versus time for a typical experiment is shown in Figure 1. For formation of the complex, replots of $\log(A_{max} - A_t)$ versus time were linear and used to determine values of k_{obs} at a number of different hydrogen peroxide concentrations. The equilibrium constant can be found by determination of the forward and reverse rate constants for the process, which can be obtained from the following equation, as demonstrated for the enzyme tyrosinase (15).

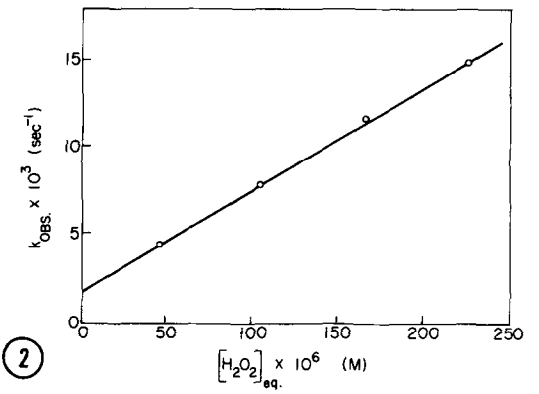
$$k_{obs} = k_1[\overline{H_2O_2}] + k_{-1} \quad (1)$$

The term $[\overline{H_2O_2}]$ is the equilibrium concentration.

Since high peroxide to enzyme ratios could not be used, initial peroxide concentrations could not be substituted in equation (1) for the unknown equilibrium values. However, by successive approximations, the binding constant was determined to be 3.3×10^4 M^{-1} and the rate constants are 58.5 $M^{-1}s^{-1}$ and 1.75×10^{-3} s^{-1} for k_1 and k_{-1}, respectively. With these parameters, the experimental results are shown in Figure 2. The points display good linearity. Based on the deviations seen with low peroxide to enzyme ratios for tyrosinase (15), this equilibrium constant should be considered a minimum value. Within the range of peroxide concentrations used in this study, no inhibition of enzymatic activity could be observed.

The dependence of enzymatic activity on pH was investigated using oxygen as the variable substrate. The results are shown in Figure 3. It is easily seen that the K_m for oxygen is insensitive to pH over the region examined. There is a random fluctuation of the K_m values with no correlation between





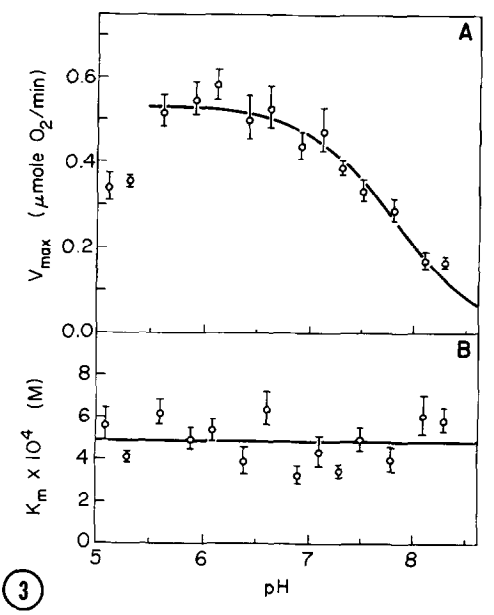


Figure 1. The absorbance changes at 350 nm versus time showing the formation and decay of a hydrogen peroxide complex of ascorbate oxidase. The enzyme concentration was 29 μM in 0.01 M phosphate (pH 7.6) at 25° C. The initial hydrogen peroxide concentration was 127 μM.

Figure 2. The relationship between the observed rate constant for formation of the hydrogen peroxide complex of ascorbate oxidase and the equilibrium peroxide concentration.

Figure 3. The pH dependence of V_{max} and of the oxygen K_m for ascorbate oxidase. The line in (A) represents a theoretical titration curve fitted to the data. The pK for this curve is 7.8. The line in (B) was determined by the method of least squares. All error bars indicate ± one standard deviation.

K_m and pH. On the other hand, the value of V_{max} drops off rapidly above pH 7. A titration curve was constructed, assuming V_{max} is zero at high pH. The best fit of this curve is shown as the solid line in Figure 3A and the resulting pK is 7.8. Thus there is a group present in the enzyme with an apparent pK of 7.8 which must be in its protonated form in order for the enzyme to function. The loss in activity which occurs below pH 5.5 is probably due to denaturation of the enzyme and loss of its copper, as well as a decreasing concentration of ionized ascorbic acid.

A complex between ascorbate oxidase and hydrogen peroxide has been demonstrated here and this complex could conceivably be a catalytic intermediate. In recent studies on fungal and Rhus laccase, a peroxide complex with that enzyme has been demonstrated to have properties similar to those reported here (7,8). Type 3 copper has been implicated as the peroxide binding site (8).

Gerwin, et al. (16) have studied the variance of V_{max} and the ascorbic acid K_m for ascorbate oxidase. They found the K_m for ascorbate to be constant in the region between pH 5 and 8.5. On the other hand, V_{max} was found to decrease above pH 7 and these workers suggested that a group with an apparent pK of about 8 was responsible for this effect. Based on shifts in this pK as a result of changing ionic strength and polyvalent anion concentrations, Gerwin, et al. (16) have concluded that the group involved is anionic in its unprotonated form.

Experiments involving the effect of pH on the K_m for oxygen have been carried out in the present work with the result that the K_m for oxygen, like the K_m for ascorbate, is insensitive to pH in the region between 5 and 8.5. The present work also confirms the decrease in V_{max} at alkaline pH and a titration curve fitted to the data yields an apparent pK of 7.8, in good agreement with the results of Gerwin, et al. (16).

Since the two K_m's for ascorbate oxidase are unaffected by pH but V_{max} is, it is possible that the rate determining step in the catalytic mechanism requires a proton from a protein group with a pK of 7.8. This is a rather un-

usual pK, being in a region where amino acid sidechains do not ordinarily ionize. Based on all the available data, the most likely sources of this pK are an unusually acidic tyrosine or cysteine, an unusually basic carboxyl group, or a water molecule bound to copper. This last possibility is very intriguing. The hexaaquocopper(II) ion has a pK of 6.8 (17). In the protein it is reasonable to assume that copper will have about four protein ligands with no more than two water molecules. Coordination through nitrogen ligands has been implicated most often for a number of copper proteins (18,19). Replacement of several water molecules of hexaaquocopper(II) ion by nitrogen ligands would be expected to cause an alkaline shift in the pK for the remaining water. It would seem quite possible to achieve a pK of 7.8 in such a system.

The results of this study, along with those of a previous paper implicating type 2 copper as a reductant binding site (20), lead to the following general proposal for the catalytic mechanism of ascorbate oxidase. Types 1 and 2 copper are primary electron accepting sites. The reduction of these two sites is followed by an essentially simultaneous transfer of both electrons to type 3 copper. A molecule of oxygen can then bind at the type 3 copper and receive these two electrons. Based on the results of the pH dependence studies, it is likely that a proton is also transfered at this point. This would result in the formation of the monoanion of hydrogen peroxide, HO_2^-, as a catalytic intermediate. Types 1 and 2 copper can again receive electrons and the second step in oxygen reduction can occur in a similar manner to the first.

Both ascorbate oxidase and ceruloplasmin are approximately twice the size of fungal laccase and each is composed of two, identical, half-molecular weight subunits (21,22,23). It appears that these two enzymes contain twice the amount of each type of copper that is present in laccase (21), although there is still some controversy on this point (24,25,26). It has been suggested that ascorbate oxidase contains two "laccase-type" active sites per molecule (21). This seems reasonable for ceruloplasmin as well. If this is so, the general catalytic mechanism proposed here for ascorbate oxidase may be applicable to all of the "blue" oxidases.

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