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The Effect of Hydrogen Peroxide on Glucose Oxidase from *Aspergillus niger**

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ABSTRACT: The effect of H_2O_2 on the flavoenzyme glucose oxidase from *Aspergillus niger* has been studied at pH 5.8 and 25°. The enzyme is inactivated by H_2O_2. The reduced form is much more sensitive to H_2O_2 and is inactivated at least 100 times more readily than the oxidized form. The rate of inactivation of the reduced form is not dependent on the concentration of D-glucose. Properties of the inactive enzyme have also been studied. The molecular weight of the inactive glucose oxidase is the same as for the untreated enzyme, whereas the spectrum differs slightly. However, no chemical alterations were detected in the free flavin-adenine dinucleotide group after it had been released from the enzyme. Amino acid analysis showed that when oxidized and reduced glucose oxidase were treated with H_2O_2 under identical conditions slightly more methionine sulfoxide was found in the reduced H_2O_2-treated enzyme than in the oxidized H_2O_2-treated enzyme. It is therefore suggested that the inactivation of the enzyme involves modification of certain methionine residues located at or near the active site. Possible mechanisms for the inactivation of the reduced enzyme are discussed.

Hydrogen peroxide has recently received increased interest in the area of enzyme chemistry because of its ability to modify certain amino acid residues in proteins. It has been shown that at acid pH values methionine is easily oxidized to methionine sulfoxide by H_2O_2 and at basic pH values tryptophan is destroyed. Koshland *et al.* (1962) have studied the effect of H_2O_2 on chymotrypsin and have shown that it oxidizes a single methionine residue situated two residues away from the active serine. This change in the enzyme resulted in a threefold increase in K_m, whereas V_{max} remained the same. Kassel (1964) and Neumann *et al.* (1962) have studied the effect of H_2O_2 on the basic trypsin inhibitor and ribonuclease. In both cases it was shown that oxidation of methionine to methionine sulfoxide had occurred. Hachimori *et al.* (1964) have investigated the effect of H_2O_2 at alkaline pH values on several proteins and found that tryptophan is destroyed.

Within the field of flavoproteins there are a number of flavoenzymes whose natural hydrogen acceptor appears to be oxygen and which therefore will produce H_2O_2 under aerobic conditions in the presence of suitable substrates. However, little is known about the effect of H_2O_2 on these proteins. Bernheim and Dixon (1928) noted some activation of xanthine oxidase with very low concentrations of H_2O_2 and decreased activity with higher concentrations of H_2O_2.

The present study was undertaken in an attempt to gain a better understanding of the effect of H_2O_2 on one of these enzymes, glucose oxidase from *Aspergillus niger* (E.C. 1.1.3.4). This enzyme catalyzes the reaction

$$\beta\text{-D-glucose} + \text{E-FAD} \longrightarrow \text{E-FADH}_2{}^{1} + \delta\text{-D-gluconolactone} \quad (1)$$

$$\text{E-FADH}_2 + O_2 \longrightarrow \text{E-FAD} + H_2O_2 \quad (2)$$

where E-FAD stands for one active site. The enzyme contains two FAD groups per molecule. It has recently been purified and the properties of it studied in some detail (Gibson *et al.*, 1964; Pazur and Kleppe, 1964; Pazur *et al.*, 1963; Pazur *et al.*, 1965).

It appears from the present work that H_2O_2 severely affects the reactivity of this enzyme. The reduced form of the enzyme is particularly sensitive to H_2O_2 and is inactivated much more rapidly than the oxidized form.

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[1] Abbreviations used in this work: FAD, flavin-adenine dinucleotide; $FADH_2$, reduced FAD; *p*-MB, *p*-mercuribenzoic acid.

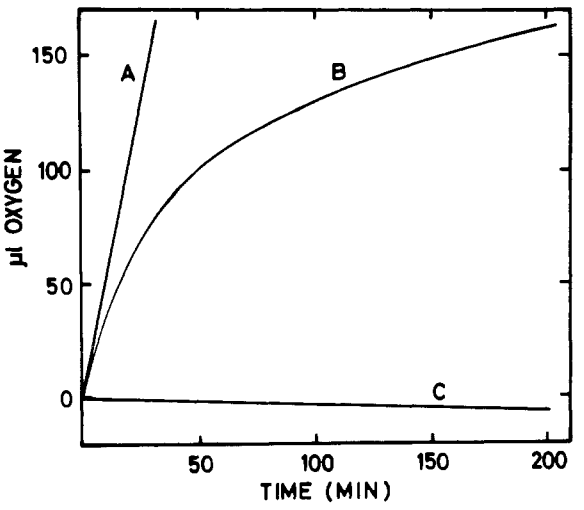

FIGURE 1: The effect of H_2O_2 on oxygen uptake. Reaction mixture consisted of: A, 0.5 mmole of D-glucose, 3.1 µg of glucose oxidase; B, 0.5 mmole of D-glucose, 3.1 µg of glucose oxidase, and 0.125 mmole of H_2O_2; and in C, 3.1 µg of glucose oxidase and 0.125 mmole of H_2O_2. Total volume was 2.5 ml and temperature was 25°. The buffer used was phosphate–citrate, pH 5.8.

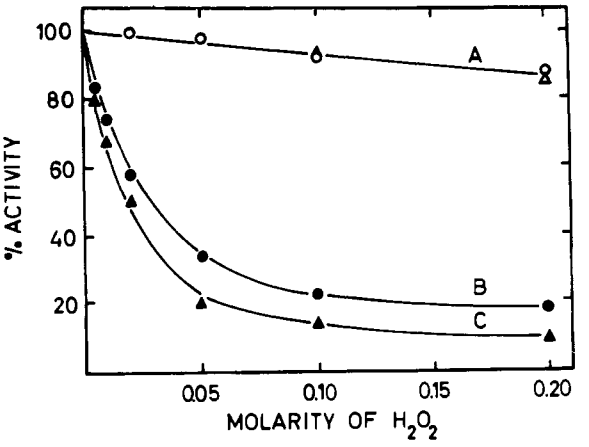

FIGURE 2: Effect of H_2O_2 concentration on the inactivation of oxidized and reduced enzyme. A, Effect on the oxidized enzyme under aerobic (○) and anaerobic (△) conditions; 3.1 µg of glucose oxidase incubated with various concentrations of H_2O_2 for 20 min at 25°; catalase, 0.1 ml, 0.01%, was then added and remaining activity measured. B, The same amount of enzyme in the presence of air, H_2O_2, and 0.208 M D-glucose. C, Effect on the same amount of reduced enzyme under anaerobic conditions in the presence of H_2O_2 and 0.208 M D-glucose. The buffer used was phosphate–citrate, pH 5.8. Further details are given in the Experimental Procedures.

Materials

Glucose oxidase was purified according to a published procedure of Pazur and Kleppe (1964). The starting material was crude glucose oxidase obtained from the Sigma Chemical Co.[2] The purified enzyme was always assayed for catalase activity by the Sigma procedure.[3] The pure glucose oxidase usually contained less than 5 Sigma units of catalase/mg of protein. One Sigma unit of catalase decomposes 1 µmole of H_2O_2/min at pH 7.0 at 25°, while the H_2O_2 concentration falls from 10.3 to 9.2 µmoles/ml of reaction mixture. In terms of per cent protein this is an exceedingly small impurity, since 1 mg of catalase contains about 40,000 units. When a dilute solution of glucose oxidase was treated with H_2O_2, the effect of this catalase impurity could be ignored. When a concentrated solution was used, KCN was added to a concentration of 10^{-3} M to inhibit the catalase. Cyanide itself does not affect the activity of glucose oxidase. The catalase used for assay of glucose oxidase activity was crystalline catalase obtained from Boehringer & Soehne. The sample of FAD was Sigma, grade III. All other chemicals used throughout this study were of analytical grade unless stated otherwise. The buffer system used was McIlvaine phosphate–citrate buffer, pH 5.8 (0.12 M Na_2HPO_4 and 0.04 M citric acid).

[2] It was verified in a personal communication from the Sigma Chemical Co. that the enzyme used was from *A. niger*.

[3] The procedure used is described in the catalog from the Sigma Chemical Co.

Methods

The glucose oxidase activity was conveniently measured by the conventional Warburg method. The total volume in the vessel was 2.5 ml and the concentration of D-glucose was 0.2 M. Readings were taken every 10 min. The oxygen electrode previously described (Dixon and Kleppe, 1965) was also used occasionally. The temperature used throughout this work was 25°.

Acid and basic hydrolysis of glucose oxidase were carried out according to the procedures of Neumann *et al.* (1962), and amino acid analyses of the hydrolysates were performed using a Technicon automatic amino acid analyzer.

Experimental Procedures and Results

Effect of H_2O_2 on Oxygen Uptake. The change in activity of glucose oxidase in the presence of H_2O_2 was conveniently studied by the Warburg manometric technique. The results of a typical experiment are shown in Figure 1. Curve A is the progress curve for oxygen uptake in the absence of H_2O_2. Curve B shows the progress curve for O_2 uptake for the same amount of enzyme in the presence of 0.05 M H_2O_2. Curve C is the oxygen liberation, not uptake, from 0.05 M H_2O_2 in the presence of the same amount of enzyme as in A and B. The oxygen liberation is due to thermal decomposition of H_2O_2 and to traces of catalase. It can be seen from curve B

that the rate of oxygen uptake decreases rather markedly in the first part of the reaction period. The fact that the enzyme was inactivated by H_2O_2 was further established by adding catalase after a certain period to destroy the H_2O_2 in the mixture, flushing the manometer vessel with air for 10 min while shaking, and measuring the activity again. The rate of oxygen uptake was found to be approximately half of the rate prior to the addition of catalase, and remained constant for at least 1 hr. This also tends to rule out the possibility that any competitive relationship existed between O_2 and H_2O_2 for the reduced flavin, at least at this concentration of H_2O_2.

Effect of H_2O_2 and D-*Glucose Concentration on the Inactivation of the Enzyme.* Figure 2 shows the change in the rate of inactivation with increasing H_2O_2 concentration under several different conditions. In these experiments a constant amount of enzyme was incubated in Warburg vessels with various concentrations of H_2O_2 in the absence and presence of D-glucose and O_2. After 20 min catalase was added to destroy the H_2O_2, and air was passed over the solutions while shaking for 10 min. The activity of the enzyme was then measured by adding D-glucose to a final concentration of 0.2 M, if this was not already present. Curve A shows the effect of various concentrations of H_2O_2 on the oxidized enzyme under aerobic and anaerobic conditions. Anaerobic conditions were maintained by letting 100% nitrogen flow through the manometric vessel while shaking 15 min prior to addition of H_2O_2, and it was continued during the whole reaction period, *i.e.*, for the next 20 min. In curve B D-glucose and oxygen (air) were present in addition to H_2O_2. In curve C D-glucose was present but no oxygen. The anaerobic conditions were maintained as described above, and under these conditions the enzyme in C should theoretically exist only in the reduced form. From these data it is apparent that the reduced form of the enzyme is particularly sensitive to H_2O_2, and low concentrations of H_2O_2 have virtually no effect on the oxidized form. However, at rather high concentrations of H_2O_2 the oxidized form is also destroyed to some extent. One must also conclude that the oxygen has no protective effect at least as long as the enzyme exists in the fully oxidized form. Regarding curves B and C the amount of D-glucose used in these experiments is far above K_m for D-glucose and should be very close to the concentration necessary for V_{max}. Thus, the differences between the two curves can be explained on the basis of kinetic factors; *i.e.*, in curve B not all the enzyme will exist in the fully reduced form at one time. The enzyme under these conditions is steadily being reduced and oxidized, and the amount of fully reduced form present will depend on kinetic constants and oxygen concentration. This difference in the rate of inactivation between B and C further supports the idea that it is the fully reduced form which is attacked rather rapidly by H_2O_2.

The effect of the concentration of D-glucose on the rate of inactivation of the reduced enzyme by H_2O_2 was also studied. The concentration of D-glucose varied from 0.005 to 0.4 M in these experiments, whereas the concentration of H_2O_2 remained constant and was 0.02 M. The results from these experiments clearly showed that the rate of inactivation of the reduced enzyme was not dependent on the concentration of D-glucose. This would seem to rule out the possibilities that the enzyme species attacked by H_2O_2 is an enzyme–substrate complex of some sort or that the attacking agent is not H_2O_2 but a D-glucose–H_2O_2 complex. It was verified by paper chromatography that H_2O_2 alone had no effect on D-glucose.

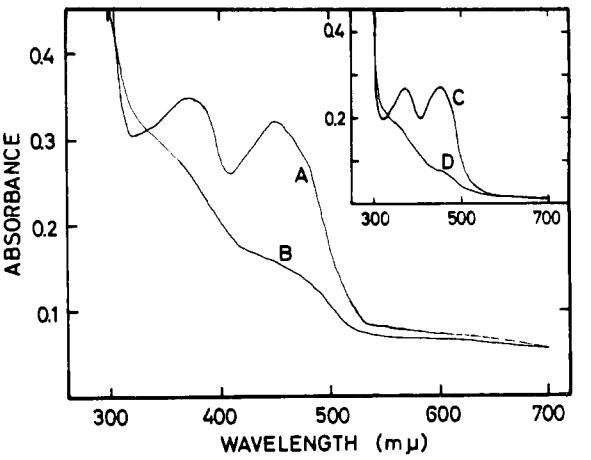

FIGURE 3: Spectrum of a sample of reduced H_2O_2-treated glucose oxidase at pH 5.8. The enzyme contained 2% of the original activity. A, Oxidized form and B, the enzyme reduced with D-glucose, recorded after 1 hr. C and D are spectra of a control sample of glucose oxidase not treated with H_2O_2; C, oxidized, and D, reduced form.

Properties of the H_2O_2-Treated Glucose Oxidase. A sample of inactive enzyme was prepared in the following way: To 10 ml of glucose oxidase in solution (0.15% protein) was added D-glucose to a concentration of 0.20 M, KCN to a final concentration of 10^{-3} M, and H_2O_2 to a final concentration of 0.1 M. The mixture was kept at 25° and the pH was maintained at 5.8 by addition of NaOH. After 1 hr, when there was little or no more change in the pH, the whole mixture was introduced on top of a Sephadex-G-25 column (30 × 3 cm). Elution was carried out by 0.01 M phosphate–citrate buffer, pH 5.8, and the enzyme was separated from H_2O_2, KCN, and D-glucose by this method. The enzyme was concentrated by freeze drying to a small volume and finally dialyzed overnight against 0.05 M phosphate–citrate, pH 5.8. It was kept in this buffer at 2°. The specific activity of the enzyme sample was 2% of the untreated sample. Further dialysis did not increase the specific activity. All attempts to reactivate the enzyme by equilibrating it for some period of time with such agents as FAD, mercaptoethanol, and cysteine failed.

The spectrum of the flavin part of the H_2O_2-treated enzyme was recorded at pH 5.8 and is shown in Figure 3, curve A. It is a typical flavin spectrum, but it differs slightly from the spectrum of the untreated enzyme,

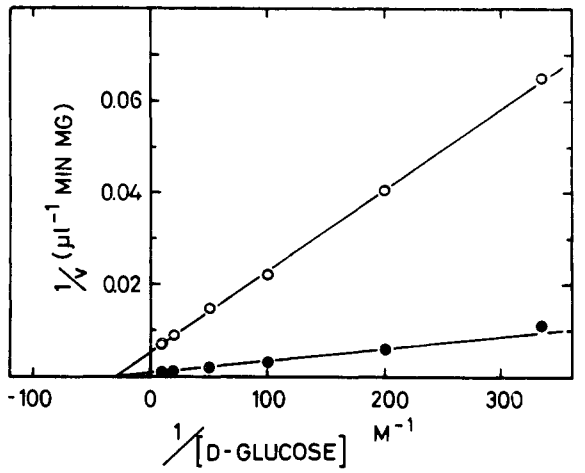

FIGURE 4: Lineweaver–Burk plots of an H_2O_2-treated enzyme sample (○) and the nontreated enzyme (●).

C (Figure 3), in that the ratio of the absorbancies of the peaks at 450 and 370 mμ are 0.9, whereas it is about 1 in the untreated enzyme. Also the absorbance in the region 550–700 mμ is somewhat larger for the modified enzyme. This difference in the spectrum could possibly be due to traces of semiquinones. The modified enzyme is reduced by D-glucose, but the rate of reduction is very small indeed compared with the nontreated enzyme. However, the reduced form still appears to be rapidly oxidized by air, suggesting that it is the reaction between D-glucose and the enzyme leading to the reduced enzyme which has been affected by the H_2O_2.

The K_m and V_{max} were measured for a partially inactivated glucose oxidase sample and control glucose oxidase. The result is shown in Figure 4. In this case the specific activity of the partially inactivated sample was 16% of the control. It can be seen that the K_m is the same for the two samples. It is only V_{max} which has changed.

The observation that the spectrum in the visible region has changed after treating the reduced enzyme with H_2O_2 would suggest that a chemical change has taken place in the FAD group. However, all attempts to demonstrate this have failed. FAD was removed from the enzyme by a modified procedure described earlier (Pazur and Kleppe, 1964) and chromatographed in two different solvent systems, 5% Na_2HPO_4 and collidine saturated with water. In both cases, the flavin from H_2O_2-treated enzyme moved identically with flavin from untreated glucose oxidase and reference FAD. Further experiments were carried out using the D-amino acid apooxidase, adding FAD from H_2O_2-treated and nontreated enzyme, and measuring the enzymatic activity on D-alanine. No significant differences were observed. The effect of H_2O_2 on reduced and oxidized FAD has also been investigated. No change in spectrum or R_F values from that of untreated FAD was noted.

Studies were carried out to determine whether or not the molecular weight had changed by H_2O_2 treatment. This was done using a Sephadex G-200 column. The effluent volume for the inactive enzyme was the same as for the control glucose oxidase. Thus, it would appear that the molecular weight is unchanged.

Amino Acid Analysis. Samples of H_2O_2-treated and nontreated glucose oxidase were subjected to acid and basic hydrolysis, and complete amino acid analyses were performed on the hydrolysates. These experiments revealed that methionine was the only amino acid affected by the H_2O_2 treatment, some of the methionine residues being oxidized to the corresponding sulfoxide residues. Table I gives the activity and the corresponding methionine sulfoxide content of control, oxidized H_2O_2-treated, and reduced H_2O_2-treated glucose oxidase. The reduced H_2O_2-treated enzyme which has lost nearly all the activity contains slightly more methionine sulfoxide than the oxidized H_2O_2-treated glucose oxidase, which has still retained most of the enzymatic activity. Thus it appears that certain methionine residues, perhaps two, are more easily converted to the sulfoxide when the flavin group exists in the reduced state than when it exists in the oxidized state on the enzyme. Furthermore, these methionine residues could be of importance in the catalytic reaction, suggesting that they may be in or near the active site(s).

Of interest is also the fact that the enzyme not treated

TABLE I: Activity and Methionine Sulfoxide Content of Glucose Oxidase.[a]

Enzyme Preparation	Activity (%)	No. of Methionine Sulfoxide Residues/Molecule[b]
Control (not H_2O_2 treated)	100	2.9
Oxidized glucose oxidase, H_2O_2 treated	74	7.1
Reduced glucose oxidase, H_2O_2 treated	0.5	8.6

[a] Glucose oxidase was treated with H_2O_2 in the following way: 10 mg of glucose oxidase was added to a mixture consisting of 10^{-3} M KCN, 0.2 M H_2O_2, 0.2 M D-glucose, when used, 0.1 M phosphate–citrate buffer, pH 5.8. The total volume was 10 ml and the mixture was kept at 25° for 30 min. The protein was then separated from other components by gel filtration on a column of Sephadex G-25, assayed for activity, concentrated by freeze drying, and subjected to alkaline hydrolysis. [b] Glucose oxidase contains 21 methionine residues, estimated on the basis of a molecular weight of 150,000 (Pazur *et al.*, 1965).

with H_2O_2 contains appreciable amounts of methionine sulfoxide. This is to be expected, however, since one of the products of the enzymatic reaction is H_2O_2. Neumann *et al.* (1962) have determined the methionine sulfoxide content of a number of nonflavoproteins, but found only traces of it in most of these.

Discussion

From the results of the present study it must be concluded that glucose oxidase from *A. niger* is inactivated by H_2O_2; the inactivation is much more rapid when the flavin groups on the enzyme exist in the reduced state than when they are in the fully oxidized state. The ease of inactivation of the reduced enzyme would suggest that the inactivation involves modification of certain groups, probably located at or near the active site.

The flavin group, removed from the inactive H_2O_2-treated enzyme, was found to be unchanged, using several methods of analysis. The inactivation of the enzyme therefore most likely is due to modification of certain amino acid residues in the protein, namely methionine residues. This view is supported by the findings from the amino acid analyses which showed that oxidation of methionine residues occurred both in the reduced H_2O_2-treated and oxidized H_2O_2-treated enzyme. Slightly more methionine sulfoxide was found in the reduced H_2O_2-treated enzyme than in the oxidized H_2O_2-treated enzyme. The inactivation does not seem to involve SH groups since the enzyme is not reactivated by mercaptoethanol and further does not react with nor is inhibited by *p*-MB or other mercurials, neither in the reduced nor in the oxidized form.[4]

At the present time several possible explanations can be put forward to account for the great difference in the rate of inactivation between the reduced and oxidized form of the enzyme. It might well be that reduced and oxidized glucose oxidase differ in conformation, and thus in the reduced form of the enzyme certain methionine residues could be more exposed to H_2O_2 than when the flavin group exists in the oxidized state. Another explanation is that the state of oxidation of the flavin group somehow influences the rate of oxidation of certain methionine residues by changing the electron density on some amino acid residues without any change in conformation. A third possibility, which also should be considered, is that H_2O_2 may react with reduced glucose oxidase to give semiquinones and OH radicals according to the following equation.

$$\text{E-FADH}_2 + H_2O_2 \longrightarrow \text{E-FADH}\cdot + \text{OH}\cdot + H_2O \quad (3)$$

The OH radicals could be the oxidizing agent rather than H_2O_2. The semiquinone would not be expected to be very stable in the presence of H_2O_2 but be oxidized rapidly to the fully oxidized enzyme. Regarding the semiquinone form of the enzyme it has recently been shown by Massey and Gibson (1964) that it is catalytically inactive. Only the fully oxidized and fully reduced forms of glucose oxidase are of importance in enzymatic catalysis.

[4] K. Kleppe, unpublished results.

Acknowledgment

The author wishes to express his appreciation to Dr. C. J. R. Thorne for performing the amino acid analyses and also to Dr. Malcolm Dixon, Dr. K. F. Nakken, and Professor A. Pihl for their interest and helpful suggestions. He is also grateful to Professor F. G. Young for providing space in his department in Cambridge and to the Norwegian Council for Scientific and Industrial Research for a research fellowship.

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