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STUDIES ON GLUCOSE DEHYDROGENASE OF *ASPERGILLUS ORYZAE*

IV. HISTIDYL RESIDUE AS AN ACTIVE SITE

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SUMMARY

1. Glucose dehydrogenase from *Aspergillus oryzae* was inhibited by Ag^+, Hg^{2+} and Cu^{2+}, but was insensitive to sulfhydryl reagents.

2. The involvement of a histidyl residue (or residues) in the activity was suggested from photoinactivation in the presence of methylene blue, the pH dependence of K_m for glucose, and titration with the histidine-specific reagent, diazo-1-H-tetrazole (DHT).

3. The inhibition by Ag^+ was reversed by dialysis and by addition of a high concentration of glucose. The activity of DHT-inhibited enzyme was not restored by dialysis, and high concentrations of glucose partly protected the enzyme from DHT inhibition only when added prior to the addition of DHT.

4. The reduction of the enzyme-bound FAD by glucose was prevented in the Ag^+- or DHT-treated enzyme, but the reoxidation of the reduced flavin by vitamin K_3 was not impaired in the treated enzyme.

5. It was concluded that a histidyl residue is involved in the reduction of the flavin by glucose, acting probably as the glucose-binding site.

INTRODUCTION

Glucose dehydrogenase of *Aspergillus oryzae* has recently been isolated as a homogeneous glycoprotein containing 1 mole of FAD per mole of enzyme, and its chemical and physicochemical properties have been studied in detail[1]. The enzyme-bound flavin has been shown to participate in the electron transfer from glucose to electron acceptors such as dyes and quinones, and a mechanism of two-electron transfer has been proposed for the oxidation and reduction of the flavin[2]. It was, however, desirable to elucidate the sites on the enzyme to which glucose and acceptor are bound. In this paper, we present evidence which suggests that a histidyl residue

Abbreviations: DCIP, 2,6-dichlorophenolindophenol; DHT, dizao-1-H-tetrazole.

is present at the active site involved in the reduction of the flavin by glucose, probably acting as the glucose-binding site.

MATERIALS AND METHODS

Enzyme preparation

The homogeneous preparation of glucose dehydrogenase described in a previous paper[1] was used. The concentration of the enzyme was estimated from the absorption peak of enzyme-bound FAD at 460 mμ, assuming a molar extinction coefficient of $11.3 \cdot 10^6$ cm^2 mole^{-1} (ref. 3).

Measurement of enzyme activity

Unless otherwise stated, the activity of glucose dehydrogenase was measured at pH 6.5 as described previously[1], using 0.2 M D-glucose as hydrogen donor and 0.1 mM 2,6-dichlorophenolindophenol (DCIP) as acceptor. One unit of the enzyme was defined as in the previous paper[1].

Photooxidation

A flask containing 0.45 ml of a solution which contained 52 μg of purified glucose dehydrogenase, 0.02% methylene blue, and 0.01 M phosphate buffer (pH 6.5) was placed at a distance of 20 cm from a 375 W spot-light lamp. The solution was kept cold with ice–water during irradiation. Control experiments were carried out in the absence of the dye or in a vessel shielded to shut out light.

Treatment with diazo-1-H-tetrazole (DHT)

DHT was freshly prepared as described by HORIKOSHI *et al.*[4]. A solution containing $9 \cdot 10^{-6}$ M glucose dehydrogenase in 2.0 ml of 0.75 M bicarbonate buffer (pH 8.8) was placed in a photometric cuvette, and titrated with DHT by stepwise addition of 0.01-ml aliquots of 30 mM DHT. After each addition, the number of histidyl residues titrated was determined from the increase in absorbance at 480 mμ, assuming a molar extinction coefficient of $1.72 \cdot 10^4$ cm^2 mole^{-1} for the bis-azohistidine residue[4]. The activity of the DHT-treated enzyme was also measured at pH 6.5 after 10 000-fold dilution. A control experiment without the addition of DHT was carried out simultaneously.

RESULTS

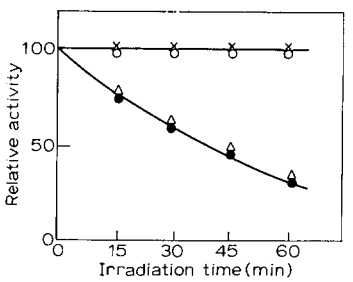
Effects of inhibitors

As reported by KURASAWA AND IGAUE[5], the reduction of DCIP by glucose catalyzed by *A. oryzae* glucose dehydrogenase was sensitive to heavy metal ions such as Ag^+, Hg^{2+} and Cu^{2+}. The inhibition by Ag^+ was most pronounced (98% inhibition by 0.02 mM $AgNO_3$ at pH 6.5). Although the sensitivity to these metal ions suggested the participation of a sulfhydryl group in the catalysis, typical sulfhydryl reagents such as *p*-chloromercuribenzoate (1 mM), *p*-chloromercuriphenylsulfonate (1 mM) and $CdSO_4$ (10 mM) failed to inhibit the activity even after preincubation of the enzyme with the reagents. These results were in agreement with the results of spectrophotometric titration of the enzyme with *p*-chloromercuribenzoate by the

method of BOYER[6], in which no sulfhydryl groups could be detected. Atebrine (3 mM), which is an inhibitor of several flavoprotein enzymes[7], also inhibited *A. oryzae* glucose dehydrogenase by 66%, but this inhibition was not reversed by the addition of FAD. The enzyme was completely insensitive to such metal chelating agents as KCN (20 mM), α,α'-dipyridyl (2 mM), *o*-phenanthroline (2 mM), EDTA (5 mM), and 8-hydroxyquinoline (1 mM) as well as to inhibitors of the mitochondrial electron-transfer pathway, including 2-nonyl-4-hydroxyquinoline-*N*-oxide (10 μg/ml), antimycin A (10 μg/ml), and Amytal (2 mM). Dicumarol (0.05 mM), and thiourea (10 mM) also showed no effects.

Effect of photooxidation

Since the sensitivity to heavy metal ions was not due to the presence of an essential sulfhydryl group, as discussed above, it was suspected that the activity was dependent upon a histidyl residue, which can also combine with Ag^+, Hg^{2+}, *etc.* This possibility was, therefore, tested by subjecting the enzyme to photooxidation in the presence of methylene blue, a technique which has frequently been used to destroy histidyl residues in proteins[8–11]. As shown in Fig. 1, irradiation of the enzyme for 60 min in the presence of methylene blue caused about 70% inhibition of the activity, and this inactivation was not affected by the presence of 0.2 M glucose. When irradiation was carried out in the absence of the dye, however, or when the vessel was shielded to shut out light, no inactivation was observed. These results suggested the involvement of a histidyl residue in the enzymatic activity, though the destruction by irradition of other residues, such as tryptophyl and tyrosyl, could not be ruled out[12–14].



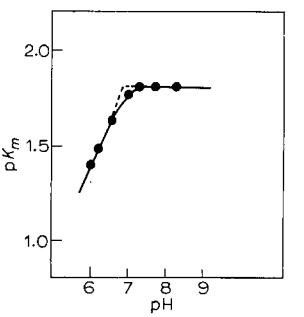


Fig. 1. Decrease in enzyme activity by photooxidation in the presence of methylene blue. The complete system contained: 0.1 M potassium phosphate buffer (pH 6.5), 0.02% methylene blue, 52 μg of the enzyme in a total volume of 0.45 ml. Irradiation was carried out for various periods of time as described in MATERIALS AND METHODS, and the residual activity was measured. △—△, complete system; ●—●, 0.2 M glucose added; ○—○, methylene blue omitted; ×—×, complete system shielded to shut out light.

Fig. 2. Dependence on pH of Michaelis constant for glucose. The reaction mixture contained 0.1 mM DCIP, glucose dehydrogenase, 0.2 M phosphate or Tris–HCl buffer of various pH's, and varying concentrations of glucose in a final volume of 1.5 ml. Michaelis constants for glucose (K_m) were determined by means of Lineweaver–Burk plots. pK_m is $-\log K_m$ (in molar concentration).

Effect of pH on Michaelis constant

Analysis of the pH dependence of the catalytic activity often gives a clue to the nature of the reactive groups in enzymes[14]. It was found that the Michealis

constant of glucose dehydrogenase for glucose, determined by means of Lineweaver–Burk plots at a DCIP concentration of 0.1 mM, was affected by pH in a characteristic way. As shown in Fig. 2, the pK_m value (—log K_m expressed in terms of molar concentration; K_m, Michaelis constant) remained constant at the alkaline side of pH 7, but decreased linearly in the acidic region. Such characteristics of the pK_m–pH curve seemed to be compatible with the assumption that a histidyl residue, rather than a cysteinyl, tyrosyl or tryptophyl residue, is present at the active site of the enzyme[13,14].

It was further found that the inhibition of glucose dehydrogenase by $AgNO_3$ was dependent on pH. This dependence could also be explained by an inhibition mechanism involving complex formation between Ag^+ and an essential histidyl group, as was also the case with invertase[15,16].

Effect of DHT

DHT is a new coupling reagent developed for specific spectrophotometric determination of protein histidyl residues[4]. This reagent also reacts slowly with tyrosyl residues, but the coupling with histidyl residues is completed at a DHT concentration permitting no reaction with tyrosyl residues[4]. The activity of glucose dehydrogenase was found to be very sensitive to the treatment with DHT. This inhibition could, however, be reversed by the presence of exogenous histidine. Curve A of Fig. 3 shows the result of spectrophotometric titration of glucose dehydrogenase with DHT. As can be seen, the absorbance increment at 480 mμ, due to bis-azohistidine which is the coupling product of histidine with DHT[4], increased in two steps; in the first step 7 histidyl residues seemed to be titrated and 2 more residues reacted in a separate step. Thus, the total number of titrable histidyl residues was 9 per mole of enzyme, in agreement with the histidine content obtained by amino acid analysis[1]. A similar titration curve was also obtained with the enzyme preparation which had been denatured in 0.05 M NaOH at 0° for 20 h, except that the critical DHT concentration required was somewhat lower. This suggested that the histidyl residues were in similar states in both the native and denatured enzyme. As shown in Curve B of Fig. 3, the glucose dehydrogenase activity was strongly inhibited on

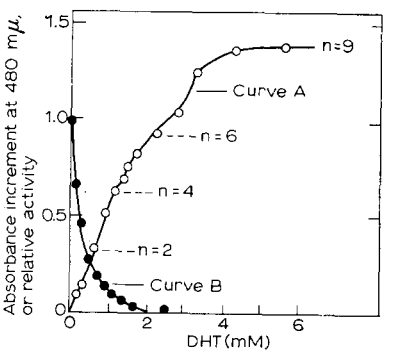


Fig. 3. Titration of glucose dehydrogenase with DHT. Glucose dehydrogenase ($9 \cdot 10^{-6}$ M) in 0.75 M bicarbonate buffer (pH 8.8) was titrated with DHT as described in MATERIALS AND METHODS. Curve A, absorbance increment at 480 mμ. Curve B, glucose dehydrogenase activity. n indicates the number of histidyl residues titrated with DHT.

addition of DHT; complete inactivation occurred when 2 mM DHT had been added. Comparison of this result with Curve A indicated that the activity could be eliminated when less than 5 histidyl residues per mole of enzyme had been blocked by DHT. From these data, it was concluded that a histidyl residue or residues are actually involved in the catalytic mechanism.

Restoration of activity by dialysis

The inhibition of the enzyme by Ag^+ was reversible as is evident from the following experiments. The enzyme (6 μg) was mixed with 0.1 mM $AgNO_3$ in 0.1 M phosphate buffer (pH 6.5) effecting almost complete loss of activity. The mixture was then dialyzed at 4° for 18 h against (a) 0.05 M phosphate buffer (pH 6.5) alone, (b) the same buffer containing 1 mM histidine, or (c) the same buffer containing 1 mM cysteine. After dialysis, the last sample was once more dialyzed against the same buffer without cysteine. Control experiments were similarly run without the inhibitor. It was thus found that the activity of the Ag^+-inhibited preparation was restored to 30, 40 and 100% of the control after dialysis against buffer alone, buffer *plus* histidine, and buffer *plus* cysteine, respectively. This was explicable, because it is known that the histidine–silver complex is considerably dissociable, and that the affinity of cysteine for Ag^+ is higher than that of histidine. The inhibition by DHT was not reversed by these methods, because of the covalent bond formation between the histidyl residue and DHT[4].

Protective effect of glucose on inhibition by Ag^+ and DHT

As shown in Table I, the inhibitory effects of Ag^+ and DHT were reduced by the coexistence of high concentrations of glucose. In the case of DHT inhibition, reversal was observed only when glucose was added prior to the addition of DHT. On the other hand, the addition of glucose to the Ag^+-treated preparation also resulted in considerable restoration of the activity. These results not only confirmed the reversibility of Ag^+ inhibition (but not DHT inhibition) but also suggested that the essential histidyl group might be the site of glucose binding to the enzyme.

TABLE I

PROTECTIVE EFFECT OF HIGH CONCENTRATIONS OF GLUCOSE ON INHIBITION BY Ag^+ AND DHT

For DHT inhibition, 6 μM DHT was added to a mixture consisting of 0.1 M phosphate buffer (pH 6.5), glucose at indicated concentration, and 0.1 μg of purified glucose dehydrogenase, and the mixture was incubated for a few minutes. The enzyme activity was then determined by adding 0.1 mM DCIP. For Ag^+ inhibition, 2 μM $AgNO_3$ was brought into contact with the enzyme in buffer prior to glucose, and after brief incubation DCIP was added to start the reaction. Final volume of the reaction mixture was 1.5 ml. Per cent inhibition was calculated by comparing the rate with that observed in a control experiment without the inhibitor.

Glucose (M)	*Per cent inhibition by*	
	DHT	*$AgNO_3$*
0.1	84	91
0.2	82	89
0.4	72	—
0.5	64	—
0.8	—	73

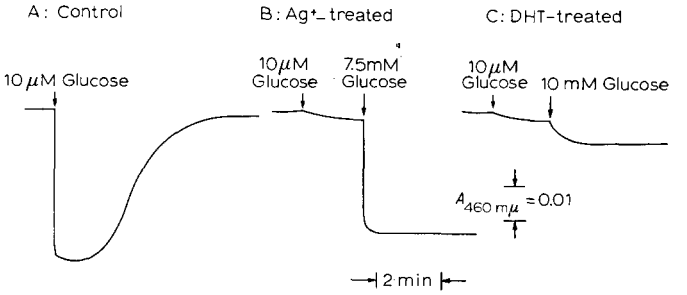


Fig. 4. Effects of Ag^+ and DHT on the reduction of enzyme-bound flavin by glucose. The initial reaction mixture (final volume, 1.5 ml) contained 50 mM phosphate buffer (pH 6.5), and 5.6 μM glucose dehydrogenase. For Expts. B and C, 0.1 mM $AgNO_3$ and 0.1 mM DHT, respectively, were also included in the mixture. The reaction was run aerobically at room temperature, and the reduction of flavin was followed by recording the change in absorbance at 460 mμ. The downward reflection of the trace indicates the reduction of flavin.

Role of histidyl residue

As reported previously[2], FAD in glucose dehydrogenase was rapidly reduced aerobically on addition of a small amount of glucose and, after a while, slowly reoxidized by autoxidation (Expt. A, Fig. 4). In the enzyme which had been pretreated with 0.1 mM $AgNO_3$, however, no appreciable reduction of the flavin was induced by the same amount of glucose, although the addition of a large amount of the hydrogen donor could reduce the flavin (Expt. B, Fig. 4). It was further found that the FAD in the DHT-inhibited enzyme was not reduced even on addition of a large amount (10 mM) of glucose (Expt. C, Fig. 4). These experiments clearly indicated that the histidyl residue is necessary for the reduction of the enzyme-bound FAD by glucose. The reversal of Ag^+ inhibition by a large amount (7.5 mM) of glucose again suggested that Ag^+ and glucose combine with the enzyme at the same site.

In the experiment shown in Fig. 5, the FAD of the enzyme (5.6 μM) was first reduced by the addition of 50 μM glucose and the reduced enzyme was treated with

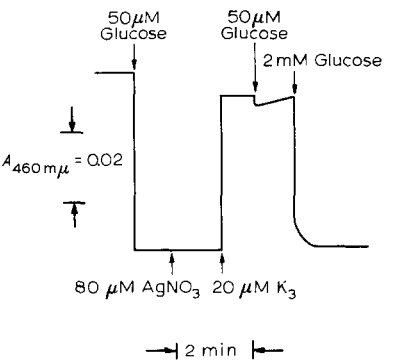


Fig. 5. Reoxidation of reduced FAD by vitamin K_3 in the Ag^+-inhibited enzyme. The initial reaction mixture (final volume, 1.5 ml) contained 50 mM phosphate buffer (pH 6.5) and 5.6 μM glucose dehydrogenase. The reaction was run anaerobically in a nitrogen atmosphere (room temperature), and the reduction and reoxidation of enzyme-bound FAD were followed by recording the change in absorbance at 460 mμ. The downward reflection of the trace indicates the reduction of flavin.

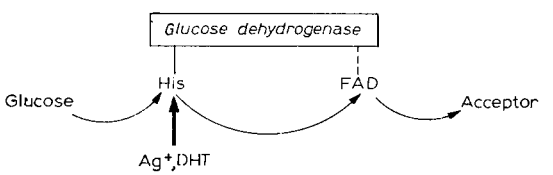
80 μM $AgNO_3$. As will be seen, further addition of 20 μM vitamin K_3, an effective acceptor for this enzyme[2], to this Ag^+-inhibited system caused rapid reoxidation of the reduced flavin. Thus, it was clear that the oxidation of reduced FAD in the enzyme by an acceptor was not affected by the Ag^+ treatment. The reoxidized flavin thus produced could not be reduced by 50 μM glucose because of the Ag^+ inhibition, but could be reduced by the addition of a large amount (2 mM) of glucose. The insensitivity of the reoxidation of reduced FAD to Ag^+ was also observed in the case of slow reoxidation by molecular oxygen.

DISCUSSION

Although the activity of *A. oryzae* glucose dehydrogenase is sensitive to heavy metal ions such as Ag^+ and Hg^{2+}, and the enzyme possesses 6 half-cysteine residues per mole[1], typical sulfhydryl reagents such as *p*-chloromercuribenzoate exert no inhibitory effects. In fact, the enzyme has no sulfhydryl groups titrable with *p*-chloromercuribenzoate. Therefore, it may be concluded as in the case of glucose oxidase from *Aspergillus niger*[17] that the glucose dehydrogenase activity is not related to sulfhydryl groups.

The results reported in this paper, on the other hand, indicate that the inhibition of glucose dehydrogenase by Ag^+ and Hg^{2+} results from complex formation between these metal ions and an essential histidyl residue or residues in the enzyme molecule. The most conclusive evidence for the role of histidyl residues is the fact that DHT, a reagent which reacts very specifically with histidine[4], inhibits the enzyme activity concomitantly with the formation of bis-azolhistidine residues. The finding that the pH–pK_m curve has a break at pH 7 also supports the conclusion, because the group most likely to dissociate in a protein in this pH region is the imidazole group[8,9,18]. Although not very conclusive, photoinactivation of the enzyme in the presence of methylene blue lends further support to the view.

Since Ag^+ or DHT inhibits the reduction of enzyme-bound FAD by glucose but shows no effect on the reoxidation of the reduced flavin by acceptors such as vitamin K_3, it is clear that the essential histidyl residue is required for the hydrogen transfer from glucose to the enzyme flavin. The inhibition of the enzyme by Ag^+ or DHT can be appreciably reduced when glucose is added prior to the inhibitor and the reduction of the FAD in the Ag^+- (but not DHT-) inhibited enzyme can proceed normally if the concentration of glucose is greatly increased. From these findings, suggesting competition between Ag^+ and glucose, it seems likely that the histidyl residue is in fact the site of glucose binding to the enzyme molecule.Although



Scheme 1. Role of the histidyl residue in the glucose dehydrogenase activity. The arrows symbolize the hydrogen flow; the thick arrow represents the inhibition.

further work is necessary to confirm this conclusion, it is of interest to recall LEWIN's[19] earlier finding that histidine combines strongly with hexoses and pentoses. It has further been suggested that the imidazole group of histidine is responsible for this combination[20,21]. It therefore seems reasonable to depict the role of the histidyl residue in the glucose dehydrogenase activity as depicted in Scheme 1.

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