0273-2289/03/110/0165/$25.00

Interaction of Glucose Oxidase With Alkyl-Substituted Sepharose 4B

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Abstract

Glucose oxidase (GOD) is often used in immobilized forms for determination of glucose. To examine the possibility of its adsorption by hydrophobic interactions, palmityl-substituted Sepharose 4B (Sepharose-lipid) was employed as an adsorptive matrix. Various conditions were used in tests to improve the limited immobilization of the enzyme observed under normal (native) conditions, including use of high concentrations of denaturing agents. Of the denaturants used, only the cationic detergent dodecyl trimethyl ammonium bromide was effective in denaturing the protein and exposing its hydrophobic sites for interaction with alkyl residues on the support. This, followed by the process of renaturation, provided catalytically active immobilized preparations. The apoenzyme, prepared by treatment of the holoenzyme with acidified $(NH_4)_2SO_4$ or thermal denaturation, was totally immobilized on the support. Furthermore, it was shown that either flavin adenine dinucleotide (FAD) or the alkyl residues, not both, may interact with the nucleotide site at any given time. Results are discussed in terms of high rigidity of GOD molecule and limited exposure of hydrophobic sites in its native structure. The observations are in accord with suggestions in the literature that the FAD pocket is a very narrow channel of hydrophobic properties, adapted to accept its natural coenzyme.

Index Entries: Glucose oxidase; adsorptive immobilization; hydrophobic matrices; molten globule; 8-anilinonaphtalene-l-sulfonate.

Introduction

Glucose oxidase (GOD) (β-D-glucose:oxygen-1-oxidoreductase, EC 1.1.3.4) catalyzes the oxidation of β-D-glucose to δ-gluconolactone, which subsequently hydrolyzes spontaneously to gluconic acid and hydrogen

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peroxide. Enzymes from different fungi are all dimers composed of two identical subunits with two molecules of flavin adenine dinucleotide (FAD) located inside deep pockets in the protein structure *(1)*. FAD, a very tightly bound prosthetic group, stabilizes the three-dimensional structure of the enzyme and cannot be removed by dialysis at neutral pH *(2)*. It has been proposed that in the absence of FAD, the protein exists mainly in the molecular conformation of a loose flexible coil *(3,4)*. On addition of the coenzyme, the protein is converted to a compact structure with enzymatic activity *(4)*. GOD from *Aspergillus niger*, used in the present study, is a dimer of mol wt of 160 kDa with a pH optimum of 5.5 and a p*I* of 4.2 *(5–7)*. There has been much interest in GOD, mainly because of its utility in glucose estimation. In this connection, the enzyme has been immobilized to various supports *(8,9)*, which have been found useful for analytical purposes, including determination of glucose in serum *(10,11)*.

Our previous studies indicated that the enzyme does not show any appreciable affinity for binding to palmityl–Sepharose 4B, a hydrophobic support employed to immobilize a number of enzymes with retention of native properties *(12)*. More recently, we reported the successful use of the process of denaturation-renaturation to improve interaction of two other proteins, which, like GOD, do not interact favorably with the hydrophobic support *(13–15)*. The present article reports our attempts to use similar approaches for improvement of immobilization of GOD. This enzyme appeared very resistant toward denaturation, and, although the apoenzyme was fully immobilized on the support, only limited improvement of adsorption of the holoenzyme was achieved. Our study provided some interesting data relevant to the general structural properties of the enzyme, especially in connection with the very limited occurrence of exposed hydrophobic patches in the protein structure, its high structural rigidity, and the nature of the FAD-binding site.

Materials and Methods

Chemicals

GOD and all other biochemicals were purchased from Sigma (St. Louis, MO). All chemicals were of analytical reagent grade. Reproducibility of the data presented herein was confirmed by repeating the experiments at least twice.

Coupling of Alkyl Glycidyl Ethers to Sepharose 4B

Palmityl and octyl glycidyl ethers were prepared according to the procedure of Ulbrich et al. *(16)*. Coupling of the glycidyl ethers to Sepharose 4B was carried out following the procedure reported earlier *(12)*, using the same amounts of Sepharose 4B and reagents. The degree of substitution was estimated by a nuclear magnetic resonance method as described previously *(17)*. The degrees of substitution of the two matrices were 31 and

3.2 mmol/mol of galactose, respectively, for octyl– and palmityl–Sepharose 4B.

Protein Concentration

Concentrations of native and denatured-renatured GOD were determined by the Lowry method *(18)*.

Fluorescence Measurements

Extrinsic fluorescence studies were carried out as outlined earlier *(13)*, using 8-anilinonaphtalene-1-sulfonate (ANS) as a fluorescence probe. Measurements were taken on a Hitachi MPF-4 apparatus. All experiments were carried out at 25°C, except as otherwise indicated, with ANS and protein concentrations of 50 µ*M* and 50 µg/mL in 0.1 *M* phosphate buffer (pH 6.0), respectively. An excitation wavelength of 350 nm was used.

Enzyme Assay

The enzymatic activity of free and immobilized GOD was determined by a colorimetric method using a coupled assay *(19,20)*. The following reagents were mixed together in a cuvet: 1 mL of 0.1 *M* sodium phosphate, pH 6.0, containing 1% *o*-dianisidine; 10 µL of a glucose solution (18% [w/v]); and 0.5 U of peroxidase. Ten microliters of GOD (2.5 U) was then added and the rate of increase in absorbance at 460 nm determined.

Preparation of Immobilized Protein

For immobilization by heat treatment of the holoenzyme, an enzyme solution at 0.1 mg/mL concentration was incubated at specific temperatures for different lengths of time. One milliliter of preheated Sepharose-lipid at the corresponding temperature was added to the enzyme solution. The mixture was shaken for another 30 min at the same temperature, subsequently cooled at 4°C, and centrifuged at 3000*g* for 5 min. The pellet was washed with 0.1 *M* phosphate buffer, pH 6.0, to remove unbound proteins. Determination of protein concentration and enzyme activity measurements were subsequently carried out for all fractions.

Treatment with 8 *M* urea, 5 *M* guanidinium hydrochloride (Gdn.Hcl), or 25 m*M* *n*-octyl-β-D-glucopyranoside was carried out for 1 h using a 0.1 mg/mL solution of the enzyme in a final volume of 1 mL. One milliliter of packed Sepharose lipid was then added, and the mixture was dialyzed for 24 h in the cold followed by washing of the pellet and activity determination as already outlined. To test immobilization by treatment with dodecyl trimethyl ammonium bromide (DTAB), 1 mL of GOD (0.1 mg/mL) in 2.5 m*M* phosphate, pH 6.5, containing the detergent at 1 m*M* concentration was incubated for 1 h at 25°C. To this, 0.25 mL of packed palmityl-Sepharose was added and the suspension was mixed for 1 h on a magnetic stirrer. The mixture was then dialyzed against 0.1 *M* phosphate buffer for 24 h in the cold. The suspension was centrifuged and

washed three times using the same buffer, and the percentage of immobilization was determined.

Catalytic Activity of Immobilized Preparations

A volume of the immobilized preparation corresponding to 0.2 mL of packed matrix was suspended in the assay mixture. To determine the rate of increase in absorbance owing to *o*-dianisidine oxidation, the suspension in the cuvet was mixed for 30 s using a very small magnet followed by rapid centrifugation for 30 s. Reading of the absorbance of the clear supernatant was performed at 460 nm. By repeating these a constant value for optical density per minute was obtained.

Preparation of Apoenzyme

Apoenzyme of GOD was prepared according to the procedure of Swoboda *(3,4)*. Saturated $(NH_4)_2SO_4$ solution at 25°C was acidified to pH 1.4 with concentrated H_2SO_4. One milliliter of GOD solution (20 mg/mL) was added dropwise with stirring to 20 mL of acidified salt solution at –5°C. The yellow supernatant was removed after centrifuging at 20,000*g* for 15 min. The precipitate was subjected to two more cycles of acidified salt treatment, centrifugation, and neutralization. The protein was redissolved in 0.1 *M* sodium phosphate buffer (pH 6.1) to a protein concentration of about 10 mg/mL.

Reactivation of Apoenzyme

Apoenzyme was incubated with varying amounts of FAD for 30 min at 25°C; and samples were withdrawn and assayed for GOD activity.

FAD-Binding Measurements

Binding of FAD to apo-GOD was measured by monitoring the changes in fluorescence at 340 nm. The excitation wavelength was 295 nm. Various concentrations of FAD were mixed with 150 µg/mL of apo-GOD, and the spectra were subsequently taken. Tryptophan fluorescence was measured on a Hitachi MPF-4 spectrofluorimeter. All measurements were made at 25°C. The buffer used was 0.1 *M* phosphate, pH 6.0.

Immobilization of Apo-GOD

Apoenzyme solution of a known concentration was incubated with 0.5 mL of Sepharose-lipid in the presence of specified concentrations of FAD at 25°C. FAD and other nucleotides (flavin mononucleotide [FMN], guanosine 5'-monophosphate [GMP], guanosine 5'-triphosphate [GTP], adenosine 5'diphosphate [ADP]) were added to the mixture before incubation with Sepharose-lipid. The enzyme solution was centrifuged, and the pellet was washed with the buffer to remove unbound proteins. Protein concentration and enzyme activity measurements were carried out for all fractions.

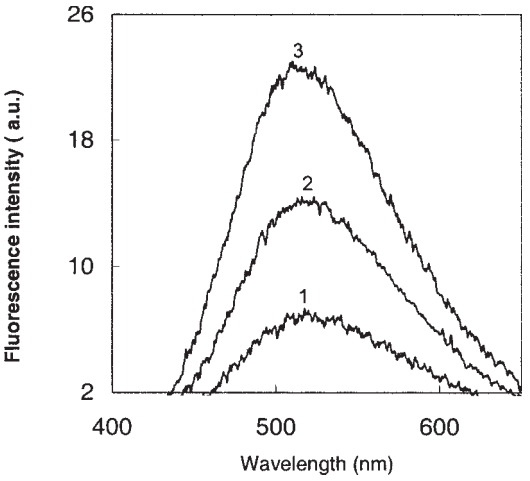

Fig. 1. Fluorescence emission spectra of 50 μ*M* ANS in presence of the following: 1, no addition; 2, native GOD; 3, GOD treated at 70°C. A protein concentration of 0.05 mg/mL was used. Excitation wavelength was fixed at 350 nm. a.u., absorbance unit.

Results

ANS Fluorescence

Possible exposure of hydrophobic clusters in the protein molecule was examined by the use of ANS as a hydrophobic reporter group. The enzyme was treated with 5 *M* Gdn.Hcl, 8 *M* urea, or 25 m*M* *n*-octyl-β-D-glucopyranoside, none of which resulted in appreciable enhancement of fluorescence. Heat treatment of the enzyme was effective and substantial enhancement was observed (Fig. 1). It should be pointed out that in this case, ANS did not show the typical high fluorescence enhancement observed on binding to other proteins. This is presumably owing to the fact that ANS itself carries a negative charge at the pH of the experiment, at which GOD (p*I* = 4.2) is also negatively charged. It is therefore suggested that had it not been for such repulsive electrostatic interactions or some kind of quenching effect, the typically higher enhancement reported for other proteins would have been attained.

Adsorption of GOD on Hydrophobic Supports

As reported earlier *(12)*, native GOD does not show any appreciable affinity for binding to the hydrophobic support. In the present study, all different attempts, including various preparations of the matrix and different batches of the holoenzyme used at different protein concentrations in the immobilization experiments, resulted in the adsorption of 10–15% of the enzyme. It is possible that because of the heterogeneity of the protein (monomer, dimer, trimer, tetramer; see ref. *21*), a certain proportion of a given form exists that has the affinity to bind the hydrophobic matrix.

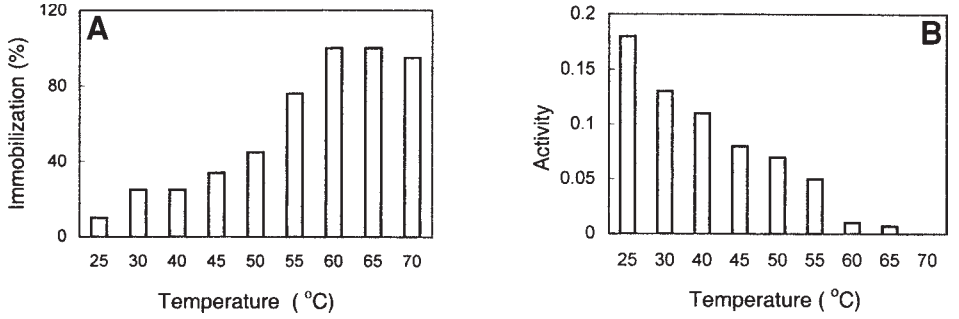

Fig. 2. Effect of temperature on immobilization and activity of GOD. GOD was preheated at various temperatures before the addition of Sepharose-lipid for immobilization. The percentage immobilization **(A)** and relative activities **(B)** were measured as described in Materials and Methods.

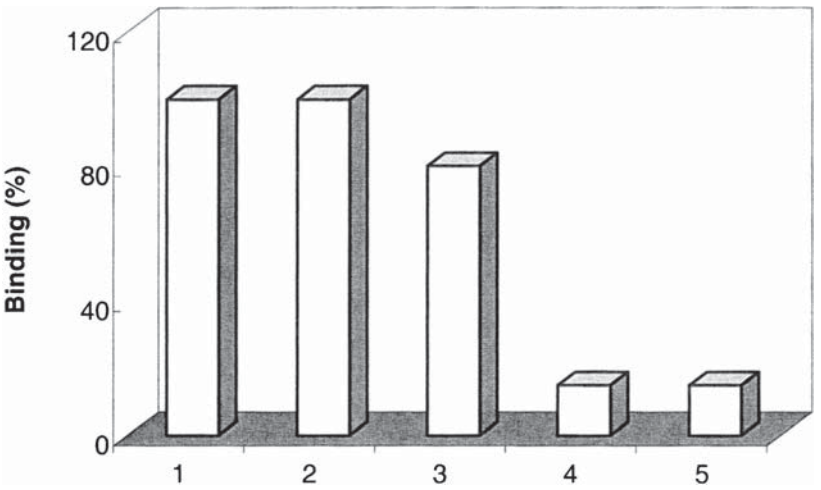

Fig. 3. Interaction of GOD (150 μg/mL) with palmityl-Sepharose and competition by FAD (0.4 μ*M*). 1, apo-GOD added to palmityl-Sepharose; 2, as for 1, followed by addition of FAD; 3, palmityl-Sepharose containing FAD, to which apo-GOD was added; 4, apo-GOD containing FAD (reconstituted holoenzyme), to which palmityl-Sepharose was added; 5, native GOD added to palmityl-Sepharose suspension. Further details were as for Fig. 2.

The use of high concentrations of Gdn.Hcl or urea was ineffective in enhancing interaction. On the other hand, heat treatment of the enzyme resulted in its immobilization, especially at elevated temperatures (Fig. 2). However, enzymatic activity was dramatically diminished, apparently owing to release of FAD *(21,22)*. In addition, use of 2-deoxyglucose, fructose, mannose, and lactose, all at 20% (w/v) concentration, did not afford protection against loss of activity. Other possibilities in this procedure related to denaturation of the enzyme followed by its immobilization were not explored.

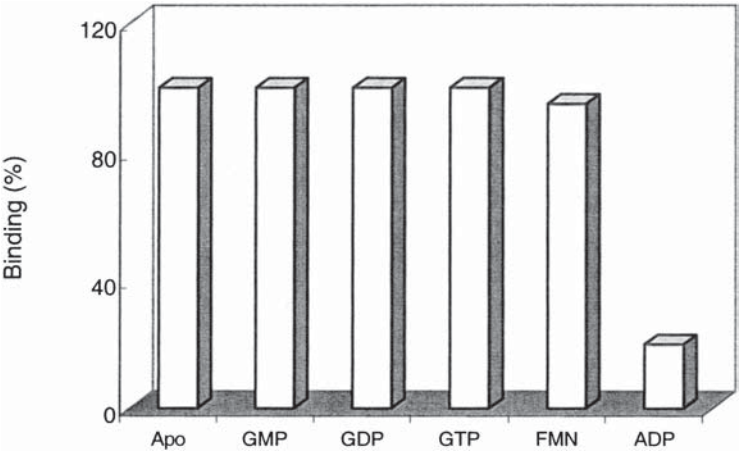

Fig. 4. Interaction of apo-GOD with palmityl-Sepharose in presence of various nucleotides. The percentage immobilization was determined as described in Materials and Methods. Apo, apoenzyme; GDP, guanosine 5'-diphosphate.

The possibility that FAD is released on heat treatment of the holoenzyme *(23)* prompted us to test the interaction of the apoenzyme with the matrix. As indicated in Fig. 3, the apoenzyme is totally immobilized and cannot bind FAD in the adsorbed form. However, the nucleotide appears to compete with alkyl residues for binding to the apoenzyme (Fig. 3). The addition of FAD to the apoenzyme (i.e., reconstituted holoenzyme) resulted in the same low degree of immobilization as for the original holoenzyme (Fig. 3). ADP was as effective a competitor as FAD, whereas FMN and some other nucleotides were ineffective (Fig. 4). As expected, full recovery of activity was attained on addition of FAD to the apoenzyme. Reconstitution was also followed using intrinsic fluorescence studies as outlined in Materials and Methods.

Denaturation by DTAB

A previous report indicated the effectiveness of this detergent in causing denaturation of GOD *(24)*. Among various approaches taken to denature the protein for improvement of immobilization, denaturation of the enzyme by the use of millimolar concentrations of DTAB was found very effective. Immobilization was achieved by treating the protein with the detergent followed by addition of the adsorbent and dialysis of the mixture. Following this procedure, 50% immobilization was achieved. The immobilized preparation obtained in this manner had a specific activity corresponding to 60% of the activity of the original native enzyme and 78% of the activity of the enzyme treated as for the sample, which was used for immobilization with the exception that no Sepharose-lipid was added. It is clear that treatment with DTAB dose not cause release of FAD.

Effect of pH

No enhancement of immobilization was achieved by using extreme pH values, previously found effective in a number of cases *(12–14)*. Thus, percentage of immobilization remained low (10–15%) when different runs were made, using a mixed buffer whose pH was adjusted between 3.0 and 10.0.

Discussion

Two major techniques are widely used to immobilize enzymes: covalent binding and adsorption. In the latter case, chemical manipulation for binding of the enzyme is not necessary, and immobilization may simply be achieved by applying an enzyme solution to a suitable adsorbent. It has been demonstrated that several proteins may be immobilized by adsorption via nonionic interactions *(12)*. Using palmityl-substituted Sepharose 4B as a hydrophobic support, GOD provided one of the exceptions in that it interacted very poorly with the adsorbent under normal conditions *(12)*. In view of recent developments in the use of reversible denaturation to improve protein immobilization by hydrophobic interaction *(13–15)*, we used similar strategies in an effort to enhance adsorption of GOD on hydrophobic supports.

As indicated in the Results, the use of high concentrations of Gdn.Hcl, urea, and *n*-octyl-β-D-glucopyranoside did not improve adsorption. However, DTAB proved to be an effective denaturating agent, and noticeable improvement of immobilization was achieved. Another approach that provided efficient binding was the use of thermal denaturation, which coincided with a substantial enhancement of ANS binding (Fig. 1). It has been shown that denaturation of GOD at 60°C and above can release FAD. Accordingly, it appears that the FAD site becomes available for interaction with alkyl residues and total immobilization is achieved. It is noteworthy that of the nucleotides tested as possible competitors, only ADP was effective. This is in accordance with previous reports on the similarity of ADP to FAD, in relation to interaction at the nucleotide-binding site of GOD *(4)*. None of the other nucleotides used in the present study were found to be effective (Fig. 4).

Access to the flavin site in the active site of the enzyme is provided by a large deep pocket that is shaped like a funnel and formed on one side by residues of the second molecule of the dimer *(25,26)*. Almost exclusively, residues 75–98 of the lid from both subunits form this side of the pocket. The flavin group is rigidly fixed, at the pyrimidine ring by hydrogen bonds involving Gly 108, Thr 110, and Met 561 and at the peteridine ring by hydrophobic contacts involving Tyr 515 and Val 106 *(27)*. It is interesting that the FAD site in the holoenzyme can accept either FAD or alkyl groups (Fig. 3). Accordingly, all attempts made in finding conditions in which interaction of FAD and adsorption would occur concomitantly failed. In the course of these experiments, it was found that interaction of FAD or

alkyl residues with the nucleotide site in the protein molecule occurs very rapidly and apparently neither of these can influence (lower) binding of the other, once complexation has been achieved. However, binding of the apoenzyme to a suspension of the matrix containing FAD was diminished by 15–20% in different experimental runs. In addition, octyl- and palmityl-Sepharose preparations behaved very similarly and no difference could be detected in this connection. No change in the extent of competition was obvious when FAD was included in the concentration range up to 0.4 μ*M*.

Resistance of GOD toward denaturation is noteworthy. It was anticipated that some of the approaches made for improvement of immobilization (e.g., use of high concentrations of urea and Gdn.Hcl, extremes of pH), although proven useful for carbonic anhydrase and urease *(13,14)*, would fail here. In other words, the denaturants apparently failed to loosen up the tertiary structure of the protein and/or to make the internal hydrophobic sites available for interaction. Treatment of the enzyme with DTAB provided the only effective approach in exposing the internal hydrophobic sites of the protein structure for interaction with the hydrophobic groups on the adsorbent. It was thus possible to obtain an immobilized preparation with catalytic activity.

As outlined recently, we were able to demonstrate formation of intermediate molten-like structures during the course of denaturation-renaturation strategies applied for urease and carbonic anhydrase *(13–15)*, both of which, like GOD, do not interact favorably with hydrophobic supports under normal conditions *(12)*. Although the approaches described here apparently failed in producing such structures for GOD, chemical modification of lysine residues, using citraconic anhydride, was found useful. This approach improved immobilization from 10–15% for the native protein to about 30% for the modified forms. Moreover, circular dichroism measurements in the far- and near-ultraviolet regions indicated the possibility of formation of molten-like structures, since the secondary structure remained unchanged and the tertiary structure was appreciably diminished (result not shown). A similar study in our laboratory indicated formation of molten-like forms of α-amylase on modification with the same modifier *(28)*. We are in the process of completing the modification studies involving citraconic anhydride for GOD, with the aim of improving immobilization with retention of catalytic activity.

In conclusion, the results presented herein clearly indicate the occurrence of very limited hydrophobic sites in native GOD available for interaction with alkyl residues on a hydrophobic support. Although total immobilization of the apoenzyme was achieved, only limited improvement in protein adsorption was attained by denaturation of the holoenzyme, providing further evidence for high rigidity of the protein structure.

Acknowledgment

Financial support for this work was provided by the Research Council, University of Tehran.

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