THE IMMOBILIZATION OF GLUCOSE OXIDASE VIA ACTIVATION OF ITS CARBOHYDRATE RESIDUES

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SUMMARY. The immobilization of glucose oxidase, a glycoenzyme from Aspergillus niger consisting of 16% carbohydrate, has been achieved by oxidizing its carbohydrate residues with periodic acid followed by coupling the activated enzyme to water-insoluble p-aminostyrene. At pH 5.6 and 25°, approximately 60% of the carbohydrate residues are oxidized, but the enzyme retains full activity. No oxidation of any amino acid residue is evident. The enzyme-polymer conjugate derived from this activated enzyme retains full activity and even shows a slightly enhanced thermal stability at 60° compared with the soluble native and oxidized glucose oxidases.

INTRODUCTION. Enzymes may be immobilized by a variety of chemical or physical techniques (1). Until now, all chemical techniques have involved the modification of the amino acid residues of an enzyme, even though the enzyme may have contained other functional groups which could be employed, e.g., the carbohydrate residues of glycoenzymes. Thus, glycoenzymes such as glucose oxidase (2-4) or glucoamylase (5) have been immobilized by chemical modification of only their amino acid residues. Because our work and that of others (6-9) on the function of the carbohydrate residues of glucose oxidase revealed that the sugar moieties do not seem to be implicated in catalysis, we reasoned that it would be more desirable to bond glycoenzymes covalently to water-insoluble polymers by these catalytically nonessential carbohydrate groups rather than by amino acid groups--some of which are responsible for substrate binding and catalysis. This communication gives our preliminary results on this approach to the "non-amino acid immobilization" of enzymes.

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MATERIALS AND METHODS. Glucose oxidase (EC 1.1.3.4) from Aspergillus niger was obtained from the Worthington Biochemical Corp., Freehold, N.J., (lot no. GOP2LA) as a salt-free lyophilized powder. Periodic acid, H_5IO_6, was purchased from Matheson, Coleman and Bell and p-aminostyrene from Polysciences, Inc.

Glucose oxidase activity was measured spectrophotometrically at 460 nm by the coupled peroxidase--o-dianisidine system using D-glucose in 100 mM phosphate buffer, pH 6.0, at 25° (3,10-12). The activity of immobilized enzyme derivatives was determined as previously described (13).

The concentration of glucose oxidase was determined spectrophotometrically at 450 nm (50 mM acetate buffer, pH 5.6) using the extinction coefficient of 1.41×10^4 M^{-1} cm^{-1} (14,15). The concentration of catalytically active enzyme was determined spectrophotometrically using the differential molar extinction coefficient at 450 nm of 1.31×10^4 M^{-1} cm^{-1} by anaerobic titration of the enzyme with glucose (2,14,16). Protein content of enzyme-polymer conjugates was determined by amino acid analysis using the described procedure (13). The amount of protein was determined from the amount of Ala, Val, and Glu found and by assuming that the mol wt of the enzyme is 150,000 and that there are 108 Ala, 79 Val, and 99 Glu residues per molecule (7). Total amino acid analysis of soluble native and oxidized glucose oxidase was performed by the Biochemical Data Corp., Highland Park, N.J.

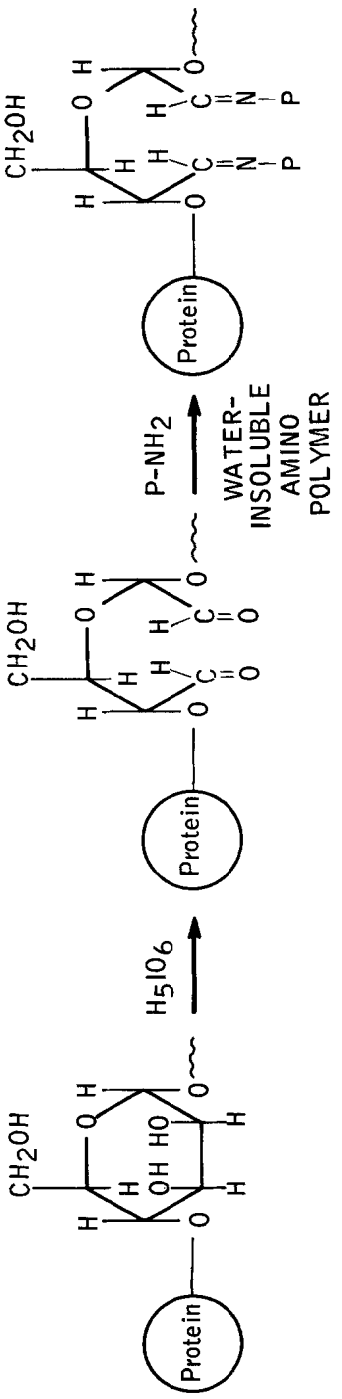
Oxidation of Glucose Oxidase with Periodic Acid - To a stirred solution of glucose oxidase (40.2 mg, 2.68×10^{-7} mole, in 20 ml 50 mM acetate buffer, pH 5.60) in a thermostated vessel at 25° protected from light was added 0.4 ml of a periodic acid solution (9.12 mg, 4.00×10^{-5} mole). The yellow solution was stirred for 4 hr after which 0.025 ml of ethylene glycol, 4.48×10^{-4} mole, was added and stirred for an additional 0.5 hr. The solution was transferred to an Amicon Model 202 ultrafiltration cell equipped with an XM-50 filter at 50 psi N_2 pressure and dialyzed with

50 mM acetate buffer, pH 5.59, until no further dialyzable material came forth. The conversion of periodic acid after the 4 hr of reaction with glucose oxidase, based on the absorbance change at 223 mm (17), was 61.6%. The oxidized enzyme was stored at 5°.

Coupling of Oxidized Glucose Oxidase to p-Aminostyrene - To a 5 ml solution of oxidized glucose oxidase (10 mg) was added 250 mg finely powdered p-aminostyrene. The suspension was adjusted to pH 9 with 0.1 N NaOH, stirred at ca. 25° for 1 hr and then filtered with a Millipore 0.45μ filter. The solid was washed with 1 l of H_2O and 1 l of 1 M NaCL in 50 mM phosphate buffer, pH 6.4. After several ml of the H_2O wash, no further activity was detected in the wash. The original filtrate exhibited high activity; the NaCl wash exhibited no activity.

RESULTS AND DISCUSSION. As outlined in Eqn. 1, this method of immobilization consists of two steps: (1) activating a glycoenzyme via periodate oxidation and (2) contacting the modified "aldehydic" enzyme with an amino-containing water-insoluble polymer. In this chemical method, as well as in others, it is desirable to bond the enzyme to the polymer through only nonessential groups of the enzyme (i.e., those groups not necessary for catalysis or substrate binding). Our approach is based on being able to oxidize the carbohydrate residues of glycoenzymes without losing activity. Although the exact role of these residues in glycoenzymes remains to be established, the evidence presented to date (8,18) and below argues against their critical involvement in catalysis.

Periodate oxidation is a well established procedure for modifying carbohydrate residues in polysaccharides (19,20) and glycoproteins (8,21) although with the latter there may be some oxidation of Cys-, Cys, Tyr, Trp, and Met (22-24). Oxidation of glucose oxidase with periodic acid, as described, resulted in the oxidation of ca. 60% of the carbohydrate residues of the enzyme, which is composed of 16% carbohydrate having 128 mannose, 19 glucosamine, and 3 galactose residues per 150,000 daltons (7).


NATIVE
H_5IO_6
OXIDIZED
$P\text{-}NH_2$
WATER-INSOLUBLE AMINO POLYMER
WATER-INSOLUBLE
Protein
CH_2OH
C=N—P

EQUATION 1

Table 1

Specific Activities of Glucose Oxidases

Glucose Oxidase	Specific Activity (units/mg protein)
Native	91.9[a,b]
Oxidized	92.5[a,b]
p-Aminostyrene-bound	93.1[c]

(a) Based on the amount of protein as determined by anaerobic spectral titration with glucose. This method measures only catalytically active protein.
(b) No difference in specific activity between the native and oxidized enzyme was noted when the protein concentration was determined by using the extinction coefficient at 450 nm. This method measures total protein.
(c) Based on protein as determined by amino acid analysis. Protein loading was 5.87 mg enzyme/g of enzyme-polymer conjugate.

Amino acid analysis revealed that no periodate-sensitive amino acids were oxidized. Likewise, no changes were observed in the spectral properties (ultraviolet, visible, and circular dichroism) of the oxidized enzyme compared with the native enzyme (unpublished results). Most important and germane to this communication, the specific activity of the oxidized enzyme was identical to that of the native enzyme (Table 1). There was neither a loss of activity nor precipitation of the enzyme as had been reported by Pazur et al. (7). Since no activity was lost upon oxidation, it is tempting to suggest that the carbohydrate residues are not involved in catalysis. However, this is too simplistic, for 40% of the residues are not oxidized (presumably the more sterically hindered or periodate-resistant carbohydrate residues). Further, although the activity of glucose oxidase was not affected, the activity of other glycoenzymes has been diminished by periodate oxidation, e.g., horseradish peroxidase (25) or α-amylase (26).

Coupling of oxidized glucose oxidase with p-aminostyrene resulted in an active enzyme-polymer conjugate presumably through the imine linkage. The protein loading (mg of enzyme per g of enzyme-polymer conjugate) with the p-aminostyrene is 5-8 mg. The activity of the immobilized enzyme is high--in fact, within experimental error, it is equivalent to the native and oxidized enzymes.

Figure 1 shows the thermal stability of the glucose oxidases. It is interesting to note that the native and oxidized enzymes have similar stabilities (with the oxidized enzyme perhaps slightly more stable) whereas the water-insoluble conjugate is definitely more stable.

We are extending this method to other glycoenzymes, and full reports on the characteristics of the water-soluble oxidized glucose oxidase and water-insoluble enzyme-polymer conjugates will be issued shortly. Periodate oxidation could be used for the carbohydrate-containing hormones, proteins, immunoglobulins, etc.

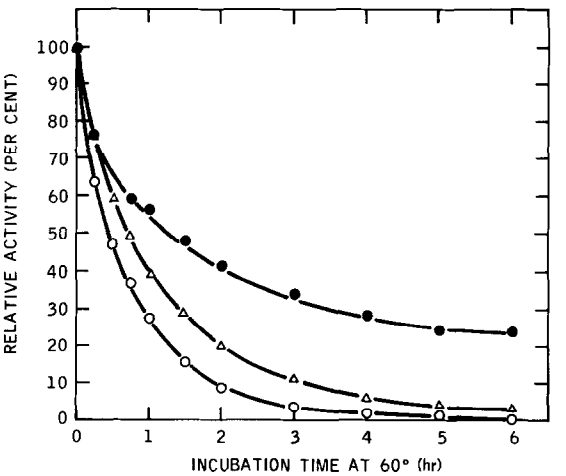


Figure 1. Thermal inactivation of soluble native (O), soluble oxidized (Δ) and p-aminostyrene-bound (●) glucose oxidase at 60° in 50 mM acetate buffer, pH 5.6. Relative activities were determined at 25° employing the standard assay. Specific activity at time zero (no heating) was taken as 100% relative activity. Native and oxidized glucose oxidase concentration was 0.4 mg/ml; the concentration of the bound enzyme was ca. 0.75 mg of enzyme-polymer conjugate/ml of buffer.

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