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FLAVIN–PROTEIN INTERACTION IN BOUND GLUCOSE OXIDASE

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Glucose oxidase was bound to Sepharose, Sephadex, gelatin, and dextran, yielding immobilized soluble and insoluble derivatives of the enzyme. The soluble preparations possessed higher enzymic activity than the analogous insoluble ones. The reversible dissociation process of the bound enzyme into apoenzyme and flavin adenine dinucleotide (FAD) was studied with the soluble and insoluble glucose oxidase in relation to enzymic activity and conformational changes as measured by circular dichroism and fluorescence methods. Bound apoenzyme was found to be more stable than the apoenzyme obtained from the unmodified glucose oxidase. The binding constant of FAD in bound glucose oxidase ($K_{diss} \approx 10^{-8}$ M) calculated from fluorescent studies was lower than that of FAD in the native enzyme ($K_{diss} \approx 10^{-10}$ M). The circular dichroism measurements indicated that dextran-bound glucose oxidase has a conformation similar to that of the native enzyme.

INTRODUCTION

Glucose oxidase (EC 1134), an enzyme that catalyzes the transfer of hydrogen atoms from glucose to oxygen, belongs to the family of flavoproteins. It contains two FAD[1] molecules as coenzyme per molecule of protein, of mol.w. 150,000 (1–4). FAD is very tightly bound and cannot be removed by dialysis at neutral pH. It undergoes reduction and oxidation in the catalytic cycle without dissociation from the enzyme. However, the flavin is not covalently bound to the protein, and may be dissociated by acid treatment or denaturing agents (5,6). The apoprotein may then be recombined with the flavin, and the renaturation of the flavoenzyme can be followed by optical methods and recovery of enzymic activity.

Some of the recent investigations on flavoproteins have focused attention on the stabilization effect of flavin on protein conformation in solution. In the absence of flavoproteins, the apoprotein undergoes conformational changes (5–10).

[1]The abbreviations used in this paper are: GO, glucose oxidase: GOG, glucose oxidase–gelatin; GO–Sephadex and GO–Sepharose, glucose oxidase bound to Sephadex and Sepharose, respectively; GOD, glucose oxidase bound to dextran; FAD, flavin adenine dinucleotide; apoGO, apenzyme of glucose oxidase; apoGOD, apoenzyme of dextran-bound glucose oxidase; CD, circular dichroism.

The binding of glucose oxidase to carrier enables us to study the kinetics of conformational change occurring during the removal of the flavin and its rebinding in a well-characterized heterogenous system. Recent studies with flavoproteins linked to insoluble macromolecules have emphasized the biological significance of bound enzymes as models of biological structures (11–13).

The effect of binding of glucose oxidase to soluble and insoluble carriers, as well as the behavior of the bound enzyme during the reversible dissociation process, were investigated in relation to enzymic activity, conformational changes as measured by circular dichroism, and fluorescence characteristics.

MATERIALS

GO, highly purified grade, was obtained from Worthington Biochemical Corp. (Freehold, New Jersey). FAD was obtained from Nutritional Biochemical Corp., Cleveland, Ohio. Sephadex G-200, Sepharose 4-B, and Dextran T-2000 were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). Gelatin powder from swine skin, type I, was obtained from Sigma (London) Ltd. (Wembley, England). Glutardialdehyde as 70% aqueous solution was purchased from Ladd Res. Industry, Inc. (Burlington, Vermont).

METHODS

Assay of Glucose Oxidase

The enzymic activity of native and bound GO was determined according to Swoboda (5). Thus, the oxidation of glucose at pH 6.2 was followed by monitoring the initial rate of oxygen uptake, using an oxygen electrode (Yellow Springs Instruments, Yellow Springs, Ohio).

Determination of Protein Concentration

Protein concentration was determined from the absorbance at 280 nm using $E^{1\%}_{1\,cm} = 18$, or by the Lowry method (14).

Circular Dichroism Measurements

The CD spectra were measured at room temperature with a Cary 60 Spectropolarimeter equipped with the Model 6002 CD attachment. The

enzyme concentration was 0.5 mg/ml, and cells of 0.5 cm and 1 cm light-path length were used. The results reported as mean residue ellipticity (θ) in degree $cm^2\ dmol^{-1}$ were calculated by using a mean amino acid residue weight M = 115.

Fluorescence Measurements

Fluorescence intensity and spectra were determined with a Hitachi–Perkin Elmer Model MPF III Spectrofluorometer. The measurements were performed at 25°C in 0.1 M sodium phosphate buffer, pH 6.2.

Immobilization of Glucose Oxidase

Binding to Sepharose and Sephadex. Water-insoluble derivatives of glucose oxidase were prepared as described by Axen et al. (15). Sepharose 4B or Sephadex G-200 (10 ml packed volume) was reacted with a solution of 250 or 500 mg CNBr for 15 min. The washed activated carriers were suspended in 0.1 M phosphate buffer, pH 7.5, and immediately reacted with the enzyme (2 mg/ml packed gel).

Binding to Dextran. Dextran T-2000 (mol.wt. 2×10^6), 1 g suspended in 50 ml water, was stirred at room temperature until a clear solution was obtained. Then 50 ml 2 M K_2CO_3 was added, and the reaction mixture was cooled in an ice bath. A solution of CNBr (0.5 g) in 0.5 ml acetonitrile (16) was added in one portion, and the reaction mixture was stirred for an additional 15 min. The pH was then brought from approximately 10.8 to 7.5 by addition of solid sodium dihydrogen phosphate. The reaction mixture was dialyzed against 0.01 M sodium phosphate buffer, pH 7.5, for 1 h. To the dialyzed activated dextran (1 g), a solution of glucose oxidase (50 mg) was added. The coupling reaction was carried out overnight in the cold room in the dialysis tube with two changes of dialysis media, in order to remove unreacted CNBr. The water-soluble GOD thus obtained was separated from the unreacted GO by ultrafiltration technique on Amicon ultrafiltration cell Type 12, using Diaflo membrane ×M 300 [GO mol.wt. = 150,000 (4)].

Preparation of Glucose Oxidase–Gelatin (GOG) Derivatives. GO (7 mg) was dissolved in 0.1 M sodium phosphate buffer (1 ml), pH 7.0, and gelatin powder (7.0 or 0.5 mg) was added. The mixture was heated to 39°C to dissolve the gelatin, then cooled to room temperature. After 15 min, glutardialdehyde (0.015 ml 70% aqueous solution) was added, yielding soluble and insoluble GOG preparations.

(*a*) *Soluble glucose oxidase–gelatin.* The reaction mixture containing GOG 14:1 (wt/wt) was stored for 18 h at room temperature, and

subsequently was dialyzed overnight against 5 liters of the same buffer, and finally for another day against water. The dialyzed solution was lyophilized.

(*b*) *Insoluble glucose–oxidase gelatin.* The gel-like material obtained by reacting GO with an equal amount of gelatin was washed several times with the same buffer until no enzymic activity was found in the washings.

Covalent Binding of FAD to Sepharose 4B. Sepharose 4B (6 ml packed gel) was activated with CNBr (150 mg) for 6 min, according to Axen et al. (15). After being washed with 300 ml 0.1 sodium bicarbonate, the gel was divided into two parts:

(1) Activated Sepharose (3 ml) was treated with ε-aminocaproic acid (400 mg) in 4 ml 0.1 M bicarbonate, pH 8.5, stirred for 15 min, and washed sequentially with 0.1 M bicarbonate, 0.01 M HCl, H_2O, and finally with 80% pyridine (17). To the ε-aminocaproyl–Sepharose thus obtained, suspended in 80% pyridine, FAD (20 mg) dissolved in 2 ml water and dicyclohexyl carbodiimide (1.2 g) dissolved in 18 ml pyridine were added, and the reaction mixture was stirred at room temperature for 5 days. The reaction mixture was washed with water, ethanol, water, and 80% pyridine.

(2) Activated Sepharose (3 ml) was reacted with 20 mg FAD dissolved in 0.1 M phosphate buffer, pH 8.0, for 5 days, and washed as described above.

The amount of FAD bound in the preparations described above was determined, after hydrolysis with 0.5 M HCl at 100°C for 1 h, by spectrophotometric measurement (at 450 nm), using FMN as standard ($O.D._{450}^{1cm}$ for 10 γFMN/ml is 0.24).

Dissociation of Native and Bound Glucose Oxidase

Native and bound GO were dissociated to apoenzyme and FAD by the following methods:

(1) Samples containing 1 mg active enzyme immobilized on Sepharose or Sephadex were centrifuged off and incubated for 5–10 min with 0.01 M hydrochloric acid–potassium–chloride buffer, pH 1.4. After incubation, the reaction mixture was diluted to 5 ml with water, centrifuged, and washed with 0.1 M phosphate buffer, pH 6.2, and checked for enzymic activity.

(2) Samples containing 1 mg active enzyme bound to Sepharose were incubated with various concentrations of guanidine HCl at pH 6.8 for different lengths of time. After incubation, the samples were washed with 0.1 M phosphate buffer, pH 6.2, and the residual enzymic activity was determined.

(3) Soluble apoenzyme bound to dextran was prepared as follows: One ml of GOD containing 1 mg active enzyme was brought to pH 1.4 by adding 0.05 ml 0.01 M HCl–KCl buffer, pH 1.5, and immediately passed through a

Sephadex G-25 column (1 × 20 cm), which was preequilibrated with the buffer described above. Eluate samples of 2 ml were collected in test tubes containing 2 ml 0.2 M Na_2HPO_4 (final pH, 6.2). The absorbancy at 280 nm was measured, to locate the tubes containing protein. The solution containing apoenzyme was concentrated by ultrafiltration at 4°C, using Diaflo membrane P30.

(4) Apoenzyme from the native GO was prepared according to Swoboda (5).

Reconstitution of the Immobilized Enzyme

Reconstitution of the Enzyme from Bound Apoenzyme and Free FAD. Samples of the bound apoenzyme were incubated with FAD (molar ratio of FAD to apoenzyme >2 : 1) in 0.1 phosphate buffer, pH 6.2, at room temperature (5). Aliquots were removed from the reaction mixture after different incubation times, and enzymic activity was measured as described previously.

Reconstitution of the Enzyme from Bound FAD and Free Apoenzyme. Unbound apoenzyme was incubated with insoluble FAD preparations under conditions similar to those described above. After different incubation times, the excess of apoenzyme was removed by washing, and the enzymic activity of the reconstituted enzyme was measured.

RESULTS AND DISCUSSION

Preparation of Immobilized Glucose Oxidase

In order to overcome the difficulties induced by the conformational instability of the apoenzyme obtained by removal of FAD from the native enzyme, GO was bound to various carriers.

Two different ways were used for preparation of immobilized GO: (1) binding of the intact enzyme to insoluble and soluble carriers, followed by separating of bound apoenzyme; (2) binding of FAD to insoluble carriers, yielding an insoluble coenzyme, able to reconstitute a bound holoenzyme.

The enzymatic properties of GO bound to soluble and insoluble carriers were compared, and the results are summarized in Table 1. It can be seen from this table that the yields of active immobilized enzyme on binding of GO to soluble carriers were much higher than with insoluble carriers. Thus, the soluble GOG conjugate contained about 80% of the activity added, whereas the insoluble GOG conjugate retained only 15%. Similarly, the soluble GOD was obtained in 70% yield (based on activity), whereas the yields of GO–Sepharose and GO–Sephadex were 2.5–15%.

TABLE 1. **Immobilization of Glucose Oxidase**

Enzyme preparations	Binding conditions	Enzyme added per g carrier (mg)	Residual activity in supernatant (%)	Acitivity retained in immobilized preparations (%)
Insoluble preparations				
1. GO–Sepharose	Sepharose 4B[a] was activated with 50 mg CNBr/ml carrier.	50	85	15
2. GO–Sepharose	Sepharose 4B was activated with 23 mg CNBr/ml carrier.	50	97.5	2.5
3. GO–Sepharose	Sepharose 4B was activated with 25 mg CNBr/ml, and the binding was carried out in the presence of 0.1 M glucose.	50	95	5
4. GO–Sephadex	Sephadex G-200 was activated with 25 mg CNBr/ml carrier.	50	96.4	3.6
5. GOG	Intermolecular cross-linking using 70% glutardialdehyde at pH 7.0.	1000	50	15
Soluble preparations				
6. GOD	Dextra T-2000 was activated with 500 mg CNBr/g carrier.	50	30	70
7. GOG	The same conditions of binding as described for insoluble preparation.	70	0	80

[a]It was assumed that 1 ml packed gel contains 40 mg dry material.

Water-insoluble FAD was prepared by direct attachment to CNBr-activated Sepharose or to ε-aminocaproyl-Sepharose. The amount of FAD in the products was found to be 3 mg/g dry polymer in both preparations, corresponding to 2% of the amount of FAD used in the reaction. The coenzymatic function of the bound FAD was determined by incubation with soluble apoenzyme, followed by the determination of recovered enzymic activity. The recombination of the apoenzyme with insoluble FAD on ε-aminocaproyl-Sepharose yields an insoluble glucose–oxidase with relatively poor activity (10% as related to the same amount of soluble FAD). No detectable enzymic activity was found after incubation of apoenzyme with insoluble FAD directly bound to Sepharose.

Dissociation and Reasscoation of Bound Glucose Oxidase on Insoluble Carriers

It has been shown that FAD coenzyme of GO is not covalently bound to the protein and can be removed under acidic conditions from the complex to form the apoenzyme, which is enzymatically inactive (5). Bound apoenzyme resulting from incubation of insoluble GO on Sepharose or Sephadex at pH 1.4 seems to be more stable than the apoenzyme obtained from native enzyme using the same treatment.

On addition of the FAD in the appropriate conditions (5), the insoluble preparation regained 45–60% of its activity; under the same conditions, the native GO recovered only 26% of its enzymic activity (see Table 2). Another way of obtaining a bound apoenzyme was achieved by incubation of insoluble GO–Sepharose with guanidine hydrochloride. Similarly to the soluble enzyme (6), the insoluble preparation was denatured concomitantly with the release of FAD. The results are summarized in Table 3. On addition of external FAD, reassociation with the bound apoenzyme was observed. Optimal results were obtained with insoluble GO–Sepharose treated with 2.0 M guanidine HCL overnight at 4°C, which yielded a recovery of 90% of the original enzymic activity (see Table 4).

Attempts to obtain insoluble apoenzyme–gelatin using one of the methods described above failed, as FAD could not be separated from the immobilized GOG preparation.

The experiments described above proved that the apoenzyme so obtained remained attached to the insoluble carriers, displaying a higher stability than the soluble one. Similar to the native enzyme, the removal of FAD from the bound enzyme is also a reversible process. However, the study of the conformational changes occurring in the aprotein moiety during dissociation and association of coenzyme by spectral methods is limited in the insoluble system.

TABLE 2. **Reassociation of native and Insoluble Glucose Oxidase Preparations**

Time (min)	Regain of activity[a] Preparations[b] 2	3	4	Native GO
0	0	0	0	0
10	0	—	—	6
18	—	38	—	—
30	40	38	31	9
45	40	38	—	—
60	—	38	—	—
120	50	—	40	16
150	52	38	—	—
240	—	—	55	26
Overnight	60	45	60	26

[a] Enzymic activity retained by insoluble GO preparations after incubation at pH 1.4 followed by raising the pH to 6.2 with addition of FAD.
[b] The numbers correspond to preparations from Table 1.

Attempts to study the fluorescence changes of GO bound to insoluble carrier using the method and the setup of Gabel et al. (18) did not meet with success. This was due mainly to the extensive scattering of both the exciting and the emitted light by the carrier, which could be only partially overcome by a higher protein concentration in the protein–carrier conjugates. However, we were not successful in our effort to insolubilize GO with a high

TABLE 3. **Dissociation of GO-Sepharose Preparation[a] in the Presence of Guanidine HCl[b]**

Concentration of guanidine HCl	Residual activity (%)[c]
2 M	68
3 M	60
4 M	36
6 M	0

[a] Preparation No. 2 (see Table 1).
[b] The reaction was carried out at room temperature for 10 min.
[c] Residual activity was determined after washing of the samples from guanidine HCl.

TABLE 4. **Recovery of Enzymic Activity of GO–Sepharose Preparation after Incubation with 2 M Guanidine HCl**

Time of incubation with FAD (h)	Recovery of enzymic activity[a] (%)
0	0
1	48
3	60
24	79
40	90

[a]The enzymic activity was determined after washing from guanidine and resuspending in 0.1 M phosphate buffer, pH 6.2, containing excess FAD.

protein content per ml bed volume. Binding of GO to soluble carriers was undertaken in order to enable us to use optical methods such as fluorescence of CD as tools for the study of flavin–protein interaction in the bound enzyme.

Dissociation and Association of Bound Glucose Oxidase on Soluble Carriers

Though higher enzymic activity was obtained by binding GO to soluble carriers (see Table 1), difficulties arose in the preparation of soluble bound apoenzyme derivatives. The soluble GOG preparation was precipitated by the regular acid ammonium sulfate treatment (5), but it was found that FAD did not dissociate from the complex. The GOD preparation was not precipitated by acid ammonium sulfate, but was found to dissociate into its components when the pH was lowered to 1.4. Separation of bound soluble apoenzyme from FAD was achieved on a Sephadex G-25 column. The yield of the immobilized holoenzyme obtained after incubation of soluble apoGOD with FAD (see the "Methods" section) was, however, low, and only 30% of the expected activity was regained.

Circular Dichroism Measurements

The CD spectra of native GO and GOD recorded under identical conditions of scanning, pH, and temperature are illustrated in Fig. 1. The right panel presents the spectral region from 300 to 240 nm, where side-chain chromophores of the protein (19) and FAD coenzyme possess

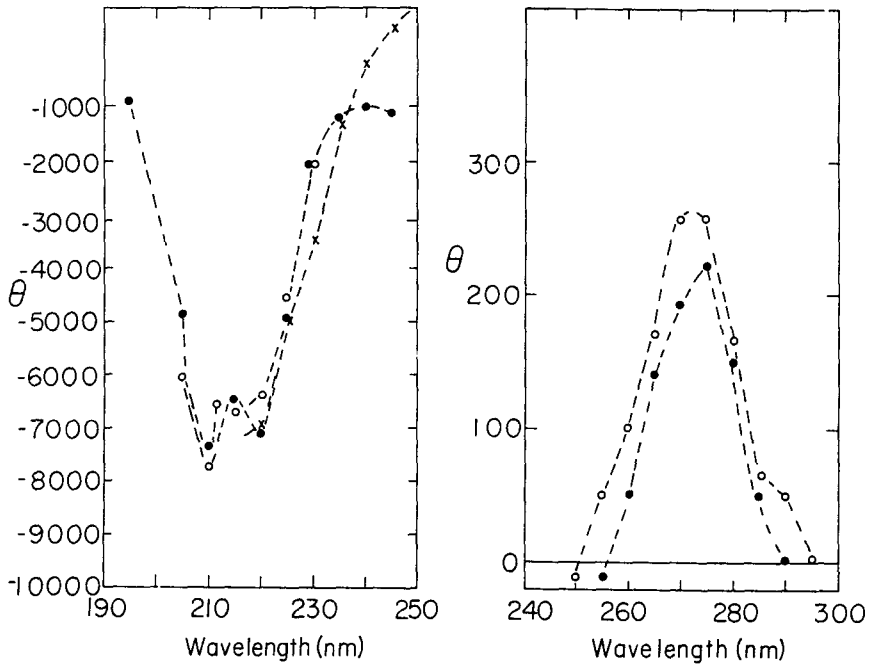

FIG. 1. CD Spectra of Go (•---•) and GOD (○---○) at pH 6.2 in 0.1 M phosphate buffer. The ellipticity values were calculated per amino acid residue. In the left panel, the spectrum of apoGOD is also included (×---×).

absorption maxima. Both preparations show positive maxima in this region, native GO at 275 nm ($\theta = +222$) and GOD at 272 nm ($\theta = +265$). The left panel in Fig. 1 shows the spectra in the region from 250 to 190 nm. Two maxima are observed, one at 215–220 nm and the other at 210 nm, with some molar ellipticities, within the expected experimental errors. Apoenzyme derived from GOD has a CD spectrum that coincides with that of the holoenzyme, as presented in Fig. 1. CD measurements indicate that after immobilization of the enzyme on dextran, no important change occurs in the conformation of the protein.

Fluorescence Measurements

The fluorescence spectra of apoGO and apoGOD are illustrated in Fig. 2. The apoenzyme of the native GO exhibits a typical tryptophan fluorescence with an emission maximum at 340 nm, characteristic of most proteins (20,21). The fluorescence spectrum of apoGOD is red-shifted to 345 nm,

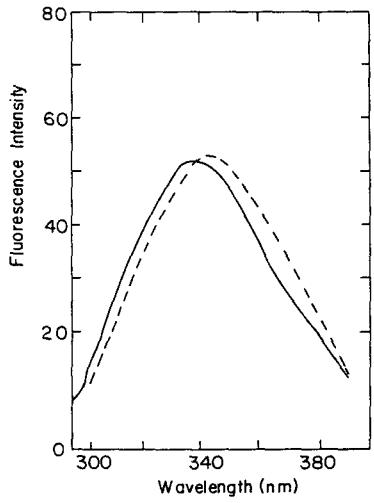

FIG. 2. Fluorescence spectra of apoGO (——) and apoGOD (– – –). The spectra were recorded by excitation at 280 nm. Intensities are not comparable.

indicating an increase in hydrophilicity of the tryphophan environment after binding of GO to dextran.

Quenching of fluorescence intensity of the apoenzyme on addition of FAD was used to monitor the binding process of coenzyme to apoenzyme preparations. The quenching fluorescence profile of apoGOD after addition of FAD, which is presented in Fig. 3, indicated a K_{diss} of the same order of magnitude as the concentrations of components ($K_{diss} \approx 10^{-8}$ M). The

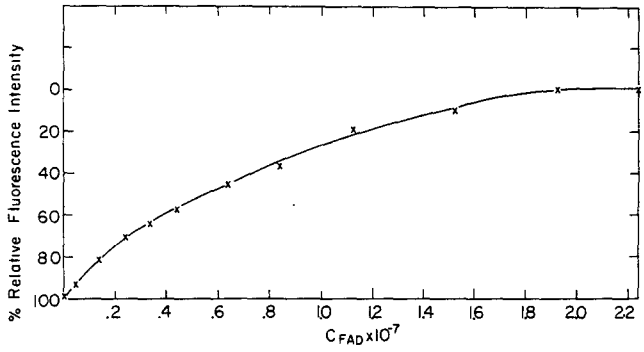

FIG. 3. Protein fluorescence titration of apoGOD with FAD. 1.2×10^{-8} M apoGOD in 0.1 M phosphate buffer, pH 6.2, was titrated with 2.10^{-8}–2.10^{-7} M FAD. The relative fluorescence intensity is expressed in arbitrary units, assuming that the fluorescence of apoGOD is 100.

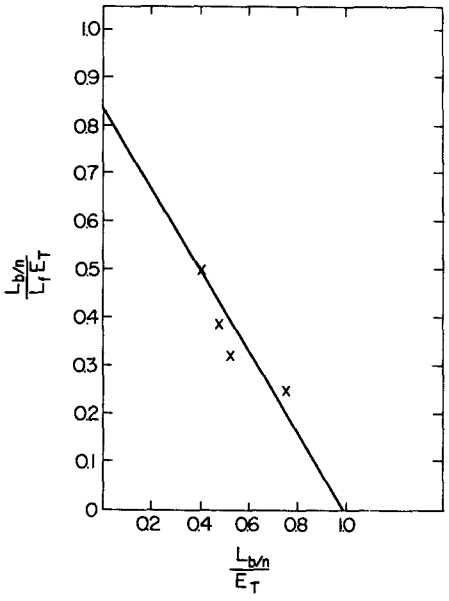

FIG. 4. Scatchard plot for the binding of FAD to apoGOD. The data were calculated from the results shown in Fig. 3.

apparent dissociation constant was evaluated from the translation of this curve to a Scatchard plot (22), as follows:

$$\frac{L_b}{L_f \cdot E_t} = \frac{n}{K_{diss}} - \frac{L_b}{K_{diss} \cdot E_t}$$

in which L_b and L_f are the concentrations of the bound and free ligand; E_t is the total enzyme concentration; n is the number of equivalent FAD binding sites; and K_{diss} is the dissociation constant of the complex.

From the slope of this plot (Fig. 4), a dissociation constant of $K_{diss} = 8 \times 10^{-8}$ M per each binding site was estimated. A decrease in FAD binding affinity compared with FAD binding to apoGO (K_{diss} of apoGO $\approx 10^{-10}$ M) (5,21) occurred after immobilization of GO on dextran. Such a decrease could be the result of conformational changes of the bound apoenzyme or of chemical changes of the group(s) involved in the binding of FAD to apoenzymes.

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