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Conjugation of Glucose Oxidase from *Aspergillus niger* and Rabbit Antibodies Using *N*-Hydroxysuccinimide Ester of *N*-(4-Carboxycyclohexylmethyl)-Maleimide

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Glucose oxidase from *Aspergillus niger* was conjugated with rabbit immunoglobulin G or its monovalent fragments (Fab'). The enzyme was treated with *N*-hydroxysuccinimide ester of *N*-(4-carboxycyclohexylmethyl)-maleimide to introduce maleimide groups, which were then allowed to react with thiol groups of reduced IgG or Fab'. More than 40% of immunoglobulin G, Fab' and enzyme used could be conjugated without self-coupling. The enzyme activity decreased about 26 and 15% upon conjugation with immunoglobulin G and Fab', respectively, and the ability of antibody to bind to antigen was well preserved in conjugates. Conjugate preparations purified by gel filtration contained little free form of immunoglobulin G, Fab' or enzyme. Both the cross-link and enzyme activity in Fab' conjugate were stable at pH 6–7 at 4 °C for at least 6 months.

Several maleimide compounds have been synthesized for cross-linking of proteins [1]. We selected one of them, *o*-phenylenedimaleimide, for the conjugation of antigens and antibodies with β-D-galactosidase from *Escherichia coli*. Thiol groups were generated in antigens or antibodies either by mercaptosuccinylation [2] or by reducing disulfide bonds [3,4] and treated with excess of *o*-phenylenedimaleimide. Maleimide groups thus introduced into antigens and antibodies were then allowed to react with thiol groups of β-D-galactosidase. Kitagawa and Aikawa have directly introduced maleimide groups into proteins using *N*-hydroxysuccinimide ester of *m*-maleimidobenzoic acid [5]. However, both thiol and maleimide groups are labile under conditions used in these studies.

The present paper describes the preparation of rabbit IgG-glucose oxidase and Fab'-glucose oxidase conjugates using *N*-hydroxysuccinimide ester of *N*-(4-carboxycyclohexylmethyl)-maleimide under the condition where both thiol and maleimide groups are stable.

Abbreviations. IgG, immunoglobulin G; $F(ab')_2$, divalent fragments obtained by digesting IgG with pepsin; Fab', monovalent fragments obtained by reducing $F(ab')_2$.

Enzymes. β-D-Galactosidase (EC 3.2.1.23); glucose oxidase (EC 1.1.3.4); peroxidase (EC 1.11.1.7); pepsin (EC 3.4.23.1).

MATERIALS AND METHODS

Synthesis of N-*(4-Carboxycyclohexylmethyl)-maleamic Acid*

A solution of 9.8 g (0.1 mol) of maleic anhydride and 15.7 g (0.1 mol) of 4-(aminomethyl)-cyclohexane-1-carboxylic acid (*trans*) (Mitsui Toatsu Chemicals, Tokyo) in 120 ml of diglyme was stirred at 25–30 °C for 3 h and cooled to 5 °C. The crystalline precipitate formed was washed with a small volume of cold diglyme and with 20–30 ml of cold water and dried. The crude product was 20.5 g (80.4%) and melted at 178–182 °C (decomposition). [Recrystallization from methanol or acetone yielded white crystals, m.p. 188.0–190.0 °C (decomposition) with a yield of 70%.] Anal. Calcd. for $C_{12}H_{17}NO_5$: C, 56.46; H, 6.71; N, 5.49. Found: C, 56.32; H, 6.90; N, 5.30.

Synthesis of N-*(4-Carboxycyclohexylmethyl)-maleimide*

A solution of 20.5 g (80 mmol) of crude *N*-(4-carboxycyclohexylmethyl)-maleamic acid and 3.2 g of sodium acetate (anhydrous) in 40 ml of acetic anhydride was stirred at 50 °C under the atmosphere of nitrogen for 2 h and poured into 600 ml of water. The mixture was stirred at 65–70 °C for 2–3 h.

Complete decomposition of excess acetic anhydride resulted in a clear brown solution. Then, 2 g charcoal was added and removed by filtration, while the mixture was hot. The filtrate was concentrated to 300 ml *in vacuo*, cooled and allowed to stand overnight. The crystals formed were washed with 50 ml cold water and dried at 70 °C overnight to yield 6.4 g (33.6%), m.p. 144.0–146.0 °C. Anal. Calcd. for $C_{12}H_{15}NO_4$: C, 60.75; H, 6.37; N, 5.91. Found: C, 60.24; H, 6.34; N, 5.65.

Synthesis of N-*Hydroxysuccinimide Ester*

A solution of 2.9 g (12 mmol) of *N*-(4-carboxycyclohexylmethyl)-maleimide and 1.9 g (16 mmol) of thionyl chloride in 30 ml of dry benzene was refluxed at 80 °C. After 1 h, benzene and thionyl chloride were removed under vacuum, leaving a residue, crude *N*-(4-chloroformylcyclohexylmethyl)-maleimide (3.0 g, 95.7%). *N*-Hydroxysuccinimide (1.41 g, 10 mmol) in 30 ml of diglyme was mixed with sodium (0.28 g, 12 mmol), heated gradually with stirring and kept at 100.0–110.0 °C for 1 h to yield sodium salt of *N*-hydroxysuccinimide. After cooling the mixture to 40–45 °C, *N*-(4-chloroformylcyclohexylmethyl)-maleimide (3.0 g) in 10 ml diglyme was added dropwise over a period of 5 min. The reaction was continued at 40–45 °C for 5 h. Sodium chloride precipitated was removed and diglyme was evaporated under vacuum. The residue was mixed with 50 ml of water, washed with a small volume of water and dried overnight at 65–70 °C. The product weighed 3.1 g (75.8%), and melted at 164.0–180.0 °C. Recrystallization from acetone raised m.p. to 187.5–189.0 °C. Anal. Calcd. for $C_{16}H_{18}N_2O_6$: C, 57.48; H, 5.43; N, 8.38. Found: C, 57.66; H, 5.67; N, 8.21.

Assay and Purification of Glucose Oxidase

Glucose oxidase activity was determined using *o*-dianisidine and horseradish peroxidase [6] in the presence of 0.1% Triton X-100 [7]. One unit of glucose oxidase was defined as that which oxidizes 1 μmole of glucose per minute. The enzyme preparation (Boehringer Mannheim, grade II) was purified by gel filtration on a Sephadex G-200 column using 0.1 M sodium phosphate buffer pH 7.0. The specific activity increased from 129 to 225 U per mg protein and a single band was obtained on disc gel electrophoresis, which was performed by using 7% polyacrylamide gel in 0.05 M Tris-glycine buffer at pH 8.6.

Introduction of Maleimide Groups into Glucose Oxidase

To the purified enzyme in 1.3 ml of 0.1 M sodium phosphate buffer pH 7.0 (10.5 mg/ml, 69 μM), 20 μl of *N*-hydroxysuccinimide ester of *N*-(4-carboxycyclohexylmethyl)-maleimide in dioxane (15 mg/ml, 47 mM) was added with stirring nine times at 5-min intervals. The reaction mixture was incubated at 30 °C with stirring for various periods of time and chromatographed on Sephadex G-25 using 0.1 M potassium phosphate buffer pH 7.0 containing 1 mM EDTA. The maleimide-glucose oxidase was pooled and concentrated in the cold.

Conjugation of the Maleimide-Glucose Oxidase with Reduced IgG and Fab'

Reduced IgG and Fab' containing a small amount (one thirtieth in weight) of fluorescein-labelled reduced IgG and Fab' were incubated with the maleimide-glucose oxidase, which contained 2.2–4.5 maleimide groups per molecule, in 0.1 M sodium phosphate buffer pH 7.0 containing 1 mM EDTA at 4 °C under an atmosphere of nitrogen for 16–22 h. The final concentrations of the enzyme, reduced IgG and Fab' in the incubation mixture were 25–100, 22–84 and 46–150 μM, respectively. Then, the reaction mixture was incubated with 1 mM mercaptoethylamine at room temperature for 20 min and chromatographed on a column (1.5 × 40 cm) of Sephadex G-200 for IgG conjugate or Sephadex G-150 for Fab' conjugate using 0.1 M potassium phosphate buffer pH 7.0. Fractions 26–34 for IgG conjugates and 22–29 for Fab' conjugates were pooled, concentrated and subjected to repeated gel filtration for further purification.

The amount of conjugates was expressed as units of glucose oxidase activity.

Determination of the Content of Free Enzyme in the Conjugate Preparation

IgG-glucose oxidase or Fab'-glucose oxidase conjugates (about 0.75 units) in 0.1–0.2 ml was passed through a goat anti(rabbit IgG) IgG-Sepharose 4B column (0.3 × 3 cm) at a flow rate of 1 ml/h using 0.1 M potassium phosphate buffer pH 7.0 containing 0.15% bovine serum albumin, and the enzyme activity in 5 ml of the effluent was determined to compare with that before the application. The enzyme activity of IgG and Fab' conjugates was recovered 96.7 (range: 94.3–100, $n = 9$) and 92.8 (range: 90.3–96.5, $n = 9$)%, respectively, in the effluent from a normal goat IgG-column.

Test for the Ability of Antibody Conjugates to Bind to Antigen

Conjugates of glucose oxidase with anti(human IgG) IgG and anti(human IgG) Fab' were passed through a human IgG-Sepharose 4B column to measure the enzyme activity in the effluent as described

above. The enzyme activity of normal IgG and Fab' conjugates was recovered 97.0 (range: 92.1–100, $n = 6$) and 97.2 (range: 94.5–100, $n = 2$)%, respectively, in the effluent.

Test for the Stability of Cross-Link

Fluorescein-labeled Fab' conjugate (about 2.1 units) after storage was chromatographed on a Sephadex G-150 column (1.0 × 40 cm), and the release of Fab' from the conjugate was monitored by measuring fluorescence intensity of fractions from the column.

Other Methods

Thiol and maleimide groups were determined by using 4,4'-dithiodipyridine. The amounts in mg and mol of IgG, its fragments and glucose oxidase were calculated from their absorbance at 280 nm, absorption coefficients and molecular weights. (The absorption coefficient and molecular weight of glucose oxidase were taken to be 1.67 $ml \cdot cm^{-1} \cdot mg^{-1}$ [8] and 153000 [9], respectively.) IgG was prepared by fractionation with Na_2SO_4 and passage through a diethylaminoethyl-cellulose column and digested by pepsin to obtain $F(ab')_2$. IgG was coupled to CNBr-activated Sepharose 4B following the instruction of Pharmacia. Details of these methods were described previously [3,4]. Reduced IgG and Fab' were prepared by reduction of IgG and $F(ab')_2$, respectively, with 2-mercaptoethylamine as described previously [3,4] except that buffers used contained 1 mM EDTA. The content of anti-human IgG in IgG fraction used was determined by comparing absorbance at 280 nm of the effluent from a human IgG-Sepharose column with that before application.

RESULTS

Stability of Maleimide and Thiol Groups

Although maleimide groups of *o*-phenylenedimaleimide and *m*-maleimidobenzoic acid were decomposed more than 50% in 0.1 M sodium phosphate buffer at pH 7.0 at 4 °C within 30 h, *N*-(4-carboxycyclohexylmethyl)-maleimide was stable under the same condition for 64 h.

The average numbers of thiol groups in the molecule of reduced IgG and Fab' prepared in the presence of EDTA were 1.67 (range: 1.40–1.93, $n = 9$) and 1.10 (range: 1.07–1.12, $n = 3$), respectively. Within 40 h of incubation in 0.1 M sodium phosphate buffer at pH 6–7 at 4 °C, thiol groups of reduced IgG and Fab' decreased 63–90 and 15–25%, respectively, but less than 7 and 3%, respectively, in the presence of EDTA.

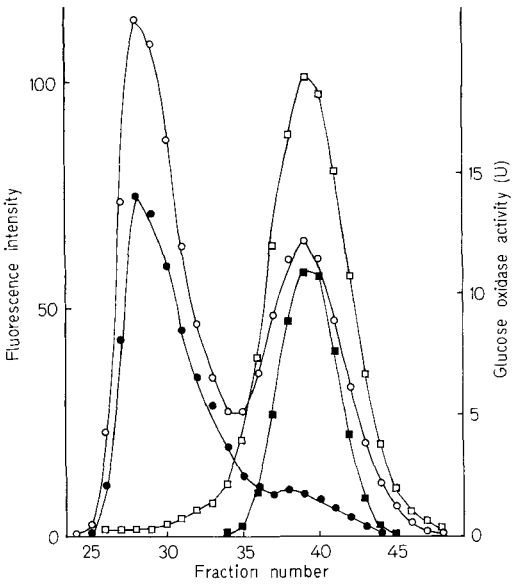

Fig. 1. *Elution profiles of IgG-glucose oxidase conjugate, reduced IgG and maleimide-glucose oxidase from Sephadex G-200 column.* Open and closed circles indicate the fluorescence intensity and enzyme activity, respectively, for the elution profile of the conjugate. Open and closed squares indicate the fluorescence intensity of reduced IgG and the activity of maleimide-glucose oxidase, respectively, which were separately incubated under the conditions for the conjugate for 20–24 h. The volume of each fraction was 0.8 ml, and fluorescence intensity was measured by adjusting that of 0.1 μM fluorescein to a scale of 100

Introduction of Maleimide Groups into Glucose Oxidase

When glucose oxidase was allowed to react with *N*-hydroxysuccinimide ester of *N*-(4-carboxycyclohexylmethyl)-maleimide for 1, 2 and 4 h after the first addition of the reagent, the average number of maleimide groups introduced per enzyme molecule was 2.2 (range: 2.0–2.4, $n = 4$), 4.1 (range: 3.6–4.5, $n = 4$) and 5.3 (range: 3.9–7.1, $n = 4$), respectively. The enzyme activity after the reaction was 100.5 (range: 97.5–100.5, $n = 14$)% of that before the reaction.

Recovery of Glucose Oxidase, IgG and Fab' in Conjugates

Fluorescence intensity of the mixture for conjugation did not change at all, when 51–65% ($n = 8$) of IgG and Fab' added were conjugated. So, the recoveries of glucose oxidase, IgG and Fab' in conjugates were calculated from the enzyme activity and fluorescence intensity in each elution profile (Fig. 1 and 2), and values for the enzyme recovery were corrected by the decrease in the enzyme activity upon conjugation, which is decribed below. The recoveries of the enzyme and IgG in the conjugate were 72–89 and 46–58% ($n = 3$), respectively, when the enzyme

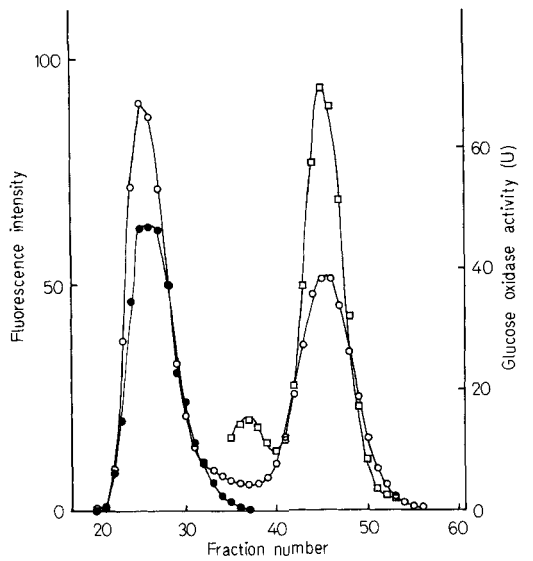

Fig. 2. *Elution profiles of Fab'-glucose oxidase conjugate and Fab' incubated in the absence of EDTA.* Open and closed circles indicate the fluorescence intensity and enzyme activity, respectively, for the elution profile of the conjugate. Open squares indicate the fluorescence intensity of Fab' which was incubated under the condition for the conjugation for 40 h except that EDTA was not added. The volume of each fraction was 0.96 ml, and fluorescence intensity was measured by adjusting that of 0.1 μM fluorescein to a scale of 100

and IgG were incubated with a molar ratio of 0.5 : 1, 45 – 62 and 48 – 63% ($n = 4$), respectively, with a molar ratio of 1 : 1 and 30 – 34 and 62 – 65% ($n = 3$), respectively, with a molar ratio of 2 : 1. When glucose oxidase and Fab' were conjugated with a molar ratio of 0.25 – 2 : 1, 38 – 59% ($n = 7$) of Fab' used was recovered in the conjugate and the recovery of the enzyme could not be estimated from elution profiles. The calculation from the enzyme activity and absorbance at 280 nm gave similar values for the recoveries.

Glucose Oxidase Activity in Conjugates

Glucose oxidase activity of the reaction mixtures after the conjugation was 81, 84 and 76%, respectively, of that before the conjugation and the recovery of the enzyme activity in conjugates calculated from elution profiles was 78, 64 and 83%, respectively. From these values, the enzyme activity was calculated to decrease 26 (24, 25 and 29)% upon conjugation with IgG. When glucose oxidase was almost completely conjugated with Fab', the enzyme activity decreased to 85.3 (range: 76 – 98, $n = 6$)%. Namely, the enzyme activity decreased about 15% upon conjugation with Fab'.

Purity of Conjugates

The enzyme activity in fractions 26 – 33 obtained by repeating gel filtration 2 – 3 times was bound 99.2 – 99.7% ($n = 3$) to an anti(rabbit IgG) IgG-Sepharose 4B column when the enzyme and IgG were conjugated with a molar ratio of 0.5 : 1 and 94.1 – 99.2% ($n = 7$), with a molar ratio of 1 or 2 : 1. When the enzyme and Fab' were conjugated with a molar ratio of 0.25 : 1, the enzyme activity in fractions 22 – 25 and 36 – 31 obtained by the second gel filtration was bound 99.4 – 99.6 and 98.5 – 98.8% ($n = 2$), respectively, to a column of anti(rabbit IgG) IgG-Sepharose 4B. However, the binding decreased to 97.5 – 99.5 and 96.8 – 97.2% ($n = 2$), respectively, with a molar ratio of 0.5 : 1 and to 95.1 – 96.0 and 90.4 – 91.8% ($n = 2$), respectively, with a molar ratio of 1 : 1. Free forms of IgG and Fab' were almost completely separated from conjugates, since IgG and Fab' were not polymerized during the conjugation reaction (Fig. 1 and 2).

Ability of Anti(Human IgG) IgG-Glucose Oxidase Conjugate to Bind to Human IgG

Anti(human IgG) IgG-glucose oxidase conjugates were purified twice by gel filtration, and fractions obtained by the second gel filtration were divided into 2 or 4 pools. Glucose oxidase activity in both IgG and Fab' conjugate pools was bound to a human IgG-Sepharose 4B column approximately as much as expected from the percentage of anti(human IgG) IgG in the IgG fraction used (19%) and the average number of IgG or Fab' molecules conjugated per enzyme molecule, which was calculated from the enzyme activity and fluorescence intensity in conjugate pools. This indicates that the ability of antibody was well preserved in conjugates.

Stability of Cross-Link and Glucose Oxidase in Conjugates

When conjugates were stored in 0.01 M sodium phosphate buffer, pH 6.0, 6.5 or 7.0, containing 0.1 M NaCl, 0.05% NaN_3 and 0.05% bovine serum albumin, neither release of Fab' from conjugates nor change in the enzyme activity was detected at all for at least 6 months.

DISCUSSION

N-(4-Carboxycyclohexylmethyl)-maleimide was readily synthesized from 4-(*aminomethyl*)-cyclohexane-1-carboxylic acid by using maleic and acetic anhydrides and shown to be stable at pH 7, while all attempts to prepare maleimido acids (e.g. maleimidoacetic acid), which are equally stable, by standard cyclization procedures have failed [10]. By using *N*-hydroxysuccinimide ester of the maleimide, rabbit antibody and glucose oxidase were conjugated at neu-

tral pH with their moderate recoveries and little self-coupling, and both enzyme and antibody activities were well preserved in the conjugates, although conjugates obtained were heterogeneous. Such conjugates have been shown to be useful for immunoenzymometric assay because of their high purity (unpublished results). It is worthwhile to note that the present procedure is also applicable to other enzymes which are resistant to the treatment with the maleimide ester, since enzyme-labelling procedures with glutaraldehyde [11] and periodate [12] which have been widely used suffer from disadvantages such as extremely low recovery of enzyme, high degree of polymerization and/or extremely limited applicability (almost exclusively to horseradish peroxidase).

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