Synthesis of Glucose-Sensitive Insulin-Releasing Protein Devices

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A glucose-sensitive insulin-releasing protein device was synthesized by connecting insulin to glucose oxidase (GOD) through a disulfide linkage. Insulin was released from the device by reductive cleavage of the disulfide linkage with electrons generated upon oxidation of glucose by GOD. Some protein devices containing different amounts of insulin were synthesized by varying the feed concentration in the synthetic process. The amount of released insulin increased with the increase of insulin content in the protein device. Although using urea enhanced the amount of insulin coupled to GOD, the efficiency of insulin release did not increase. The fluorescence quenching of the protein device containing fluorescence-labelled insulin with acrylamide showed that insulin was incorporated into the interior domain of the GOD molecule by addition of urea.

It is very interesting for synthetic chemists to design and synthesize "molecular devices". Proteins are useful materials for constructing such devices. We have been using a protein to construct a synthetic drug delivery system.[1,2] Drug delivery systems are necessary for efficient use of medical drugs. These systems are generally categorized into site- and time-specific controls. The former is an organ-specific targeting system such as missile drug therapy. The latter is a slow release or a stimulus-responsive release of drugs. For this stimulus-responsivity various intelligent materials have been designed and synthesized[3,4] and were reviewed by one of us recently.[5] In this research field, glucose-sensitive insulin-release is one of the most important topics.[6,7]

The glucose-sensitive insulin-releasing system we have developed is illustrated in Fig. 1.[2] The protein device is composed of glucose oxidase (GOD) conjugated to insulin by a disulfide linkage which is reductively cleavable with electrons generated upon oxidation of glucose by GOD to release the insulin moiety. We demonstrated that the protein device released insulin by this mechanism, and that the released insulin had about 80% biological activity of native insulin in the previous investigation.[2] In the present investigation, several protein devices were synthesized by the varying synthetic conditions and their properties were investigated by spectroscopic methods.

Experimental

Materials. Glucose oxidase (EC 1.1.3.4) from *Aspergillus niger* (G-8135, type X, 128 IU/mg protein) and insulin from bovine pancreas (I-5500, 24.4 IU/mg protein) were purchased from Sigma Chemical Co. (St. Louis, MO). 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB), a water-soluble carbodiimide (WSC), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride, β-D(+)-glucose, urea, acetone, and anhydrous ether were purchased from Nacalai Tesque Inc. (Kyoto, Japan). 4-Bromomethyl-7-methoxycoumarin (MMC) and 10% methanolic hydrogen chloride solution were purchased from Tokyo Kasei Co. (Tokyo, Japan).

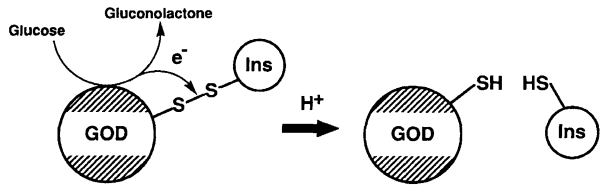

Fig. 1. Principle of the glucose-sensitive insulin-releasing protein device. GOD and Ins represent glucose oxidase and insulin, respectively.

Phosphate-buffered saline (PBS, pH7.0) was prepared by dissolving NaCl (8 g), KCl (0.2 g), KH_2PO_4 (0.2 g), Na_2HPO_4 (1.15 g), $MgCl_2$ (0.1 g), and $CaCl_2$ (0.1 g) in 1 dm^3 of distilled water.

Synthesis of Insulin/GOD Hybrid. The protein hybrid was synthesized by the previously reported method[2] as shown in Fig. 2. In order to prevent inter- and intramolecular crosslinking reactions of proteins in the WSC-activated reaction between insulin and DTNB or between DTNB-insulin and GOD, the carboxyl groups of insulin (500 mg) were esterified in 10% HCl–methanol (40 ml) at room temperature for 24 h. The insulin methyl ester was precipitated from the reaction solution by adding anhydrous ether. This precipitate was then dissolved in PBS. The fractions, which had 210-nm absorbance, were collected by gel permeation chromatography (GPC) on a column of Sephadex G-15 (Pharmacia LKB, Upsala, Sweden; eluent, PBS).

Insulin containing the fluorescent probe MMC was synthesized as follows. Insulin (300 mg) was mixed with the fluorescent halide MMC (300 mg) in an acetone solution (200 ml) at 60 °C for 1 h. The reaction product (MMC-insulin) was purified by the same method using the above-mentioned GPC column.

The amount of esterified groups in the proteins were determined using ADAM (9-anthryldiazomethane). ADAM (1 mg) was dissolved in 50 μl of acetone and the solution was diluted by addition of 450 μl of methanol. Sample solution (25 μl) containing insulin or the insulin derivative (0.05 mg) and the ADAM solution (25 μl) were mixed and the mixture was left standing for 1 h. The product was purified by a column packed with Sephadex G-10. The amount of ADAM coupled to the proteins was determined by measuring the fluorescence at 412 nm when the compound was excited at 365 nm.

The esterified insulins were connected to the disulfide compound DTNB in an aqueous solution containing WSC (10 wt%) at 4 °C for 12 h. The reaction products were purified by using the same method as above. The amount

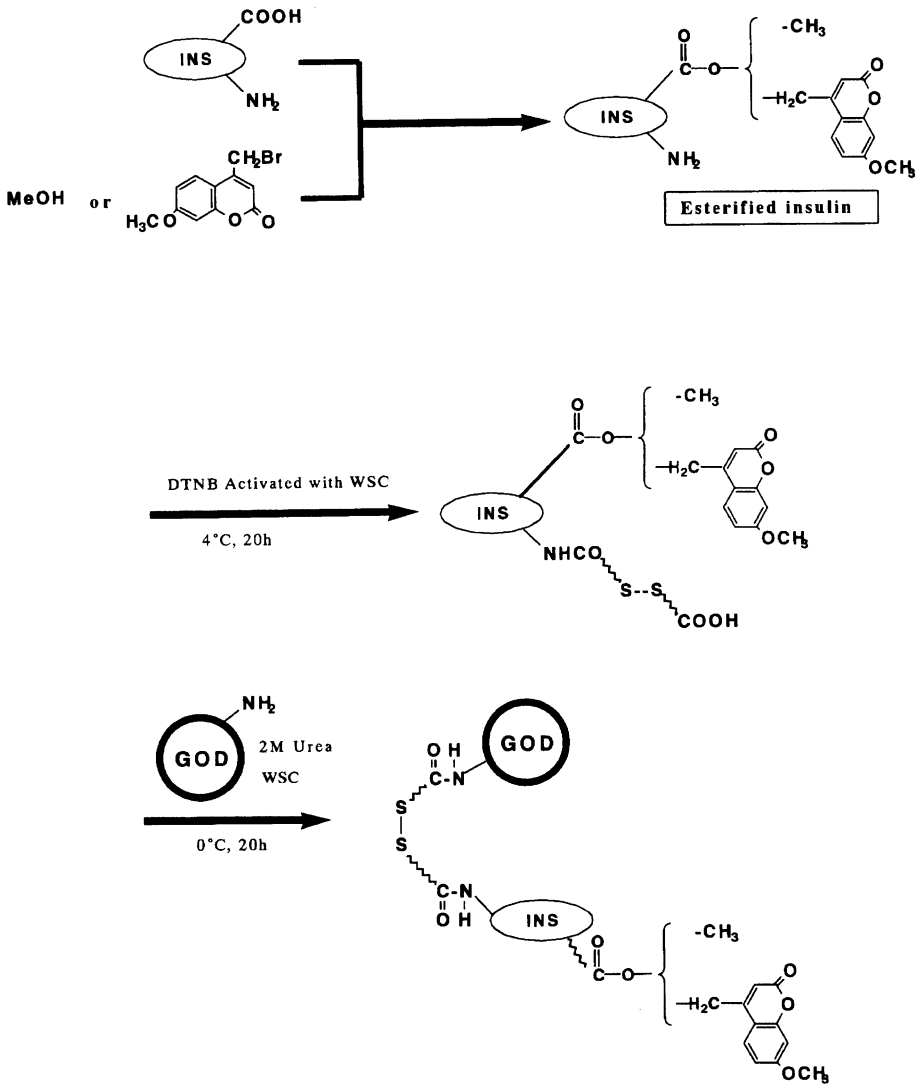

Fig. 2. Synthetic scheme of insulin/glucose oxidase hybrids. GOD, Ins, and WSC represent glucose oxidase, insulin, and water-soluble carbodiimide, respectively.

of coupled DTNB was determined by elemental analysis of sulfur. The sulfur content in the modified insulin increased by coupling to DTNB.

These insulins esterified and coupled to DTNB were connected to GOD in an aqueous solution containing WSC (10 wt%) in the presence or absence of urea at 0 °C for 24 h. The reaction products were purified by eluting with PBS through a GPC column packed with Sephadex G-100 (Pharmacia LKB, Upsala, Sweden), freed from urea and salts by dialysis, and freeze-dried to obtain the insulin/GOD hybrids. The content of each component in the hybrids was determined by elemental analysis of sulfur. The sulfur content in the hybrids depended on the content of DTNB-coupled insulin.

Spectroscopic Measurements. Circular dichroism (CD) and fluorescence spectra were recorded on a JASCO J-600 spectropolarimeter and on a Hitachi F-3010 fluoropolarimeter, respectively.

For fluorescence quenching experiments, an aqueous solution of acrylamide (3 M (M=mol dm^{-3}), 20 ml), which was recrystallized from acetone, was added to a PBS solution of the MMC-insulin/GOD hybrids (5 mg ml^{-1}). The fluorescence intensity of the MMC fragment at 400 nm with an excitation at 314 nm was measured.

Determination of Released Insulin. An aqueous glucose solution of a known concentration was added to a PBS solution containing the insulin/GOD hybrid during incubation at 37 °C. The solution after the addition of the glucose solution was fractionated by eluting through a JASCO 880-PU HPLC column (Tokyo, Japan) packed with JASCO Biofine PRC-PO with H_2O/CH_3OH (7/3 v/v) as an eluent at an elution rate of 1 ml min^{-1}, monitoring at 280 nm with a UV detector. The concentration of released insulin was determined by using a calibration curve, which was obtained by using insulin solutions of known concentrations as previously reported.[2)]

Results and Discussion

Esterification of Insulin. Forty five percent of the original six carboxyl groups in insulin was esterified by the reaction with methanol, measuring the remain-

ing carboxyl groups in the insulin derivative by ADAM. From the elemental sulfur analysis, 1.2 molecules of DTNB were coupled to one molecule of the modified insulin by WSC. In the case of MMC coupling, the same percentage of carboxyl groups was used for the coupling and the same amount of DTNB was incorporated.

Covalent Bonding of DTNB-Insulin to GOD. Insulin methyl ester, coupled with DTNB, was bonded to GOD in varying molar ratios, and the amount of the modified insulin incorporated into GOD was determined and is shown in Table 1. It was found that with increasing feed concentration of the modified insulin in the reaction with GOD, the amount of the modified insulin incorporated into GOD increased. It was also found that more modified insulins were introduced to GOD in the coupling reaction in the presence of urea. The effect of urea may be due to a conformational change of GOD to make more amino groups available for the coupling reaction.

In 1987 Degani and Heller synthesized ferrocene-incorporated GOD and demonstrated that this incorporated ferrocene efficiently mediated electrons.[8] They used urea to incorporate ferrocenes into the interior domain of GOD, because the active site FAD in GOD locates there. Figure 3 shows the CD spectra of aqueous GOD solutions containing varying concentrations of urea. The negative molar ellipticity approaches zero with increasing urea concentrations. However, it is also shown that the molar ellipticity recovers the original value of the native GOD by removing urea from the solution containing GOD and urea by ultrafiltration. These results suggest that a deteriorative change of conformation may not occur on GOD upon treating to 2-M urea.

The amount of MMC- and DTNB-coupled insulin incorporated into GOD was the same as that of DTNB-coupled insulin methyl ester. This result demonstrates that the presence of the fluorescence probe does not affect the binding of insulin with GOD.

Fluorescence of Hybrids. The fluorescence spectra of the fluorescent-labelled insulin derivatives are shown in Fig. 4. Unmodified insulin (A) was not fluorescent in the 400-nm region. MMC-insulin (B) showed a fluorescence at about 400 nm. The final product of the insulin modification, the MMC-insulin/GOD hybrid (C), was also fluorescent, but the fluorescence spectrum

Table 1. Properties of Insulin/Glucose Oxidase Hybrids

Sample	Ins/GOD in feed (mol/mol)	Urea treatment	Ins/GOD in hybrid (mol/mol)	Insulin release/glucose addition[a] (%)
1	10/1	+	6/1	7.5
2	3/1	+	2/1	6.0
3	3/1	−	1/1	4.5

a) The value means the molecule number of insulin released by 100 molecules of added glucose.

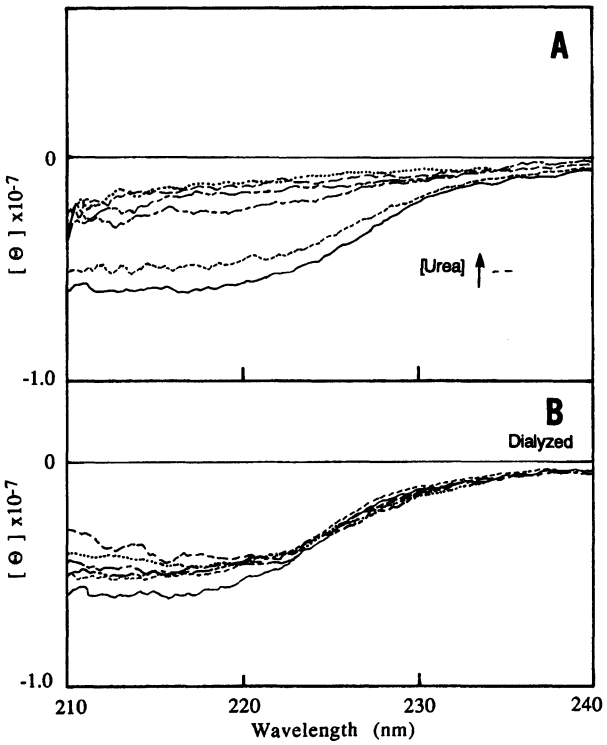

Fig. 3. CD spectra of aqueous glucose oxidase solution containing various concentrations of urea (A) and those after removing urea by ultracentrifugation (B), the urea concentration being zero (—), 2 M (---), 4 M, (-·-), 6 M, (-···-), 8 M (---), and 10 M (····).

became somewhat broad and the fluorescence peak was blue-shifted. These changes observed with the hybrid indicate a binding of the MMC-insulin to a hydrophobic domain of GOD.

Fluorescence spectra of the MMC-insulin/GOD hybrid were measured in the presence and absence of different concentrations of acrylamide, and the Stern–

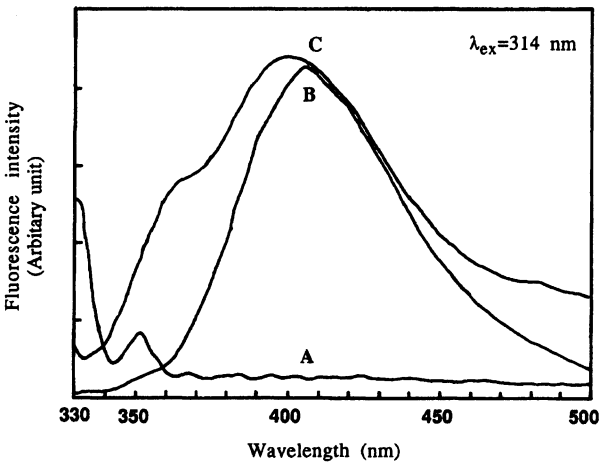

Fig. 4. Fluorescence spectra of insulin (A), MMC-insulin (B), and MMC-insulin/glucose oxidase hybrid (C) in PBS (pH 7.0).

Volmer plot is shown in Fig. 5. The acrylamide quenching of the MMC-insulin/GOD hybrids decreased with increasing content of insulin in the hybrids. Assuming that insulin was coupled in the interior domain of GOD as reported by Degani and Heller for ferrocene coupling,[8] it is considered that the increasing feed concentration of insulin enhanced incorporation of insulin in the interior domains of the protein, as illustrated in Fig. 6.

Insulin Release from the Hybrids. Aqueous glucose solution was added to a PBS solution containing the insulin/GOD hybrid. Insulin was released rapidly upon addition of glucose as shown in Fig. 7. A protein hybrid having more insulin incorporated released more insulin on addition of the same quantity of glucose. However, the efficiency of insulin release decreased with the increase of insulin content in the hybrids as shown in the last column of Table 1. Samples 1 and 2 needed 3.6 and 1.5 times more glucose to cleave one disulfide bond (to release one insulin molecule) than Sample 3, respectively. The difference should have been caused by protein denaturation. Incorporation of a large amount of macromolecular insulin into GOD should suppress renaturing of the enzyme after removing urea, although recovery was apparently observed in Fig. 3. This has also been reported in the incorporation of low-molecular-weight ferrocene.

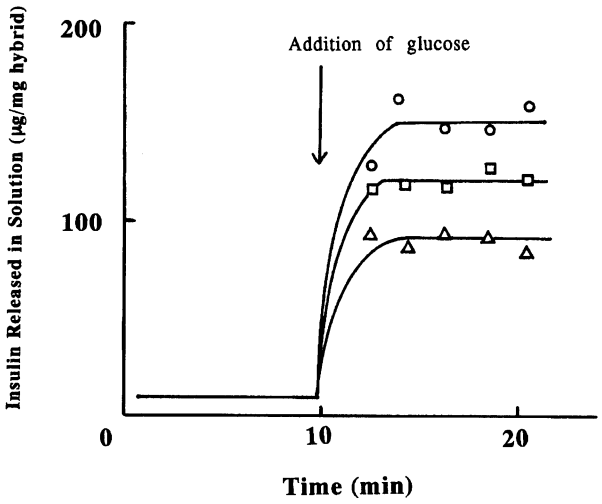

Fig. 7. Insulin release from protein devices (sample 1, ○; sample 2, □; sample 3, △) by addition of 2 mM glucose.

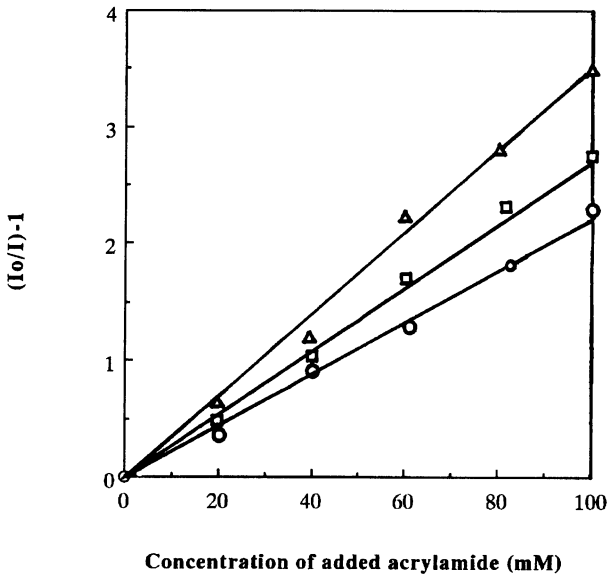

Fig. 5. Fluorescence quenching of MMC-insulin/glucose oxidase hybrid (sample 1, ○; sample 2, □; sample 3, △; for notations see Table 1) with acrylamide at room temperature.

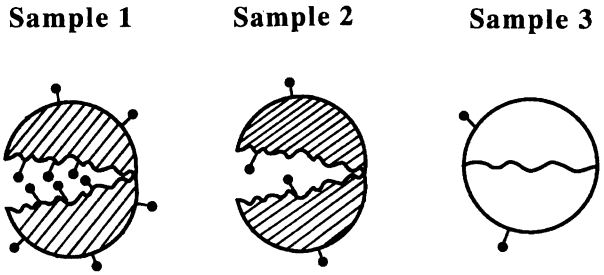

Fig. 6. Schematic drawing of synthesized protein devices. The increase of insulin content in the hybrids should be caused by incorporation in the interior area of the hybrids; notations are given in Table 1.

This study demonstrated that several types of protein hybrid can be made by varying the conditions during the synthetic procedures. For practical uses, this protein device should be encapsulated by a semipermeable membrane through which the released insulin can permeate, or should be modified with poly(oxyethylene), to be immuno-isolated from biocomponents such as immunoglobulin in the living body. This type of protein modification is considered to be promising for the design of a nano-machine.

References

1) Y. Ito, D. J. Chung, and Y. Imanishi, *Artif. Organs*, **14**, 234 (1990).
2) Y. Ito, D. J. Chung, and Y. Imanishi, *Bioconjugate Chem.*, **5**, 84 (1994).
3) Y. Ito, S. Kotera, M. Inaba, K. Kono, and Y. Imanishi, *Polymer*, **31**, 2157 (1990).
4) Y. Ito, M. Inaba, D. J. Chung, and Y. Imanishi, *Macromolecules*, **25**, 7313 (1992).
5) Y. Ito, "Synthesis of Biocomposite Materials," ed by Y. Imanishi, CRC Press, Boca Raton (1992), p. 137.
6) Y. Ito, M. Casolaro, K. Kono, and Y. Imanishi, *J. Controlled Release*, **10**, 195 (1989).
7) D. J. Chung, Y. Ito, and Y. Imanishi, *J. Controlled Release*, **18**, 45 (1992).
8) Y. Degani and A. Heller, *J. Phys. Chem.*, **91**, 1285 (1987).