Site-Specific Modification of Interleukin-2 by the Combined Use of Genetic Engineering Techniques and Transglutaminase

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ABSTRACT: Exploring a new method for the site-specific incorporation of functional groups into proteins, we have studied the combined use of genetic engineering techniques and enzymatic methods. Specifically, a short peptide for use as a substrate of guinea pig liver transglutaminase (TGase) is introduced at the N terminus of human interleukin-2 (hIL-2). The expressed chimeric protein (rTG1-IL-2) is chemically modified at a glutamine site in the appended sequence by TGase-catalyzed transamination with two amines, monodansylcadaverine (MDC), or a constructed derivative of poly(oxyethylene) (POE3). For the TGase-catalyzed modifications with MDC and POE3, 1 mol of donor was incorporated per mole of rTG1-IL-2, respectively. N-Terminal sequence analysis of MDC-modified rTG1-IL-2 (MDC−rTG1-IL-2) showed that the Gln-4 residue in the chimeric protein was site-specifically modified with MDC. On the other hand, tryptic mapping of POE3-modified rTG1-IL-2 (POE3−rTG1-IL-2) by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS) suggested that one of the Gln sites in the appended sequence was modified with POE3. The POE3−rTG1-IL-2 retained full bioactivity relative to the unmodified molecule and rhIL-2. This methodology could be a new and general route for the site-specific modification of proteins.

The introduction of probes, biologically active molecules, and polymers into proteins is of paramount importance for applied uses of proteins in many areas. For example, the use of fluorescent probes is of importance in the study of proteins and protein environment interactions (Gonzalez-Ros et al., 1983). The introduction of metal chelating agents into proteins is useful for *in vivo* diagnostic applications and for adding multiple functions to biologically active proteins (Sieving et al., 1990; Sato et al., 1995). On the other hand, covalent attachment of suitable hydrophilic polymers to proteins yields biologically active conjugates. For example, poly(oxyethylene) (POE)[1] polymers can reduce the immunogenicity of modified proteins and the elimination of relatively small proteins via the kidney (Abuchowski et al., 1977a,b). Such conjugates are prepared using techniques that employ random derivatization of lysine residues (Brinkley, 1992), but the overall utility of these methods is limited, due to the heterogeneity of the products. Therefore, the development of site-specific modifications of proteins is essential for sophisticated use of the conjugated products.

Recently, genetic engineering techniques have allowed the introduction of cysteine residue on the defined site of proteins, using site-directed mutagenesis techniques, and the creation of site-specific disulfide- or thioether-linked conjugates with thiol-selective derivatives, such as maleimide and iodoacetamide (Kuan et al., 1994; Chikoti et al., 1994). Although this type of coupling is superior to other modification methods with respect to specificity and rate of reaction, there are some limitations to its general application. First, the unmodified or modified form of a genetically engineered mutant may have an effect on its structure and function, as compared to that of the native protein. Second, a cysteine mutant may form disulfide isomers with intact cysteine residues and decrease the yield of the properly folded form. Third, *N*-maleimide derivatives, although considered to be sulfhydryl group-specific, may react at a much slower rate with amino and imidazoyl groups in the range of pH 7− 8.

Another approach to a site-specific modification is the introduction of an aminooxy-functionalized group into the N-terminal-introduced reactive glyoxyl group in proteins (Mikolajcjik et al., 1994; Gaertner et al., 1996). However, this method is not generally applicable because the incorporation is restricted to N-terminal serine and threonine residues, and because the modification reaction must be done in two steps, including a harsh periodate oxidation reaction. To try to overcome these limitations of the current methods, we have pursued the development of a new methodology for site-specific modifications of proteins.

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[1] Abbreviations: IL-2, interleukin-2; hIL-2, human IL-2; rhIL-2, recombinant human IL-2; TG1-IL-2, chimeric protein of IL-2 for site-specific modification; rTG1-IL-2, recombinant TG1-IL-2; MDC, monodansylcadaverine; POE, poly(oxyethylene); POE3, polyoxyethylene substrate (MW = 3000); MDC−rTG1-IL-2, MDC-modified rTG1-IL-2; POE3−rTG1-IL-2, POE3-modified rTG1-IL-2; POE3−Boc, α-(carboxymethyl)-ω-methoxy(polyoxyethylene) modified with *N*-Boc-1,5-diaminopentane; TGase, guinea pig liver transglutaminase; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; CTLL, cytotoxic T lymphocyte line; MALDI-TOFMS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; HOBt, 1-hydroxybenzotriazole; DCC, dicyclohexylcarbodiimide; TFA, trifluoroacetic acid; Gu-HCl, guanidine hydrochloride; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; MeOH, methanol; EtOH, ethanol; CHCA, α-cyano-4-hydroxycinnamic acid; UV, ultraviolet; CD, circular dichroism; NMR, nuclear magnetic resonance; PTH, phenylthiohydantoin.

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The basic premise for this work lies in the specificity of the transglutaminase. Transglutaminase catalyzes the reaction

$$\text{R-CONH}_2 + \text{R}'\text{-NH}_2 \overset{Ca^{2+}}{\rightleftharpoons} \text{R-CONHR}' + \text{NH}_3 \quad (1)$$

in which R-$CONH_2$ represents the acceptor, a Gln residue in proteins, and R′-NH_2, the donor, an alkylamine; *in vivo*, the common amine donor is probably a protein-bound lysine side chain (Folk, 1983), and an amide bond cross-linkage is formed between the proteins. It appears that transglutaminase, especially guinea pig liver transglutaminase (TGase), has very stringent sequence specificity and structural requirements for the amine acceptor site around the Gln residues (Gorman et al., 1980, 1984). According to the specificity determinant for proteins, substrate Gln residues for TGase must first satisfy an accessibility criterion, either by being in a highly flexible region of the polypeptide chain or by being clearly exposed to the solvent in a more structured region of the polypeptide chain (Coussons et al., 1992). Using these properties, Yan and colleagues have selectively introduced glycosyl units at Gln residues in β-casein (Yan et al., 1984). Furthermore, Coussons and co-workers prepared a Gln mutant of yeast phosphoglycerate kinase and selectively modified this mutant with monodansylcadaverine (MDC) by TGase (Coussons et al., 1991). Although this methodology is unique for site-specific modification, the design of the mutation for the modification may be difficult to apply to some proteins.

On the basis of this information, we have developed a strategy for the site-specific incorporation of functional groups into proteins by the combined use of genetic engineering techniques and TGase. That is, a short substrate sequence for TGase is introduced at the N terminus of a model protein, and the expressed chimeric protein is chemically modified with alkylamines at a Gln site in the appended sequence by TGase. To confirm our strategy, we have constructed a model system. The protein selected for this study is human interleukin-2 (hIL-2), a lymphokine with several immunoregulatory functions (Smith, 1988). The sequence selected for the preparation of the chimeric protein (named rTG1-IL-2) is Pro-Lys-Pro-Gln-Gln-Phe-Met, derived from Substance P, which is known to be a very good substrate *in vitro* for TGase (Lorand et al., 1984). For modification studies, we have used two model substrates. One is MDC, which is a useful fluorescent probe for studying the proteins and possesses a suitable primary amine for the substrate in the TGase-catalyzed reaction (Takashi, 1988; Yan et al., 1984; Coussons et al., 1991). The other is a synthetic POE derivative (POE3, MW = 3000) with a straight chain alkylamine at one end, which is a useful polymer for bioconjugate chemistry and is designed to be a good substrate for TGase (Zalpsky et al., 1995). The TGase-catalyzed incorporation of these two substrates was monitored by analytical reversed-phase HPLC for MDC-modified rTG1-IL-2 (MDC−rTG1-IL-2) and by SDS−PAGE for POE3-modified rTG1-IL-2 (POE3−rTG1-IL-2). To determine the site of modification in rTG1-IL-2, N-terminal sequence analysis was carried out for MDC−rTG1-IL-2 and POE3−rTG1-IL-2. In addition, tryptic mapping of POE3−rTG1-IL-2 by MALDI-TOFMS was used to confirm the attachment site of POE3 (Billeci et al., 1993; Huberty et al., 1993).

MATERIALS AND METHODS

Enzymes and other reagents were purchased from the following sources. TGase, MDC, and L-1-(tosylamido)-2-phenylethyl chloromethyl ketone-treated trypsin were from Sigma Chemical Co., St. Louis, MO. α-(Carboxymethyl)-ω-methoxypoly(oxyethylene), with an average molecular weight of 3000, was purchased from Nippon Oils & Fats Co., Ltd., Japan. The high-level expression plasmid of the hIL-2 gene, pT13SNco, was provided by Ajinomoto Co., Inc. (Tonouchi et al., 1988). rhIL-2 was purified from inclusion bodies, as described by Tsuji et al. (1987), which were also provided by Ajinomoto Co., Inc. Synthetic DNAs, $^{5'}$CGTTAAATGCCAAAACCTCAGCAGTT$^{3'}$ (Fr. A) and $^{5'}$CATGAACTGCTGAGGTTTTGGCATTTAA$^{3'}$ (Fr. B), were obtained from Takara Shuzo Co., Ltd., Japan. Other chemicals were of reagent grade.

Synthesis of POE3−Boc. The carboxylic acid group terminus of α-(carboxymethyl)-ω-methoxypoly(oxyethylene) was modified with *N*-Boc-1,5-diaminopentane (Fluka, Buchs, Switzerland) according to the procedure of König et al. (1970). α-(Carboxymethyl)-ω-methoxypoly(oxyethylene) (MW = 3000, 3.5 g, 1.17 mM) was dissolved in 8 mL of dry *N*,*N*-dimethylformamide. After HOBt (0.789 g, 5.85 mM) and DCC (1.21 g, 5.85 mM) were sequentially added to the reaction solution, the mixture was stirred under an atmosphere of nitrogen at room temperature for 5 h. To the reaction mixture was added *N*-Boc-1,5-diaminopentane (0.708 g, 3.51 mM), and the resulting mixture was stirred under an atmosphere of nitrogen at room temperature for 36 h. After the solvent was removed at reduced pressure, the residue was purified by column chromatography on silica gel (10/1 CH_2Cl_2/MeOH) and yielded 2.83 g (76%) of POE3−Boc as colorless crystals: R_f 0.72 (10/1 CH_2Cl_2/MeOH); ^{1}H-NMR ($CDCl_3$, 400 MHz) δ (ppm) 3.98 (2H, s), 3.70−3.60 (335H, m), 3.38 (3H, s), 3.36−3.31 (2H, m), 3.15−3.07 (2H, m), 1.58−1.28(6H, m), 1.44 (9H, s).

Synthesis of POE3. After POE3−Boc (2.7 g, 0.85 mmol) was dissolved in 7 mL of TFA, the mixture was stirred at room temperature for 12 h. The solvent was removed at reduced pressure, and the colorless residue was dissolved in 10 mL of MeOH. The solution was then neutralized by the addition of sodium methoxide and was concentrated in a vacuum. The residue was purified by column chromatography on silica gel (10/3/1 CH_2Cl_2/MeOH/H_2O) and yielded 1.59 g (61%) of POE3 as colorless crystals: R_f 0.57 (10/3/1 CH_2Cl_2/MeOH/H_2O); ^{1}H-NMR ($CDCl_3$, 400 MHz) δ (ppm) 4.01 (2H, s), 3.72−3.60 (311H, m), 3.38 (3H, s), 3.34−3.29 (2H, m), 3.00−2.96 (2H, t), 1.76−1.69 (2H, m), 1.63−1.57 (2H, m), 1.48−1.42 (2H, m).

Construction of the Fusion Gene Encoding TG1-IL-2. The plasmid pTTG1IL2 encoding TG1-IL-2, consisting of a short substrate sequence with seven amino acids (Pro-Lys-Pro-Gln-Gln-Phe-Met) and hIL-2, was prepared by digesting pT13SNco at its *Cla*I and *Nco*I sites, removing the resulting fragment, and replacing it with the synthetic DNAs, $^{5'}$CGTTAAATGCCAAAACCTCAGCAGTT$^{3'}$ (Fr. A) and $^{5'}$CATGAACTGCTGAGGTTTTGGCATTTAA$^{3'}$ (Fr. B). The mutant sequence was screened by restriction fragment analysis of plasmid DNA isolated from overnight cultures of transformed HB101 cells. Plasmid sequencing was performed using the Dyedeoxy terminator cycle sequencing

kit and an automated sequencer (Applied Biosystems, Foster City, CA).

Expression and Purification of rTG1-IL-2. *Escherichia coli* HB101 was transformed with the plasmid (Boyer et al., 1969). The culture of *E. coli* and the induction of the *trp* promoter by indole acrylic acid were done using the method of Sato et al. (1987). Inclusion body preparation, solubilization, renaturation, and protein purification were done according to the method used for hIL-2 (Tsuji et al., 1987). Inclusion bodies of the chimeric protein were prepared from the *E. coli* extract, solubilized in 6 M Gu-HCl, and renatured with 10 mM reductive glutathione and 1 mM oxidative glutathione (pH 8.0). The renatured protein was purified by a four-step chromatography procedure. The solution was desalted by passage through a Sephadex G-25 column (Pharmacia-LKB, Uppsala, Sweden) equilibrated with 0.05 M sodium acetate buffer (pH 6.0). The desalted solution was applied to a CM-Sepharose FF column (Pharmacia-LKB) equilibrated with the same buffer, and rTG1-IL-2 was eluted with 0.5 M sodium acetate buffer (pH 6.0). Fractions containing rTG1-IL-2 were collected and applied directly to a YMC-Pack C8-AP column (20 × 250 mm; YMC Co., Ltd., Japan), which was diluted with a linear gradient of 48% to 80% acetonitrile containing 0.1% TFA, at a flow rate of 9.5 mL/min for 60 min. Following reversed-phase chromatography, the peak fractions with an absorbance at 280 nm were pooled and applied to a Sephadex G-25 column equilibrated with 0.05 M sodium acetate buffer containing 0.25 M NaCl (pH 5.0).

CD Spectra. CD spectra of rTG1-IL-2 and rhIL-2 were recorded using a JASCO J-720 spectropolarimeter at room temperature in 5 mM ammonium acetate (pH 5.0). The cell path lengths for the near-UV and far-UV spectra were 10 and 1 mm, respectively, and the protein concentrations were 25.7 μM for rTG1-IL-2 and 26.7 μM for rhIL-2, respectively, which were determined from their A_{280nm} values, using a molar extinction coefficient ϵ_{280} of 1.2×10^4 M^{-1}. Spectra presented are the average of four scans. CD data are expressed as molar ellipticity $[\theta]$ (deg·cm^2·dmol^{-1}) calculated per molecular weight, not per mean residue weight, using protein concentration ([M]).

MDC Modification of rTG1-IL-2 and Collection of MDC−rTG1-IL-2. An enzyme reaction was carried out with the following concentrations of reagents in a total volume of 4.0 mL of 0.1 M Tris/HCl (pH 7.5) at 25 °C for 4 h : MDC, 1.0 mM; $CaCl_2$, 10 mM; protein, 7.5 μM; and TGase, 0.08 unit. The amounts of MDC bound to rTG1-IL-2 and rhIL-2 were analyzed under denaturing conditions by the method of Takashi (1988). Aliquots (60 μL) of the reaction mixture were obtained at predetermined times, up to 4 h, and diluted with 3 volumes of 8 M Gu-HCl in 0.1% TFA to terminate the reactions. These samples were centrifuged for 1 min in an Eppendorf microfuge at 12 000 rpm and were analyzed by analytical reversed-phase HPLC using a known concentration of MDC as the standard. The concentrations of rTG1-IL-2 and rhIL-2 labeled with MDC were measured by the Coomassie method, using a Bio-rad protein assay kit (Bio-Rad Laboratories, Inc.). In addition, 200 μL aliquots of the reaction sample were obtained at predetermined times, diluted with 3-fold volumes of 0.1% TFA, and analyzed.

At the end of the reaction, 250 μL of the reaction mixtures of rTG1-IL-2 and MDC were applied directly to a YMC-Pack C8-AP column (4.6 × 250 mm; YMC Co., Ltd.) and run with a linear gradient of 48 to 80% acetonitrile containing 0.1% TFA, at a flow rate of 1 mL/min for 30 min. Following reversed-phase chromatography, peak fractions with their absorbance at 280 nm and their fluorescence at 500 nm when exited at 355 nm were pooled and used for the N-terminal sequence analysis.

POE3 Modification of rTG1-IL-2 and Purification of POE3−rTG1-IL-2. The TGase-catalyzed POE3 modification of rTG1-IL-2 was carried out with the following concentrations of reagents in 0.1 M Tris/HCl (pH 7.5) at 25 °C for 12 h: POE3, 3.5 mM; $CaCl_2$, 10 mM; rTG1-IL-2, 7 μM; TGase, 0.3 unit/mL. In a control experiment, modification of rhIL-2 was also carried out with the same reagent concentrations. Blanks were prepared in the absence of POE3. At the end of the incubation, the reaction mixtures were analyzed by SDS−PAGE.

To remove the residual free POE3, reaction mixtures of rTG1-IL-2 and POE3 were adsorbed onto a Sep-Pak C_8 cartridge (Millipore Co., Milford, MA) that was previously washed with methanol and equilibrated with 8% acetonitrile containing 0.1% TFA. After a wash with 16 mL of an 8% acetonitrile solution, the sample was eluted with 7 mL of 80% acetonitrile containing 0.1% TFA. The eluted fractions were directly applied to a CM-Sepharose column (Pharmacia-LKB) equilibrated with 0.05 M sodium acetate buffer (pH 4.5), and separation of native and modified forms of rTG1-IL-2 was accomplished by eluting with the same buffer containing 0.5 M NaCl. Each fraction was collected, and the different protein peaks were separately pooled and analyzed by reversed-phase HPLC and SDS−PAGE.

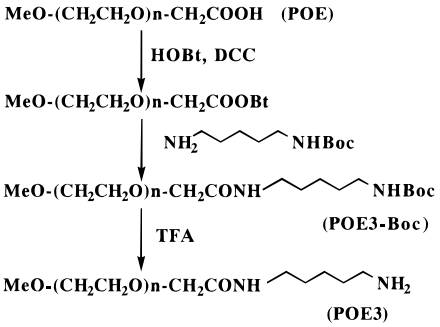
Analytical HPLC. The solutions of rTG1-IL-2, rhIL-2, MDC−rTG1-IL-2, and POE3−rTG1-IL-2 were injected onto a YMC C8-AP column (4.6 × 250 mm; YMC Co., Ltd.) and eluted at 1 mL/min with a linear gradient of 48 to 80% acetonitrile containing 0.1% TFA in 30 min. The concentrations of unmodified and modified forms of rTG1-IL-2 and rhIL-2 were determined by measuring the peak area at 280 nm, which was previously calibrated for rTG1-IL-2 and rhIL-2 by using a molar extinction coefficient ϵ_{280} of 1.2×10^4 M^{-1} (Goodson et al., 1990). The concentrations of MDC bound to rTG1-IL-2 and rhIL-2 were determined fluorometrically by measuring the peak area of proteins at 500 nm when exited at 355 nm, which was previously calibrated for MDC (Fink et al., 1992).

SDS−PAGE Analysis. SDS−PAGE was performed with the Pharmacia phast system using a 20% (w/v) acrylamide gel under reducing conditions. One-fourth volume of sample loading buffer [10 mM Tris-HCl buffer (pH 8.0), 10% glycerol, 1% SDS, 5% 2-mercaptoethanol, and 0.00125% bromophenol blue] was added to the samples. Each sample was then heated for 3 min at 90 °C, and 1 μL of the sample was electrophoresed. After electrophoresis, protein bands were detected by the silver nitrate procedure, according to the recommendations of the manufacturer.

N-Terminal Sequence Analysis. The sequences of the N-terminal amino acids of rTG1-IL-2, MDC−rTG1-IL-2, and POE3−rTG1-IL-2 were determined using a PSQ-1 protein sequencer (Shimazu Co., Ltd., Japan). The phenylthiohydantoin (PTH) derivatives produced at each cycle were identified by HPLC reference to standard derivatives of the amino acids.

Tryptic Digestion of POE3−rTG1-IL-2. Tryptic digestions of POE3−rTG1-IL-2 and rTG1-IL-2 were done according

Scheme 1: Synthesis of POE3



to the method used for human IL-2 (Tsuji et al., 1987). One hundred micrograms of reduced and S-carboxymethylated POE3−rTG1-IL-2 and rTG1-IL-2 were respectively suspended in 200 μL of 0.05 M ammonium hydrogen carbonate buffer (pH 7.9) and treated with $^1/_{50}$ (w/w) amount of L-1-(tosylamido)-2-phenylethyl chloromethyl ketone-treated trypsin (Sigma) for 4 h at 37 °C.

Sample Preparation for Mass Spectrometry. Tryptic peptides of modified and unmodified rTG1-IL-2 were lyophilized to remove the buffer and dissolved in 0.1% TFA to a concentration of 10 pmol/μL. POE3 was also dissolved in 0.1% TFA to the same concentration. CHCA was used as a matrix to assist in the ionization and evaporation processes. To 0.5 μL of each sample solution was added 1.0 μL of a CHCA solution (10 mg/mL, in 50% EtOH and 0.1% TFA). All samples were deposited directly on the laser target and dried at ambient temperature.

Mass Spectrometry. All spectra were obtained using a Kompact MALDI III time-of-flight mass spectrometer (Kratos Analytical, Manchester, U.K.). A nitrogen laser operating at 337 nm was used for laser desorption.

In Vitro Biological Assays. The bioactivities of rhIL-2, rTG1-IL-2, and POE3−rTG1-IL-2 were determined by using the IL-2-dependent murine cell line CTLL-2 (Stern et al., 1984). The amount of IL-2 activity was determined in unit per milligram by using the rhIL-2 standard (Genzyme Co.) and was then expressed as the percent residual bioactivity as compared to that of the purified rhIL-2. All samples were tested in quadruplicate.

RESULTS

Synthesis of POE3. The synthesis of POE3 is shown in Scheme 1. It is suggested that TGase can recognize a wide variety of alkylamines as donors, showing high affinity for straight chain aliphatic amines with a chain length of six carbons (Lorand et al., 1979). Thus, POE3, in which one end of this linear polymer is modified to introduce an alkylamine group, has been constructed. Analysis by ^{1}H-NMR demonstrated that the product was a 1/1 binding product of α-(carboxymethyl)-ω-methoxypoly(oxyethylene) and 1,5-diaminopentane.

Construction of pTTG1IL2. pTTG1IL2, a direct expression plasmid of rTG1-IL-2, was constructed from pT13SNco by replacement of the *Cla*I/*Nco*I fragment with synthetic DNA (Figure 1). pT13SNco is a high-level expression plasmid of the human IL-2 gene, which is partly synthetic to allow it to have many restriction sites, as well as a *trp* promoter/operator and a *trp*A terminator.

Expression and Isolation of rTG1-IL-2. The ligation mixture was used to transform *E. coli* strain HB101. The

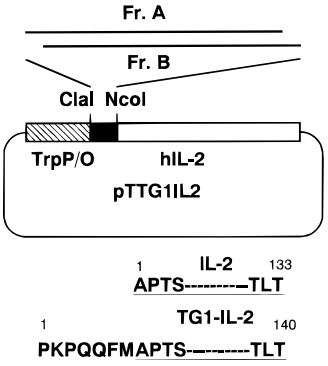


FIGURE 1: Construction of plasmid pTTG1IL2 and structure of rTG1-IL-2. The open area represents the human IL-2 cDNA, and the solid area represents the cDNA for the short substrate sequence. The thin line represents the *trpP/O* region, and Fr. A and B represent synthetic DNA fragments.

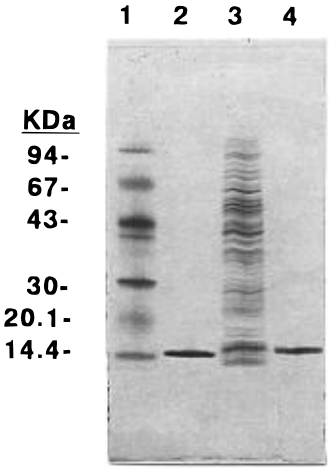


FIGURE 2: SDS−PAGE analysis of purified rTG1-IL-2: lane 1, molecular weight markers; lane 2, purified rhIL-2; lane 3, inclusion bodies of HB101/pTTG1IL2; and lane 4, purified rTG1-IL-2.

ligation construct was confirmed by agarose gel electrophoresis of *Cla*I/*Nco*I-digested plasmid DNA isolated from overnight cultures of the transformed cells. Five out of six clones had the unique *Cla*I/*Nco*I restriction site, resulting in an 83% mutation frequency (data not shown). Single-stranded DNA of one clone from the five containing the *Cla*I/*Nco*I site was sequenced. DNA sequence analysis showed that pTTG1IL2 contained the TG1-IL-2 gene encoding human IL-2 with the short appended sequence on its N terminus. The chimeric protein was accumulated in the cells as inclusion bodies by growing HB101/pTTG1IL2 as a liquid culture. The preparation and purification of the rTG1-IL-2, chimeric protein from inclusion bodies, were carried out as described in Materials and Methods. The inclusion body fraction was recovered, solubilized in 6 M Gu-HCl, and renatured using a glutathione redox system. Following a four-step chromatography procedure, about 50 mg of rTG1-IL-2 was purified from the lysate of 7.2 L of the *E. coli* culture. Figure 2 shows the SDS−PAGE analysis of the purified rTG1-IL-2. Purified rTG1-IL-2 gave only a single peak by analytical reversed-phase HPLC (data not shown) and ran as a single band at 16 kDa (lane 4), which is larger than rhIL-2 (lane 2; 15 kDa) in SDS−PAGE.

CD Spectra. hIL-2 is a member of the structurally related four-helix bundle cytokine superfamily and shows a typical CD spectrum for α-helices (Cohen et al., 1986; Bazan et al., 1992). Thus, CD is a valuable tool in monitoring the

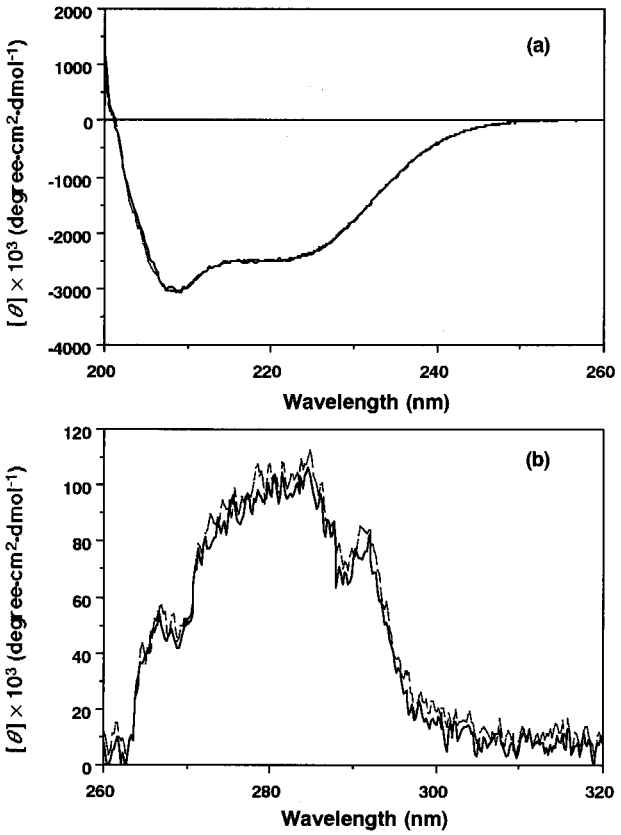


FIGURE 3: CD spectra of rTG1-IL-2 and rhIL-2. Spectra were recorded in 5 mM ammonium acetate buffer (pH 5.0): (a) far-UV spectra and (b) near-UV spectra. Lines (— and - - -) represent rhIL-2 and rTG1-IL-2, respectively. The protein concentrations were 26.7 μM for rhIL-2 and 25.7 μM for rTG1-IL-2, respectively, which were determined from their A_{280nm} values, using a molar extinction coefficient ϵ_{280} of 1.2×10^4 M^{-1}.

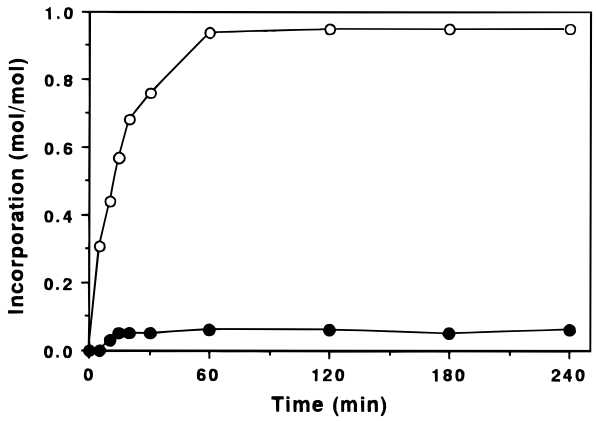


FIGURE 4: Kinetics of the TGase-catalyzed labeling of proteins. rTG1-IL-2 (○) and rhIL-2 (●) were incubated with MDC and TGase under the conditions described in the text.

Table 1: N-Terminal Sequence Analysis of rTG1-IL-2, MDC−rTG1-IL-2, and POE3−rTG1-IL-2[a]

		recovery (%)[b]		
cycle no.	residue	rTG1-IL-2	MDC−rTG1-IL-2	POE3−rTG1-IL-2
1	Pro	114	101	107
2	Lys	137	123	141
3	Pro	104	83	91
4	Gln	98	5	6
5	Gln	106	64	24
6	Phe	105	96	108
7	Met	105	99	106
8	Ala	100	100	100

[a] 100 pmol of rTG1-IL-2, MDC−rTG1-IL-2, and POE3−rTG1-IL-2 were inserted into a PSQ-1 protein sequencer, and the recoveries of the first eight amino acid residues were determined for each. [b] The values shown are relative recoveries of PTH-amino acids. The values are normalized to Ala-8 = 100%.

conformational change of hIL-2. Comparison of the far-UV CD spectra of the chimeric protein (rTG1-IL-2) with that of rhIL-2 showed that both spectra were identical, indicating that two proteins had very similar overall secondary structures (Figure 3a). Likewise, the near-UV CD spectra (Figure 3b) of rTG1-IL-2 and rhIL-2 were also virtually identical, confirming that the two proteins possess similar tertiary structures. These results suggest that addition of a seven-residue tag at the N terminus does not affect the overall structure of hIL-2.

MDC Modification of rTG1-IL-2. Reversed-phase HPLC analysis indicated that the protein peak of rTG1-IL-2 was highly fluorescent due to the incorporation of MDC. A control experiment using rhIL-2 under the same conditions showed only a small amount of fluorescence was incorporated over a 240 min period. Figure 4 shows the time course of incorporation of MDC into rTG1-IL-2 and rhIL-2. There was a rapid incorporation of MDC into rTG1-IL-2, to 0.95 mol/mol after 60 min, and there was no significant change until 240 min thereafter, indicating that 1 mol of MDC per rTG1-IL-2 is incorporated by TGase. This result suggests that the labeling occurs in a highly specific manner.

In contrast, only a small amount of incorporation ($\leq$0.05 mol/mol) was observed over 30 min in the case of rhIL-2, and there was no significant change in the extent until 240 min. This result indicates that the six Gln groups in rhIL-2 do not couple with MDC. The small amount of incorporation must be due to the partial unfolding of rhIL-2, because of the lack of any increase of the incorporation ratio of MDC from 0.05 mol/mol.

From the results of the stoichiometric labeling, it is assumed that either Gln-4 or Gln-5 in the appended sequence is modified with MDC. To determine the site of modification, N-terminal sequence analysis was carried out for MDC−rTG1-IL-2 (Table 1). It shows that the yield of PTH-Gln at cycle 4 was low (5%), suggesting that the Gln-4 residue was MDC-modified. In contrast, the yield of PTH-Gln at cycle 5 was 64%. A small decrease of the yield at cycle 5 would be due to the absence of the carryover effect of the preceding Gln-4 residue in sequencing the modified protein by Edman degradation. Thus, we conclude that MDC couples site-specifically at Gln-4.

POE3 Modification of rTG1-IL-2. POE3-modified rTG1-IL-2 and rhIL-2 were incubated for long periods of time with TGase, under the conditions reported in Materials and Methods, and were analyzed by SDS−PAGE (Figure 5). Under these conditions, no significant polymerization due to intermolecular ϵ-(γ-glutamyl)lysine cross-links was seen for rhIL-2 (lane 2) and rTG1-IL-2 (lane 4) in the absence of POE3. The high-molecular weight band observed at about 80 kDa could be due to TGase. In the presence of POE3, however, the rTG1-IL-2 band migrating at 16 kDa was almost undetectable, and a new single band was observed at approximately 21 kDa, which presumably represents the single POE3-modified product of rTG1-IL-2 (POE3−rTG1-IL-2) (lane 5). In contrast, there was no new band for the

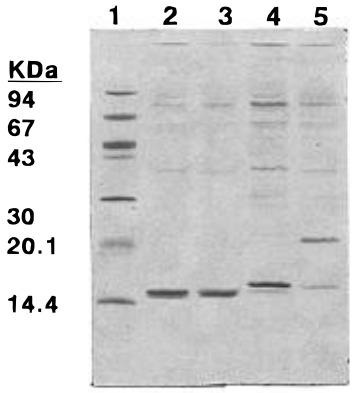


FIGURE 5: SDS−PAGE analysis of the TGase-catalyzed incorporation of POE3 into rTG1-IL-2 and rhIL-2: lane 1, molecular weight markers; lane 2, reaction mixture of rhIL-2 and TGase; lane 3, reaction mixture of rhIL-2, POE3, and TGase; lane 4, reaction mixture of rTG1-IL-2 and TGase; and lane 5, reaction mixture of rTG1-IL-2, POE3, and TGase.

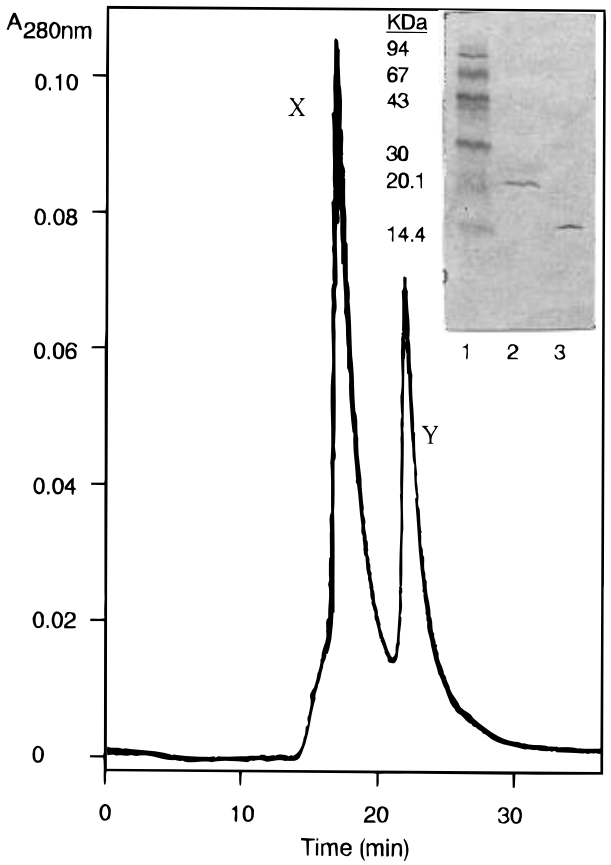


FIGURE 6: Purification of POE3−rTG1-IL-2 on the CM-Sepharose column: X, peak containing POE3−rTG1-IL-2; and Y, peak containing unmodified rTG1-IL-2. The peak protein fractions from the iron-exchange chromatography were analyzed by SDS−PAGE.

reaction mixture of rhIL-2 and POE3 (lane 3), suggesting that none of the six Gln residues in rhIL-2 couples with POE3. Attachment of one POE3 molecule to rTG1-IL-2 has caused the greater increase (about 5000 Da) in size than predicted from its molecular mass (about 3000 Da), presumably due to the hydration of POE3.

Separation of the reaction products of rTG1-IL-2 and POE3 was achieved with a CM-Sepharose column (Figure 6). The protein peaks X and Y were separately examined by SDS−PAGE (Figure 6). The electrophoretic pattern demonstrated that the protein in peak Y migrated at 16 kDa,

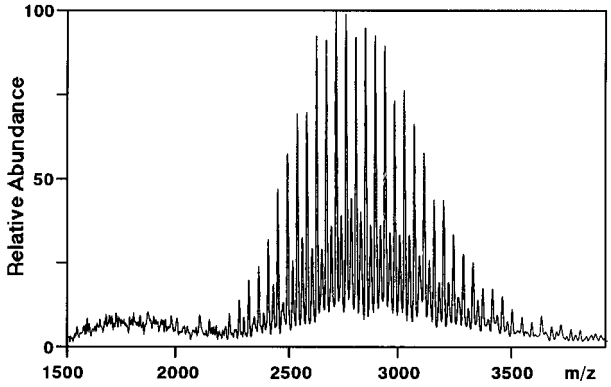


FIGURE 7: MALDI-TOFMS spectrum of POE3 with a CHCA matrix.

corresponding to the size of rTG1-IL-2 (lane 3). On the other hand, the observance of protein bands from peak X, migrating at approximately 21 kDa (lane 2), corresponds to the size of POE3−rTG1-IL-2. Fractions of peak X (yield of 13%) were collected and stored at 4 °C until use for peptide mapping and biological activity tests. The ratio of POE3−rTG1-IL-2/rTG1-IL-2 was lower in the HPLC profile in Figure 6 than in the SDS−PAGE in Figure 5, because of preferential loss of POE3−rTG1-IL-2 due to the failure to absorb on the column.

Sequence Analysis of POE3−rTG1-IL-2. The MALDI-TOFMS spectrum of POE3 in the CHCA matrix is shown in Figure 7. POE3 showed broad signals between *m*/*z* 2200 and 3400, which were assigned using $[CHCA + H]^+$ (190.2) as an internal standard and $[insulin + H]^+$ (5734.6) as an external standard. The difference of each signal was about 44, which is equal to the molecular weight of 1 unit of POE3 (CH_2CH_2O).

The tryptic peptides of reduced and carboxymethylated POE3−rTG1-IL-2 and rTG1-IL-2 were directly analyzed by MALDI-TOFMS (Figure 8). The mass of each signal was assigned using $[CHCA + H]^+$ (190.2) and $[T2 + T3 + T4 + H]^+$ (3193.8) as internal standards, and the assignment was shown in Table 2. As shown in Figure 8b, T1 was observed at 1635.6 in the spectrum of tryptic rTG1-IL-2. In the spectrum of tryptic POE3−rTG1-IL-2, T1 was not observed, and broad signals (labeled T-POE), which presumably represent the POE3-modified fragments, were observed between *m*/*z* 4200 and 5000 (Figure 8a). These results suggest that T1 was site-specifically modified with POE3, because the difference in the molecular masses of T-POE and T1 is almost equal to that of POE3 (about 3000 Da). Since the presence of the bulky POE3 moiety might inhibit the action of trypsin on neighboring peptide bonds, the other spectrum profile for POE3−rTG1-IL-2 was not completely consistent with that for rhIL-2. In addition, the single measurement could not yield complete coverage of the expected fragments as reported by Huberty et al. (1993). However, the fact that an appearance of distinct new broad signals and a disappearance of T1 was observed in the modified rTG1-IL-2 suggested that the major site of modification was being examined.

In contrast, N-terminal sequence analysis showed that the yields of PTH-Gln at cycle 4 and cycle 5 were 6 and 24%, respectively (Table 1). These data raise a question as to whether the POE3 molecule couples at both Gln residues. However, the double incorporation of POE3 is not consistent

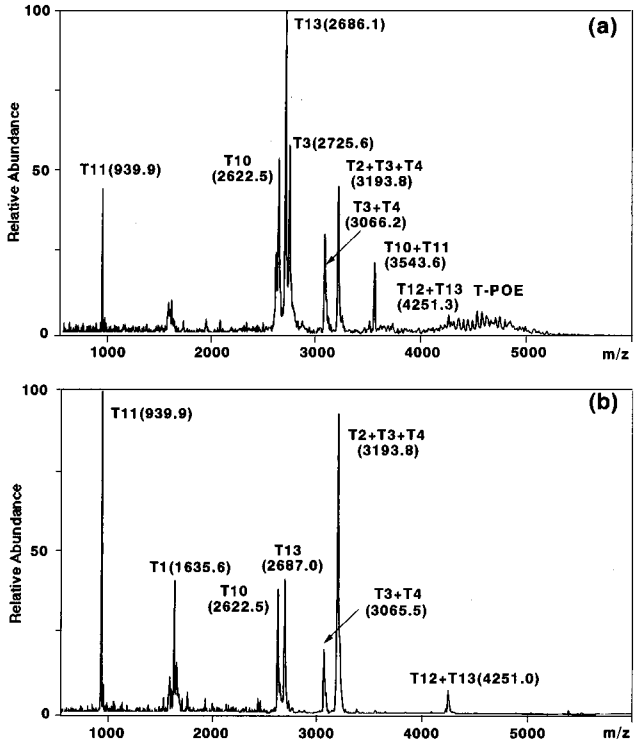


FIGURE 8: Comparison of representations of the MALDI-TOFMS spectra of the tryptic digests of reduced and S-carboxymethylated POE3−rTG1-IL-2 (a) and rTG1-IL-2 (b) with a CHCA matrix. T*n* indicates the assigned tryptic peptide of rTG1-IL-2 given in Table 2.

Table 2: Predicted Tryptic Fragments of Reduced and Carboxymethylated rTG1-IL-2

amino acid residue no.	tryptic peptide	amino acid sequence	MH^+ (Da)
1−15	T1	PKPQQFMAPTSSSTK	1634.8
16	T2	K	147.1
17−39	T3	TQLQLEHLLLDLQMILNGINNYK	2724.5
40−42	T4	NPK	358.2
43−45	T5	LTR	389.3
46−50	T6	MLTFK	639.4
51−55	T7	FYMPK	685.3
56	T8	K	147.1
57−61	T9	ATELK	561.3
62−83	T10	HLQCLEEELKPLEEVLNLAQSK	2621.4
84−90	T11	NFHLRRR	939.5
91−104	T12	DLISNINVIVLELK	1582.9
105−127	T13	GSETTFMCEYADETATIVEFLNR	2685.2
128−140	T14	WITFCQSIISTLT	1570.8

with the 1/1 stoichiometric labeling of rTG1-IL-2. In fact, a small increase of the yield of each PTH-Glu at cycle 4 and 5 (18 and 12%, data not shown) was observed. This increase would be due to the TGase-catalyzed hydrolysis at both Gln residues (Folk, 1983). Considering the rest of the total recovery of (PTH-Gln + PTH-Glu) as POE3-modified, POE3 may couple mainly at Gln-4 and partly at Gln-5.

In Vitro Biological Assays. The bioactivities of rhIL-2, rTG1-Il-2, and POE3−rTG1-IL-2 are shown in Table 3. In the control, purified rhIL-2 has an activity of 4.2×10^6 units/mg. The bioactivities of rTG1-IL-2 and POE3−rTG1-IL-2 are 4.0×10^6 units/mg (94%) and 4.2×10^6 units/mg (100%), respectively, indicating that modified and unmodified forms of the chimeric protein retain full bioactivity relative to rhIL-2.

Table 3: IL-2 Bioactivity of rTG1−IL-2 and POE3−rTG1-IL-2

protein	bioactivity	
	units/mg[a]	% of rhIL-2
rhIL-2	4.2×10^6	100
rTG1-IL-2	4.0×10^6	94
POE3−rTG1-IL-2	4.2×10^6	100

[a] Determined on murine CTLL-2 cells.

DISCUSSION

The TGase-catalyzed reaction offers a potential method for the selective introduction of functional groups into proteins under mild conditions. Although this method has been used for acceptor proteins that have established sites of alkylamine modification (McKee et al., 1972; Barsigian et al., 1988; Ikura et al., 1980; Cordella-Miele et al., 1990), no attention has been paid to the creation of chimeric proteins designed to possess a built-in substrate sequence. According to the information about the specificity of the TGase-catalyzed modification of proteins, the substrate Gln residues tend to be located in a highly flexible region at the terminus (Aeschlimann et al., 1992). Thus, we have described a strategy for the site-specific modification of proteins with this enzyme.

We have designed a chimeric protein of hIL-2 (rTG1-IL-2) with a short substrate sequence at the N terminus, on the following assumptions. (i) Six Gln residues in hIL-2 are not available as substrates for TGase. (ii) The addition of the sequence to the N terminus does not affect the bioactivity or the original structure of hIL-2. From the X-ray data, it is known that the N-terminal region of hIL-2 is very flexible (Brandhuber et al., 1987). Furthermore, studies of structure−activity relationships have revealed that the N terminus of hIL-2 is not essential for biological activity (Cohen et al., 1986).

Our CD and IL-2 bioactivity studies showed that the overall structure of rTG1-IL-2 must be similar to that of rhIL-2. In fact, the addition of the full sequence of Substance P (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Gly-Leu-Met) to the N terminus of hIL-2 revealed that almost 50% of the IL-2 bioactivities was retained on a molar basis (data not shown). Thus, we have selected the essential part of the Substance P molecule consisting of the seven-residue sequence (Pro-Lys-Pro-Gln-Gln-Phe-Met), to avoid a conformational change of hIL-2.

From the results of the TGase-catalyzed MDC and POE3 modifications, the lack of incorporation into native rhIL-2 means that the six Gln side chains, located at amino acid positions 11, 13, 22, 57, 74, and 126 (Robb, 1984), are not in the exposed region and/or not within an acceptable sequence for the TGase-catalyzed reaction. Thus, rhIL-2 is a suitable model protein for confirming our strategy for the site-specific modification. The results of the 1/1 stoichiometric labeling of rTG1-IL-2 suggest that the appended tag must maintain the flexibility of the peptide in an exposed segment of the polypeptide chain.

N-Terminal sequence analysis of MDC−rTG1-IL-2 showed that the Gln-4 residue in the chimeric protein was modified with MDC in a site-specific manner. It is reported that the recovery of PTH-amino acid at cycle *n* is carried over to that at cycle $n + 1$ in the Edman degradation studies (Yan et al., 1984). Then, it seems difficult to recover the PTH-

Gln at cycle 5 quantitatively due to the carryover effect of the preceding Gln-4 residue. Thus, we have tried to estimate the content of the modified Gln residue and propose the reasonable attachment site(s) of each alkylamine on the basis of the recovery of cycle 4 only. The attachment site of MDC seems reasonable, on the basis of the following observation. In our preliminary experiment, FABMS (fast atom bombardment mass spectrometry) was employed to characterize the TGase-catalyzed reaction product of Substance P with a model donor substrate, according to the method of Porta et al. (1988). Substance P has a sequence similar to the appended tag in rTG1-IL-2, as described before. The FAB spectrum of the modified peptide demonstrated that only the first of the two Gln residues, which corresponds to the position of Gln-4 in rTG1-IL-2, is site-specifically modified with the donor (data not shown).

In contrast, the attachment site of POE3 was assumed to be mainly at Gln-4 and partly at Gln-5. This result is not consistent with that of MDC−rTG1-IL-2. The reason for this finding is not immediately clear to us; the most likely explanation is as follows. It is known that the catalysis of TGase takes place in an acylation−deacylation pathway; this reaction proceeds through the formation of a Michaelis-type of acyl−enzyme intermediates and subsequent transfer of acyl groups to the alkylamines (Folk et al., 1977; Lorand et al., 1979; Folk, 1983). In order for reactive groups to participate in intermolecular cross-linking, three macromolecules (protein substrate, alkylamine, and the enzyme) must come into contact in a highly oriented fashion at some stage of the reaction, indicating that the reaction of POE3 occurs via the acyl−enzyme intermediates. Then, the bulky poly(oxyethylene) chains in POE3 may influence the affinity to each intermediate and change the specificity of TGase for each Gln substrate. In fact, this affinity of POE3 seems lower than that of MDC on the basis of the following observations.

(i) We were unable to push the incorporation reaction of rTG1-IL-2 with POE3 to its completion in spite of the longer incubation time and larger amounts of the alkylamine substrate.

(ii) Some TGase-catalyzed hydrolysis was observed at Gln-4 and Gln-5, occurring in competition with TGase-catalyzed aminolysis at both residues.

On the other hand, tryptic mapping of POE3−rTG1-IL-2 by MALDI-TOFMS also suggests that one of the Gln sites in the appended sequence is modified with POE3. As shown in the peptide mapping, the extreme sensitivity of MALDI-TOFMS as well as the ability to analyze the digest mixtures means that only small amounts of material are required and time-consuming purification steps can be omitted. To study the optimal conditions for MALDI of the digests, MALDI-TOFMS would be a useful method to determine the attachment site of protein−polymer conjugates.

The constructed MDC−rTG1-IL-2 has a useful fluorescent probe in the appended sequence at the terminus. Thus, the conjugate can allow monitoring of significant processes in which hIL-2 participates and also can serve to identify cell-surface receptors. By the use of various alkylamine derivatives of fluorescent molecules, different fluorophores can be specifically located on the protein.

The attachment of the POE3 molecule to rTG1-IL-2 does not affect the bioactivity of hIL-2. In addition, rTG1-IL-2 was selectively modified with a POE substrate with a higher molecular mass (20 kDa), without decreasing its bioactivity (data not shown). These observations indicate that the selective introduction of the POE substrates can improve the blood circulation lifetime of pharmacologically active proteins just like the random introduction of low-molecular mass POE chains. Thus, our enzymatic modification would be generally applicable to the preparation of POE−protein conjugates.

On the basis of these observations, the methodology described in this paper can be generally applicable to site-specific modification of some proteins in cases (a) when the chimeric protein can be easily constructed and (b) where proteins lack reactive Gln residues. This would be generally useful, as compared to that of the thiol-selective modifications used in protein conjugation studies, as detailed in the following points.

(i) This method can be applied to a wide variety of proteins without changing the original sequence of the native protein.

(ii) The chimeric protein with a short substrate sequence at its N or C terminus can be easily expressed by a genetic engineering technique.

(iii) Modification of the Gln residues in the appended sequence would not cause any effect on the structure and function of the native proteins.

(iv) The incorporation site of the TGase-catalyzed modification is limited to Gln residues.

There appear to be many interesting and practical applications for our strategy. For example, this approach can be extended to introduce other functional molecules such as polysaccharide derivatives, which can be prepared by introduction of alkylamine groups at their reducing terminus, and deferoxamine, which is a chelating agent and possesses a suitable primary amine for the substrate in the TGase-catalyzed reaction (Sato et al., 1995). Although the guinea pig liver enzyme (TGase) was employed here to demonstrate the site-specific modification, the use of other transglutaminases may offer certain advantages in the way of different specificities toward peptide-bound Gln residues. For example, another transglutaminase, factor XIIIa (thrombin-activated blood coagulation factor XIII), displays pronouncedly different structural requirements than does the liver enzyme for Gln substrates (Gorman et al., 1980; Coussons et al., 1992). Furthermore, it is possible to use different kinds of substrate sequences to introduce at the terminus of proteins. In fact, there are a lot of candidate sequences containing a substrate Gln (Coossons et al., 1991). Although we have selected the N terminus for appending the sequence, it would be possible to add the tag to the C terminus of some proteins. If a three-dimensional structure or structural model is available, the chimeric protein can be easily designed by molecular modeling.

In conclusion, we have successfully developed a new methodology for the site-specific modification of proteins.

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