New Procedures for Preparation and Isolation of Conjugates of Proteins and a Synthetic Copolymer of D-Amino Acids and Immunochemical Characterization of Such Conjugates†

Fu-Tong Liu,‡ Mark Zinnecker, Toshiyuki Hamaoka,§ and David H. Katz*

ABSTRACT: Conjugates of small haptens and a synthetic copolymer of D-glutamic acid and D-lysine (D-GL) have been shown to be very effective in inactivating hapten-specific B lymphocytes that bind determinants attached to D-GL. In order to extend the D-GL tolerance system to more complex protein antigens which are directly related to various human diseases, it is necessary to develop techniques for preparing stable conjugates of protein–D-GL which can then be isolated in pure form. Using hen egg ovalbumin (OVA) as a prototype protein, herein we describe conjugation and purification methods which involve (1) application of a recently developed coupling method employing *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS) as the coupling reagent, i.e., reaction of MBS-modified OVA with thiolated D-GL molecules generated in situ from acetyl-*S*-D-GL by hydroxylamine, and (2) introduction of biotin moieties into D-GL molecules to allow application of an avidin–biotin system for affinity chromatographic purification of conjugates. The reactions and isolations are carried out under mild conditions and in good yields. The conjugate prepared in this way retained a majority of antigenic determinants of OVA and was devoid of any nonconjugated protein, protein dimers, and oligomers which could pose a serious detriment to the effectiveness of tolerance induction. These methods were also applied to the preparation of D-GL conjugates of insulin. The conjugates were characterized by immunoprecipitin reactions and radioimmunoassays. The potential applications of these methods are discussed.

Conjugates of low molecular weight haptens and a synthetic copolymer of D-glutamic acid and D-lysine (D-GL)[1] have been shown to be very effective in inducing in experimental animals hapten-specific immunological tolerance which is highly specific and long lasting (for review, see Katz, 1974; Katz & Benacerraf, 1974). The tolerant state in such circumstances (1) is restricted to bone marrow derived lymphocytes (B cells) which are precursors of antibody-secreting cells, (2) is accompanied by a significant diminution of hapten-specific antigen-binding B cells, and (3) results in a preferential depression of the high affinity anti-hapten antibody response. Antibody responses of all immunoglobulin classes, including reaginic (IgE) antibodies responsible for local and systemic allergic reactions, are abolished by hapten–D-GL conjugates. Moreover, a very important aspect of this system is that such D-GL conjugates are highly effective in turning off ongoing antibody responses in previously sensitized individuals.

This system has been well-characterized with 2,4-dinitrophenyl (DNP)-D-GL (Katz, 1974; Katz & Benacerraf, 1974) and has been extended to the induction of tolerance to nucleoside conjugates of D-GL (Eshhar et al., 1975) which has clinical potential for abolishing anti-nuclear antibody production occurring in patients with systemic lupus erythematosus. Induction of tolerance to the major allergenic determinant of penicillin, the benzylpenicilloyl (BPO) hapten, has also been demonstrated by administering BPO-D-GL to experimental animals (Chiorazzi et al., 1976); the latter system has obvious clinical applicability in terms of treating patients with penicillin allergy. Based on the previously established knowledge in tolerance systems using hapten–D-GL conjugates, it is conceivable that larger macromolecules coupled to D-GL will have similar tolerance-inducing properties, once bound to specific immunoglobulin receptors on B lymphocytes. Therefore, we have been attempting to develop the methodology for preparation of stable conjugates of complex proteins coupled to D-GL for therapeutic use.

We have two major concerns in the preparation of protein–D-GL conjugates which will be tested in experimental animals for their biological activities and clinical applicabilities. (1) The conjugation reaction should be as mild as possible so that the antigenic determinants of the protein of interest are maximally retained. (2) The conjugate should be free of nonconjugated protein, especially protein dimers or oligomers which may be produced under the conjugation conditions and may not be easily separable from the conjugate by conventional chromatographic techniques. For all the protein–D-GL conjugates prepared, we should have a method to demonstrate conclusively the absence of nonconjugated protein, since contamination of any preparation by such molecules would pose a serious detriment to the effectiveness of tolerance induction and, more importantly, could constitute a life-threatening health hazard if such preparations were employed clinically.

Most commonly used coupling reagents such as glutaraldehyde, bis(imido esters), toluene diisocyanate, and carbodiimides are not suitable for our purpose since they react mainly by coupling amino group with amino or carboxyl group and can result in extensive self-coupling of proteins. D-GL,

† This is Publication No. 55 from the Department of Cellular and Developmental Immunology, Scripps Clinic and Research Foundation, La Jolla, California 92037. *Received June 30, 1978.* This work was supported by a grant from Miles Laboratories and the Pardee Foundation. This work has been presented in part at the 62nd Annual Meeting of the American Association of Immunologists (Liu et al., 1978).

‡ Supported by U.S. Public Health Service National Research Training Grant T32-AI-07065.

§ Present address: Institute for Cancer Research, Osaka University Medical School, Osaka, Japan.

[1] Abbreviations used: D-GL, a synthetic copolymer of D-glutamic acid and D-lysine; we chose the abbreviation D-GL to be consistent with past usage and as a space saver for the IUPAC nomenclature, poly(D-Gly^{60}-D-Lys^{40}) [(1972) *J. Biol. Chem. 247*, 323]; OVA, ovalbumin; MBS, *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester; MB-OVA (or -D-GL), *m*-maleimidobenzoyl-OVA (-D-GL); SH-D-GL (or -OVA); mercaptosuccinyl-D-GL (-OVA); PBS, phosphate-buffered saline (0.01 M sodium phosphate buffer, pH 7.2, 0.15 M NaCl); HPP, 3-(4-hydroxyphenyl)-propionyl; 2-ME, 2-mercaptoethanol; BPO, benzylpenicilloyl.

0006-2960/79/0418-0690$01.00/0

which has an abundance of amino and carboxyl groups, is particularly susceptible to this process. The ideal coupling method involves the introduction of a functional group into the protein (or D-GL) which reacts only with another functional group introduced into D-GL (or the protein). The recently reported coupling reagent *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS; Kitagawa & Aikawa, 1976) seems to be just such a reagent. Sulfhydryl groups, the other necessary reactive component, can be incorporated into D-GL (or protein) by known procedures.

We report in this paper detailed procedures for a successful preparation of the D-GL conjugate of ovalbumin, the prototype protein, using the maleimide–sulfhydryl approach. This approach was demonstrated to be effective in preparing insulin–D-GL as well. We also report an effective and reproducible strategy for absolute purification of the conjugates based on the natural affinity of the egg white protein, avidin, for the small vitamin, biotin. These procedures should be of wide applicability in preparation and purification of conjugates of protein with proteins, polypeptides, and other polymers.

Materials and Methods

Reagents

The random copolymer of D-glutamic acid and D-lysine (poly(D-Glu^{60}-D-Lys^{40}) or D-GL) was obtained from Miles Laboratories, Inc., Elkhart, IN, as the HBr salt and used as received. The polymer had an average molecular weight of 63 700 and a ratio of D-glutamic acid to D-lysine residues of 60:40. Hen egg ovalbumin (OVA), 5× recrystallized, was purchased from Pentex, Inc., Kankakee, IL. Insulin (porcine) was obtained from Schwarz/Mann, Orangeburg, NY. *m*-Maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS) and *N*-succinimdyl-3-(4-hydroxyphenyl)propionate were obtained from Pierce Chemical Co., Rockford, IL. *S*-Acetylmercaptosuccinic anhydride, avidin, and *d*-biotin were purchased from Sigma Chemical Co., St. Louis, MO. 5,5′-Dithiobis(2-nitrobenzoic acid) was obtained from Calbiochem, La Jolla, CA. *d*-[*carbonyl*-^{14}C]Biotin was obtained from Amersham/Searle Co., Chicago, IL. Biotinyl-*N*-hydroxysuccinimide ester and [*carbonyl*-^{14}C]biotinyl-*N*-hydroxysuccinimide ester were prepared from *d*-biotin and *d*-[*carbonyl*-^{14}C]biotin, respectively, as described (Bayer & Wilchek, 1974; Jasiewicz et al., 1976).

Preparative Procedures

The amount of unmodified D-GL below refers to the weighed amount and the amount of unmodified protein is determined by UV absorbance.

A. Radiolabeling of D-GL, OVA, and Insulin. In order to radiolabel D-GL with ^{125}I, the synthetic copolymer was substituted with hydroxyphenylpropionyl (HPP) as follows: 10 mg (157 nmol) of D-GL was dissolved in 1 mL of 0.075 M borate buffer, pH 8.5, and the pH of the solution was adjusted to 8.5. Fifty microliters of a solution of *N*-succinimidyl-3-(4-hydroxyphenyl)propionate (4 mg/mL, 760 nmol) in dimethylformamide was added and the mixture was stirred for 30 min at room temperature. The resulting HPP-substituted D-GL was dialyzed extensively against phosphate-buffered saline (PBS, 0.01 M phosphate buffer, 0.15 M NaCl, pH 7.2). Using ϵ(280 nm) = 1660 M^{-1} cm^{-1} for 3-(4-hydroxyphenyl)propionamide (in PBS, pH 7.2), the molar quantity of the hydroxyphenylpropionyl group was determined. HPP_4–D-GL was radiolabeled with ^{125}I using the standard Chloramine-T oxidation procedure (Greenwood et al., 1963). The specific activity of the sample was 420 μCi/mg.

OVA and insulin were labeled with ^{131}I by the solid-phase lactoperoxidase method (David, 1972; David & Reisfeld, 1974). The specific activities were 2 and 5 mCi/mg, respectively.

B. m-Maleimidobenzoylovalbumin (MB-OVA). The maleimide group was incorporated into OVA by reacting the protein with *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS, Kitagawa & Aikawa, 1976). OVA (17 mg, 380 nmol) was dissolved in 1.0 mL of 0.01 M phosphate buffer at pH 7.0. Fifty microliters of a solution of MBS (24.8 mg/mL, 4 μmol) in dimethylformamide was added. After the solution was stirred at room temperature for 30 min, the mixture was applied to a 0.9 × 40 cm column of Sephadex G-25 (Pharmacia, Piscataway, NJ) equilibrated with 0.1 M phosphate buffer, pH 6.0, and eluted with the same buffer at 4 °C. Collected fractions were monitored for absorbance at 280 nm and those containing the derivatized OVA were pooled and used directly for the conjugation reaction. In order to determine the quantity of maleimide groups incorporated, an aliquot (100 μL containing 9 nmol of protein) of the solution was taken, flushed with nitrogen, and reacted with a known amount of deoxygenated aqueous solution of 2-mercaptoethanol (2-ME, 25 μL, 35 nmol) for 20 min. Deoxygenated 0.2 M Tris buffer, pH 8.2 (1 mL), and 0.01 M 5,5′-dithiobis(2-nitrobenzoic acid) in deoxygenated methanol (100 μL) were added and the color developed in the sample after 30 min was measured at 412 nm on a Beckman Model 25 spectrophotometer (Ellman's method; Ellman, 1959). The molar quantity of maleimide groups present then equals the molar quantity of 2-ME consumed. The amount of derivatized OVA in the aliquot was determined by the Folin–Lowry method (Lowry et al., 1951) using OVA as the standard and the ratio of maleimide groups to OVA was found to be 2.4:1 (i.e., $MB_{2.4}$–OVA).

C. m-Maleimidobenzoyl-D-GL (MB-D-GL). This compound was prepared essentially as described for MB-OVA; radiolabeled D-GL was used as a tracer. The buffer used to dissolve D-GL was 0.2 M phosphate buffer, pH 7.2, and the molar ratio of MBS to D-GL used in the reaction was 5:1. The molar quantity of maleimide groups was similarly determined and the derivatized D-GL was quantitated by radioactivity. The MB:D-GL ratio was found to be 2.4:1 ($MB_{2.4}$–D-GL).

D. m-Maleimidobenzoylinsulin (MB-Insulin). This compound was prepared essentially as described for MB-OVA except that the eluent used for the Sephadex G-25 was PBS (pH 7.2). Reaction of insulin (1 mM) with 5 mol equiv of MBS gave $MB_{0.9}$-insulin.

E. Mercaptosuccinyl-D-GL (SH-D-GL). Thiolation of D-GL was effected by the reported method (Klotz & Heiney, 1962) of thiolation of proteins at the ϵ-amino groups of lysine residues. D-GL (40 mg, 627 nmol, containing trace quantities of [^{125}I]-D-GL) was dissolved in 900 μL of 0.125 M phosphate buffer, pH 7.2, and the pH of the solution was adjusted to 7.2 with 1 N NaOH. Twenty microliters of a solution of *S*-acetylmercaptosuccinic anhydride (12 mg/mL, 1.4 μmol) in dimethylformamide was added and the mixture was stirred at room temperature for 30 min, during which time the pH was maintained at 7.0 by the addition of 1 N NaOH. The solution was applied to a Sephadex G-25 column (1 × 23 cm) equilibrated with PBS, 0.01 M in Na_2EDTA, and was eluted with the same buffer at 4 °C. Effluent (4 mL) containing the derivatized D-GL (^{125}I) was collected and deoxygenated by three cycles of vacuum-bleeding in nitrogen. Deoxygenated 0.5 M hydroxylamine, pH 7.3 (400 μL), was added and the solution was incubated at 37 °C for 20 min to remove the

protecting acetyl group. The sulfhydryl groups present were quantitated by Ellman's method as described above (Ellman, 1959). The amount of the derivatized D-GL in the aliquot was determined by the radioactivity and the SH:D-GL ratio was found to be 1.2:1 ($SH_{1.2}$-D-GL).

$SH_{1.9}$-D-GL was similarly prepared by use of a molar ratio of *S*-acetylmercaptosuccinic anhydride to D-GL of 5:1.

F. Mercaptosuccinyl-OVA (SH-OVA). This compound was prepared essentially as described for SH-D-GL. The ratio of *S*-acetylmercaptosuccinic anhydride to OVA used in the reaction was 8.6:1. The ratio of sulfhydryl groups to OVA in the product was 1.1:1 ($SH_{1.1}$-OVA).

G. Biotin-D-GL. One hundred milligrams of D-GL (1.57 μmol) containing trace quantities of [^{125}I]-D-GL was dissolved in 3 mL of 0.05 M phosphate buffer, pH 7.2. The pH was adjusted to 7.2 with 1 N NaOH and 500 μL of a solution of biotinyl-*N*-hydroxysuccinimide ester (5.3 mg/mL, 7.84 μmol) in dimethylformamide was added. The mixture was stirred at room temperature for 2 h and was dialyzed against PBS and then against distilled water and finally lyophilized. The recovery (based on ^{125}I counts) was generally 90–95%.

Utilizing [*carbonyl*-^{14}C]biotinyl-*N*-hydroxysuccimide ester (and no [^{125}I]-D-GL), it was found that 90% radioactivity was incorporated under the same conditions as described above. Therefore, the ratio of biotin groups to D-GL in the product must be about 4.5:1.

H. Mercaptosuccinyl-D-GL-Biotin (SH-D-GL-Biotin) This compound was prepared from $biotin_{4.5}$-D-GL as described for SH-D-GL.

I. Preparation of Avidin-Sepharose Conjugates with Reduced Affinity for Biotin. Avidin-Sepharose conjugates were prepared by coupling 50 mg of avidin with 5 g of cyanogen bromide activated Sepharose 4B (Pharmacia) in 15 mL of 0.1 M $NaHCO_3$ and 0.5 M NaCl, pH 8.3, for 16 h at 4 °C as described in the Pharmacia booklet ("Affinity Chromatography Principles and Methods"). A suspension of the conjugate in PBS was poured into a column. To dissociate the tetramer of avidin and remove noncovalently bound subunits, the column was eluted with 30 mL of 6 M guanidinium chloride and left at room temperature overnight followed by elution with 6 M guanidinium chloride until the effluent had $A_{280} < 0.01$ (30 mL sufficed; Green & Toms, 1973). The column was reequilibrated in PBS (40 mL) and all the binding sites were saturated by equilibrating with 1.2 mM biotin in PBS (40 mL). Elution with 0.1 M glycine hydrochloride, pH 2.0 (40 mL), then removed the biotin from low affinity binding sites, and the column was finally reequilibrated in PBS for use (15-mL packed bed).

The capacity of the modified avidin-Sepharose was determined as follows. One milliliter (packed volume) of the gel was poured into a small column and an excess of [*carbonyl*-^{14}C]biotin with known specific activity was added. The column was eluted with PBS (5 mL) to remove unbound [^{14}C]biotin. The bound [^{14}C]biotin was then eluted with PBS containing biotin (1.2 mM) and was quantitated by the radioactivity. The capacity of avidin-Sepharose was 10.3 μg (42.2 nmol) of biotin/mL of gel. When [^{125}I]-D-GL-$biotin_{4.5}$ was used in place of [^{14}C]biotin, the capacity was determined to be 0.5 mg (7.8 nmol) of $biotin_{4.5}$-D-GL/mL of gel.

J. Preparation and Isolation of OVA-D-GL-(Biotin). $MB_{2.4}$-OVA (30 mg, 667 nmol) in 3.0 mL of 0.1 M phosphate buffer, pH 6.0, was mixed with acetyl-$S_{1.9}$-D-GL-$biotin_{4.5}$ (36 mg, 565 nmol, containing a trace quantity of ^{125}I-labeled molecules) in 3.8 mL of PBS, 0.01 M in EDTA (pH of the mixture: 6.4). The mixture was deoxygenated by three cycles of vacuum-bleeding in nitrogen. To the solution was added 680 μL of deoxygenated 0.5 M hydroxylamine, pH 7.3, and the mixture was stirred under nitrogen at 25 °C for 2.5 h. 2-Mercaptoethanol was added to a final concentration of 1 mM followed by *N*-ethylmaleimide to a final concentration of 2 mM. The solution was stirred at 25 °C for 20 min after addition of each reagent.

One-third of the reaction mixture was applied to a column of avidin-Sepharose (40-mL packed bed) equilibrated with PBS at 4 °C and eluted with 100 mL of PBS followed by 100 mL of 1.2 mM biotin in PBS and finally 80 mL of 0.1 M glycine hydrochloride, pH 2.0. The column was then equilibrated with PBS for reuse. The effluents were monitored for ^{125}I radioactivity and UV absorbance at 280 nm.

The biotin-eluted fractions were dialyzed to remove free biotin and were concentrated either by Amicon ultrafiltration or lyophilization (see Results section for quantitation).

K. Preparation of Insulin-D-GL (Biotin). Insulin-D-GL (biotin) was similarly prepared by reacting $MB_{0.9}$-insulin (59 μM) and acetyl-$S_{1.2}$-D-GL-$biotin_{4.5}$ (47 μM) in PBS.

L. Antisera. Rabbit anti-OVA antiserum was obtained by hyperimmunization of New Zealand red rabbits with 50–100 μg of OVA emulsified initially in complete Freund's adjuvant and subsequently in incomplete Freund's adjuvant administered subcutaneously. Rabbit anti-insulin antiserum was prepared by hyperimmunization of the same kind of rabbits first with 200 μg of insulin-KLH in complete or incomplete Freund's adjuvant as above and then with 200 μg of insulin-KLH in 4 mg of alum, administered intraperitoneally.

M. Immunoadsorbent Affinity Chromatographic Column. The conjugate of OVA with Sepharose 4B was prepared by reacting OVA with cyanogen bromide activated Sepharose 4B (Pharmacia). The column was washed with 0.1 M glycine hydrochloride, pH 2.2, until $A_{280} < 0.005$ before each use.

Antibodies with affinity for OVA were isolated by passing rabbit anti-OVA antiserum through OVA-Sepharose and eluting with 0.1 M glycine hydrochloride, pH 2.2, after washing with PBS. The acid-eluted solution was neutralized with solid tris(hydroxymethyl)aminomethane and dialyzed first against PBS and then 0.1 M $NaHCO_3$, 0.15 M NaCl, pH 8.3. The solution was used directly to conjugate with cyanogen bromide activated Sepharose 4B to give an immunoadsorbent with affinity for OVA. The column was washed with 0.1 M glycine hydrochloride, pH 2.2, before each use.

Immunoadsorbent with affinity for insulin was similarly prepared from anti-insulin antibodies isolated from rabbit anti-insulin serum by an insulin-Sepharose column.

Amino Acid Analysis

Amino acid analyses were made on a Beckman Spinco Model 121-M amino acid analyzer; protein samples were hydrolyzed in 6 N HCl in sealed and evacuated tubes at 110 °C for 24 h.

Results

Modification of OVA and D-GL: General Considerations. A summary of the preparative reactions employed in this study is presented in Figure 1. This approach is discussed in the following sections.

A. Maleimidobenzoylation. Early in these studies, we found that the maleimide group on MB-OVA and MB-D-GL was not stable at neutral pH. The maleimide content gradually decreased, most probably by reacting with the ε-amino group of lysine residues. This was especially noticeable on D-GL, which has an abundance of such amino groups. The half-life of the maleimide group on D-GL was found to be only a few

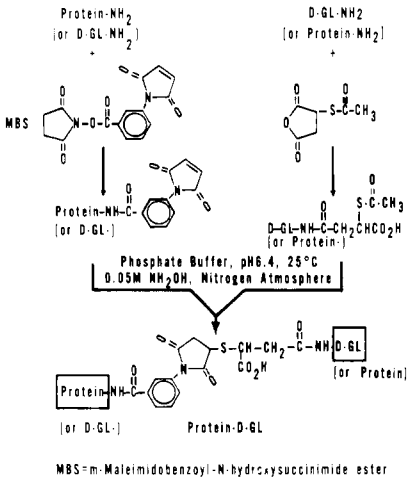

FIGURE 1: Chemical coupling of protein to D-GL using protein (or D-GL) conjugated with a maleimide group and thiolated D-GL (or protein).

hours at room temperature at pH 7.0. The reaction of maleimide derivatives with amines has been reported in the literature (Smyth et al., 1960). We found that the maleimide group on MB-OVA and MB-D-GL remained intact longer at lower pH, e.g., pH 6.0. Less than 10% of the maleimide groups on the modified OVA and D-GL reacted in 1 h at room temperature at pH 6.0. It was therefore essential that the gel filtration be run at pH 6.0, in the cold, and the sample be subsequently kept at the same pH. In addition, we took care to prepare the compound just before the conjugation reaction.

When the maleimide group is incorporated into an OVA molecule, both amino groups and sulfhydryl groups of OVA may react with the maleimide group either on the same or a different molecule. Therefore, dimerization or self-cross-linking is very likely. When MB-OVA was incubated at room temperature and at pH 6.2 for 2 h (the OVA–D-GL conjugation conditions), around 10% of OVA was dimerized as determined from the UV absorbance of the peak eluted earlier than OVA on Sephadex G-100. This represents the maximum amount of dimerization which could occur in the OVA–D-GL conjugation reaction.

B. Thiolation. In the spectrophotometric determination of sulfhydryl groups by Ellman's method, we found it to be very important to deoxygenate the reaction solution as the reagent, 5,5′-dithiobis(2-nitrobenzoic acid), was rapidly oxidized in the buffer solution used (0.2 M Tris buffer, pH 8.2) and produced color which interfered with the reading at 412 nm. Hydroxylamine seems to accelerate this oxidation, and deoxygenation of the solution is especially important in its presence if the correct SH content is to be obtained. In stringently deoxygenated solution, hydroxylamine does not interfere with this spectrophotometric determination.

Oxidation of the sulfide (OVA or D-GL) to disulfide and, therefore, the resulting formation of protein and D-GL dimers was the major problem in the thiolation of OVA and D-GL. In an investigation on thiolation of D-GL, we found that the succinylation reaction and the subsequent gel filtration on Sephadex G-25 did not have to be run under deoxygenated conditions; however, it was especially important that the solution be deoxygenated before hydroxylamine treatment. When SH-OVA was incubated at room temperture and at pH 6.2 for 2 h, 26% of the protein was dimerized as determined by chromatography on a Sephadex G-100 column.

We also found that hydroxylamine did not interfere with the reaction of the sulfhydryl group with the maleimide group and, therefore, did not have to be removed from the SH-OVA or SH-D-GL preparation prior to the conjugation reactions. As a matter of fact, generation of free sulfhydryl from the *S*-acetyl form can be performed in the presence of maleimide.

Thiolated OVA and D-GL were stable if kept in the protected form (i.e., S-acetylated). For instance, we found that acetyl-*S*-D-GL was stable for at least a week at 4 °C.

Conjugation of OVA and D-GL. There are two alternatives to coupling OVA and D-GL by the maleimide–sulfhydryl coupling method: (i) reaction of MB-OVA with SH-D-GL; (ii) reaction of SH-OVA with MB-D-GL as illustrated in Figure 1. In our preliminary studies, ^{131}I-labeled protein and ^{125}I-labeled D-GL were used as tracers in order to facilitate identification of the conjugate since it would contain both the ^{125}I and ^{131}I radiolabels.

In each reaction, the two components were mixed and the reaction solution was incubated at room temperature and pH 6.2 for 2 h under nitrogen atmosphere (solutions were deoxygenated by three cycles of evacuation-bleeding in nitrogen), and, after 2 h of reaction time, the unreacted sulfhydryl groups were blocked with *N*-ethylmaleimide (1 mM). The reaction solution in each case was applied to a Sephadex G-100 column. The fractions were counted for ^{125}I and ^{131}I radioactivity. In both approaches, we observed a peak containing both ^{125}I and ^{131}I radioactivity which was followed by a peak containing only ^{131}I. The elution positions of D-GL and its conjugates were at the void volume, closer than would be expected based on their molecular weights. This is probably related to the nonglobular nature of the D-GL molecule. In parallel experiments, in which modified OVA (MB-OVA or SH-OVA) was incubated alone under the conjugation conditions and was subjected to gel filtration on Sephadex G-100, we found that the elution position of the OVA dimers (and oligomers) formed was close to that of D-GL and the conjugate. Therefore, the first peak contained a mixture of the OVA–D-GL conjugate, D-GL and OVA dimers (and oligomers), and, for absolute purifications, we resorted to other methods such as affinity chromatography.

In both approaches, about the same amount of protein reacted as indicated by the ratio of the ^{131}I counts in the first and second peaks of Sephadex G-100 column. However, approach i is probably a better choice since it should result in less protein self-coupling as indicated in previous sections. Another possible method of conjugating OVA with D-GL is to couple MB-D-GL to unmodified OVA using the naturally occurring sulfhydryl group of OVA. When equimolar amounts of MB-D-GL and OVA were reacted, the conjugate was formed only in low yield. In this experiment, we did not use radiolabeled OVA as tracer since the sulfhydryl group of OVA is very likely converted to sulfenyl iodide upon radiolabeling (Cunningham & Nuenke, 1961). The conjugated protein in the first peak of the Sephadex G-100 column was quantitated by the Lowry–Folin method (see below for quantitation methods).

Preparation and Isolation of Biotin-Labeled OVA–D-GL Conjugate. The preparation procedures for OVA–D-GL (biotin) from MB-OVA and SH-D-GL (biotin) were described in Materials and Methods. One feature of this preparation is the generation of SH-D-GL (biotin) from its protected precursor in situ in the presence of MB-OVA. The extent of self-coupling of SH-D-GL was thereby reduced and the overall preparative procedure was simplified. Another feature of the preparation is the direct application of the reaction mixture to the avidin–Sepharose column for purification.

The separation profile is shown in Figure 2. The fractions were monitored for radioactivity and UV absorbance at 280

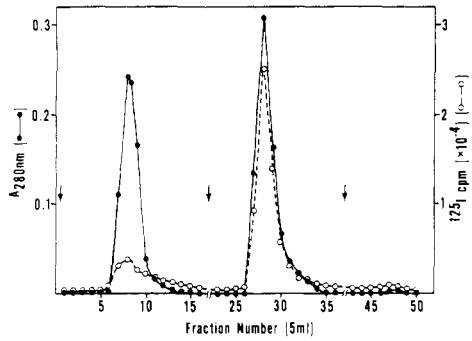

FIGURE 2: Purification of biotin-labeled OVA–D-GL conjugate by affinity chromatography on avidin–Sepharose. Conjugate preparation composed of 9.9 mg (220 nmol) of OVA and 11.9 mg (187 nmol) of D-GL was loaded on the column of avidin–Sepharose (40 mL) equilibrated with PBS at 4 °C. Arrows indicate change of eluent: (from left to right) PBS, 1.2 mM biotin in PBS, 0.1 M glycine hydrochloride, pH 2.0. Flow rate was 57 mL/h.

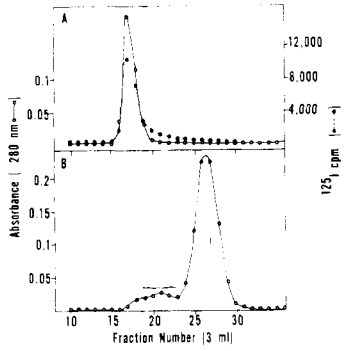

FIGURE 3: (A) Sephadex G-100 chromatography of biotin-eluted material from avidin–Sepharose column (Figure 2, second peak). The peak contained OVA–D-GL (biotin) and D-GL (biotin). (B) Sephadex G-100 chromatography of PBS-eluted material from avidin–Sepharose (Figure 2, first peak). The major peak was OVA and the material eluted before OVA contained OVA dimers and oligomers. The column (1.6 × 80 cm) was equilibrated with PBS and eluted with the same solvent at 4 °C. Flow rate was 9.6 mL/h.

nm. Elution with PBS alone gave a peak which consisted of a mixture comprised of (1) unconjugated OVA and/or OVA dimers and aggregates and (2) unconjugated D-GL or OVA–D-GL conjugates which either lacked biotin or in which the biotin molecules were unexposed for binding to avidin. Elution with PBS containing biotin (1.2 mM) then yielded the desired OVA–D-GL-(biotin) conjugate together with unconjugated D-GL-(biotin). Further elution with 0.1 M glycine hydrochloride, pH 2.0, yielded little material. The first peak contained 15% of the ^{125}I counts loaded on the column. The second peak contained 70% of the counts and third peak, 5%. Therefore, the total recovery was about 90%.

The effectiveness of the avidin–Sepharose column for purification of OVA–D-GL conjugates is demonstrated in Figure 3. Material eluted by PBS was fractionated on Sephadex G-100 (Figure 3B) and was shown to contain mainly OVA and its dimer (eluted at positions identical with those products obtained when MB-OVA was allowed to stand at room temperature for 2 h). Material eluted by biotin-containing buffer was similarly shown to be exclusively devoid of unconjugated protein (Figure 3A). It is to be noted that D-GL (^{125}I) exhibits long tailing on Sephadex G-100 (Figure 3A). Since dinitrophenylated D-GL (DNP_{10}-D-GL) did not exhibit such long tailing (fractions monitored by absorbance at 360 nm), we think this is probably due to fragmentation of D-GL molecules upon radioiodination or due to radiodecomposition of the radiolabeled D-GL (Bayly & Evans, 1966) although it is also partially due to the heterogeneity of D-GL employed.

Quantitation of Biotin-Containing Protein–D-GL Conjugate. For quantitation of OVA, we originally used ^{131}I-labeled OVA as tracer and thought that the conjugate could be identified by the presence of both ^{125}I and ^{131}I counts and that the antigenicity of the conjugate could be assessed by the ^{131}I counts precipitable by anti-OVA antibodies. However, we found that most (>70%) of the ^{131}I labels on OVA were not recovered from the avidin–Sepharose column. This is most probably due to the release of the labile radiolabels on OVA which are present as sulfenyl iodide (Cunningham & Nuenke, 1961).

There is also a problem in the quantitation of protein in the conjugate preparation by the Lowry–Folin method, since D-GL will give a Folin color. For example, a test solution (2.4 mL) containing a final concentration of 0.65 μM of D-GL had A_{700} of 0.22 compared with A_{700} of 0.75 for a solution of OVA (0.92 μM). The amounts of protein can, however, be quantitated by the Lowry–Folin method (Lowry et al., 1951) provided that the contribution from D-GL be corrected for. For example, the amount of color (A_{700}) developed in the tests for solutions containing 50 μg of D-GL and varied amounts of OVA is linear for 0–100 μg of OVA and is equal to the sum of that for isolated D-GL and OVA.

Quantitation can be more conveniently done from UV absorbance of the conjugate. MBS-modified protein does not have the same absorbance as the unmodified protein since maleimidobenzoyl (MB) groups contribute to the absorption at 280 nm. We measure the UV absorption of the conjugation reaction mixture after the maleimide groups are quenched by mercaptoethanol. Since the reaction mixture contains a known amount of protein derivatized with MB in which the maleimide moieties are saturated by coupling to SH on either D-GL or mercaptoethanol, an extinction coefficient for the modified protein can be calculated and the amount of conjugated protein from the avidin–Sepharose column can thereby be quantitated. Since proteins are probably not uniformly modified by MBS (e.g., number of MB groups on the modified proteins may follow a Poisson distribution (Gennis & Cantor, 1972)), the absorbance used here is an average absorbance. It is also assumed that there is no significant difference in the "average" absorbance between D-GL-conjugated and nonconjugated OVA.

During the development of the conjugation and purification methods reported here, we always used ^{125}I-labeled D-GL to facilitate quantitation. Biotin-modified D-GL can also be quantitated by a sensitive spectrophotometric assay for biotin-containing compounds. It is based on the use of the dye 4-hydroxyazobenzene-2′-carboxylic acid which binds to avidin to form a complex having an absorption maximum at 500 nm. Biotin can displace this dye from avidin and cause a decrease in absorption at 500 nm which is linearly proportional to the concentration of biotin (Green, 1970). We found that there was a linear relationship between the decrease of absorption and the amount of biotin–D-GL up to a maximum of 250 μg which corresponded to a ΔA_{500} of 0.55. We also found that conjugation of protein to D-GL had little effect on the binding of the biotin on D-GL to avidin (Figure 4). The amount of D-GL in the conjugate preparation can therefore be quantitated using biotin–D-GL as the standard.

Quantitated in these ways, a particular conjugate preparation obtained after avidin–Sepharose purification contained 11.0 mg (244 nmol) of conjugated OVA and 21.0 mg (330 nmol) of conjugated and nonconjugated D-GL, starting from

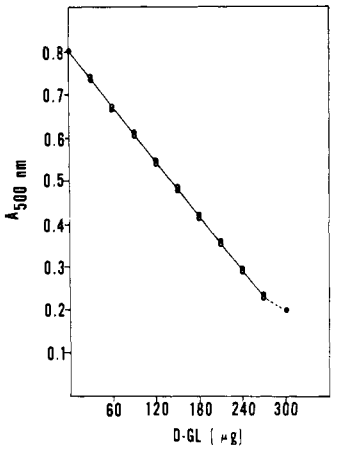

FIGURE 4: Spectrophotometric assay of biotin-labeled D-GL and OVA–D-GL conjugate. A 1-mL solution of avidin (8.3 μM) containing 4-hydroxyazobenzene-2′-carboxylic acid (HABA, 240 μM) was titrated with a solution (3 mg/mL) of biotin$_{4.5}$–D-GL (●) or a mixture of biotin$_{4.5}$–D-GL and biotin$_{4.5}$–D-GL–OVA containing an equal amount (3 mg/mL) of total biotin$_{4.5}$–D-GL (○).

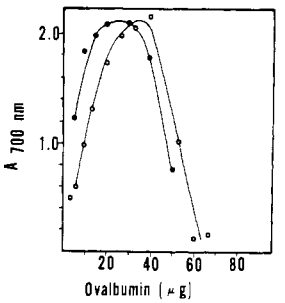

FIGURE 5: Quantitative precipitin reaction of OVA (●) and OVA–D-GL conjugate (○) with rabbit anti-OVA serum. Reactions were run with 200 μL of OVA or OVA–D-GL (the amount of OVA in the conjugate was determined as described in the text) and 100 μL of antiserum. The mixture was incubated at 37 °C for 1 h and 4 °C overnight. The precipitates were collected, washed with cold PBS, and analyzed by the Lowry–Folin method.

667 nmol of MB-OVA and 565 nmol of acetyl-*S*-D-GL. The sample was subjected to amino acid analysis to obtain a more accurate quantitation of conjugated protein. The results indicated that the actual amount of protein was less than that estimated as above with a difference of less than 10%.

Immunochemical Characterization of OVA–D-GL and Purification of the Conjugate by Immunoadsorbent Affinity Chromatography. Quantitative precipitin reaction of the OVA–D-GL preparation after the avidin–Sepharose purification with rabbit anti-OVA serum is shown in Figure 5 which illustrates that the majority of antigenic determinants of OVA were retained after conjugation.

Protein–D-GL conjugates were further purified by immunoadsorbent affinity chromatography on columns prepared with anti-protein antibodies. The column was first eluted with PBS and the desired conjugate was recovered by elution with 0.1 M glycine hydrochloride buffer at pH 2.2. Conjugates were found to be stable under these purification conditions, since they could subsequently be quantitatively readsorbed by the immunoadsorbent after neutralization. When OVA–D-GL preparation was subjected to this purification, 76% of the protein was in the acid-eluted fractions as determined from absorbance at 280 nm. This represented the fraction of conjugated OVA which retained the capacity to combine with antibodies. Together with the protein, 39 ± 3% of D-GL (quantitated by ^{125}I counts) was contained in the acid-eluted fractions. This represented the amount of D-GL conjugated

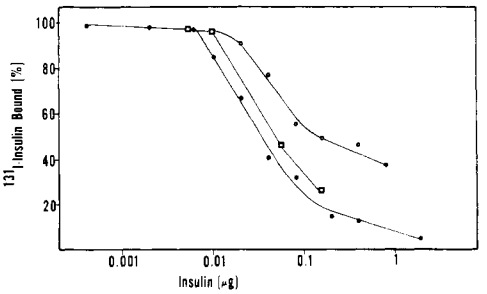

FIGURE 6: Test of insulin–D-GL antigenicity by insulin radioimmunoassay. Insulin (porcine) or insulin–D-GL was incubated with rabbit anti-insulin serum (1:40) at 4 °C for 1.5 h. ^{131}I-labeled insulin was added and the mixture was incubated at 4 °C for 1.5 h. Antigen–antibody complexes were precipitated with goat anti-rabbit γ-globulin and the supernatants were counted for ^{131}I radioactivity. Triplicates were run for each concentration of antigen. ((●) Insulin; (○) insulin–D-GL purified by avidin–Sepharose; (□) insulin–D-GL purified by avidin–Sepharose and anti-insulin immunoadsorbent.)

to OVA which retained antigenicity. We have demonstrated that these immunoadsorbent columns do not have affinity for nonconjugated D-GL.

Preparation of Insulin–D-GL Conjugate. The same conjugation and isolation procedures were applied to preparation of insulin–D-GL conjugate. The conjugate was similarly purified by avidin–Sepharose and anti-insulin immunoadsorbent columns. A preparation containing insulin and D-GL in a ratio of 0.8:1 (conjugated insulin:total D-GL) was obtained by reacting MB-insulin and SH-D-GL in concentrations designated in Materials and Methods and then subjecting the conjugate to avidin–Sepharose purification.

The affinity of D-GL-conjugated insulin for rabbit anti-insulin antibodies before and after immunoadsorbent purification was assessed by a radioimmunoassay essentially as described by Glover et al. (1967). The results are shown in Figure 6. There is a substantial loss of antigenicity of insulin upon conjugation to D-GL. The percentage antigenicity one assesses from this radioimmunoassay depends on what percent bound one chooses. At 50% bound, the antigenicity retained is 30%, in agreement with the percentage of insulin–D-GL adsorbed by the anti-insulin column. After purification by anti-insulin immunoadsorbent, insulin–D-GL exhibited 68% of the antigenicity of insulin. The difference between the inhibition of ^{131}I-labeled insulin binding by insulin and by the purified insulin–D-GL here must be reflecting the difference in the affinity of two molecules for anti-insulin antibodies.

Discussion

The coupling method we have employed is based on the reaction of a maleimide group conjugated to the protein (or D-GL) and a sulfhydryl group either present on the native protein or introduced into the protein (or D-GL) by thiolation (Figure 1), as originally developed by other investigators (Kato et al., 1975, 1976; Kitagawa & Aikawa, 1976). Using OVA as a prototype protein, we have tested three different approaches and demonstrated that conjugation by reacting MB-OVA and SH-D-GL was most satisfactory in terms of its higher efficiency and lower extent of protein self-coupling. Modified OVA with an average of two to three maleimide groups and modified D-GL with an average of one to two sulfhydryl groups were reacted in this conjugation reaction.

This method is extremely mild, has high coupling efficiency, and does not result in extensive self-coupling, intra- or intermolecular, of D-GL or protein as occurs when other commonly employed coupling reagents are used (such as glutaraldehyde, bis(imido esters), toluene diisocyanate, and carbodiimides). However, we found that dimerization of

protein was still not totally avoidable since maleimide groups also react with amino and sulfhydryl groups of another protein molecule. Therefore, a method to separate protein dimers (and aggregates) from the conjugates was needed. Affinity chromatography is clearly the method of choice. Ideally, if antibodies specific for D-GL were available, we could use an anti-D-GL immunoadsorbent as the affinity column. However, since D-GL is not immunogenic, this is not possible. In order to circumvent this problem, we have devised a method which applies the well-known affinity between a protein, avidin, and the small vitamin molecule, biotin. The use of biotin for this purpose, furthermore, would be consistent with sound and ethical medical therapeutic principles since its administration should be completely safe. Thus, we have introduced biotin into the D-GL molecule to create a probe which allows us to separate the biotin-labeled protein–D-GL conjugate from nonconjugated protein by affinity chromatography on an avidin–Sepharose column.

The avidin–biotin complex is one of the tightest biological complexes known (for review, see Green, 1975). Due to its high affinity and specificity, this system has been applied in many biological studies (see references in Liu & Leonard, 1979). However, the application of avidin–Sepharose in the affinity chromatographic isolation of biotin-containing molecules has been limited, due to the problem in recovery since the affinity of avidin to biotin is so high. To circumvent this problem we have modified the avidin–Sepharose in such a way that the biotin-labeled compounds can be recovered with high yields under very mild conditions and the affinity column can be easily recycled. A similar modification of avidin–Sepharose for the purification of biotin-containing enzymes has recently been reported (Maloy, 1977).

Using these methods, we have obtained an OVA–D-GL–(biotin) conjugate which is absolutely free of nonconjugated OVA. The ratio of OVA to D-GL in our preparations was 0.7:1. These preparations consisted of the conjugate and the free D-GL–(biotin). It is to be noted that, since there is more than one sulfhydryl group and maleimide group on D-GL and OVA, respectively, molecules containing two OVA on one D-GL or two D-GL on one OVA are probably present. For our purposes, the free D-GL–(biotin) does not have to be removed from the conjugate preparations since it has been well-established in our laboratory that D-GL is neither immunogenic nor toxic. Moreover, the introduction of small numbers of the vitamin molecule, biotin, should not induce any change in the nonimmunogenic and nontoxic properties of D-GL.

The methods we have developed can be applied to the preparation of many protein–D-GL conjugates. In general, protein is modified with *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester to give MB protein which is reacted with SH-D-GL–biotin generated in situ from acetyl-*S*-D-GL by hydroxylamine. The reaction mixture is then subjected to avidin–Sepharose affinity chromatography to separate the conjugate and D-GL from protein dimers, oligomers, or aggregates. The conjugate fractions can be further purified by immunoadsorbent affinity chromatography on a column prepared with anti-protein antibodies. This removes the nonconjugated D-GL and any conjugate containing protein with disrupted antigenic determinants from the stable conjugate. Therefore, the application of the avidin–biotin system has provided us with a convenient and rapid method for purification of our conjugates. More importantly, *it has provided us with a way to obtain conjugates which are absolutely free of detectable nonconjugated protein.*

The conjugation and purification procedures have also been applied to insulin–D-GL. The efficiency of our conjugation reaction is quite dependent on the protein employed. Insulin–D-GL was produced in high yield even though MB-insulin containing only an average of one maleimide group was used.

In this study, we have been most concerned about retention of antigenicity of proteins conjugated to D-GL. It should be also of general interest to see the effect of modification of proteins by a polymer containing large numbers of amino and carboxyl groups capable of interacting with proteins by electrostatic and hydrogen bonds on the capacity of the protein to combine with its antibodies. We have demonstrated that conjugation of OVA with D-GL does not affect its ability to form precipitating complexes with antibodies (Figure 5). The shape of the quantitative precipitin reaction curve of OVA-D-GL is similar to that of OVA. The equivalence point is shifted to a higher antigen concentration for OVA–D-GL; this can be accounted for by the presence of some conjugates containing OVA with altered antigenic determinants which are nonprecipitable by antiserum and nonadsorbable by anti-OVA immunoadsorbent.

The effect of D-GL on the affinity of the D-GL-conjugated protein for its antibodies and the effect of purification by immunoadsorbent affinity chromatography are best demonstrated in the case of insulin–D-GL (Figure 6). Insulin–D-GL prepared under our conjugation conditions retained less than 30% of the antigenicity of insulin. This is not unexpected since antigenicity of insulin is very sensitive to chemical modifications. For example, modification of phenylalanine B1 amino group of insulin by acetylation or acetoacetylation causes a marked decrease of its affinity for anti-insulin antibodies (Lindsay & Shall, 1971). After purification by anti-insulin immunoadsorbent, insulin–D-GL exhibited substantial retention of the antigenic structure of insulin. These conjugates must have D-GL conjugated at some specific site(s) It is interesting that antigenicity of insulin is not significantly disturbed by conjugation with a large molecule, such as D-GL, if it is appropriately conjugated. Since our purification procedures guarantee the absence of any nonconjugated insulin, it will be of interest to test the hormonal activity of the conjugate.

The results of our studies on the tolerogenic activity of OVA–D-GL in mice have indicated that the conjugate is, indeed, effective in suppressing reaginic (IgE) antibody responses. Detailed description of these experimental procedures and results will be presented elsewhere (Liu & Katz, 1979). The capacity of such protein–D-GL conjugates to suppress specific antibody responses would be of obvious significance to their clinical applicability for therapy in various allergic and autoimmune diseases.

Finally, conjugation, purification and quantitation procedures such as those described in this report and the concept of application of the avidin–biotin system should be widely applicable to preparation of conjugates of proteins, protein–polypeptides, and protein–polysaccharides for a variety of experimental purposes in which such substances may be of value.

Acknowledgments

We are very grateful to Dr. Hiroshi Yamamoto for helpful advice generously provided in certain aspects of this work. We also thank Anthea Hugus for excellent secretarial assistance in the preparation of this manuscript.

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Improvements in the Prediction of Protein Backbone Topography by Reduction of Statistical Errors†

Frederick R. Maxfield and Harold A. Scheraga*

ABSTRACT: We have simplified our previously published method for predicting the occurrence of residues in one of five conformational states [Maxfield, F. R., & Scheraga, H. A. (1976) *Biochemistry 15*, 5138] without sacrificing accuracy. An increase in the size of the data set from 3681 to 5082 residues led to a slight (1–2%) increase in the accuracy. In order to overcome the limitations in the accuracy due to statistical errors, we tested the usefulness of averaging the predictions for several homologous proteins at each position in the aligned sequence. When this procedure was used on 15 cytochrome *c* sequences and 24 globins, the accuracy of the predictions increased by 8 and 6%, respectively. Averaging did not improve the accuracy when only a few homologous sequences were available or when there was only a slight variability in the amino acid sequence. The improved accuracy from the use of homologous proteins and the slight improvement from an increase in the size of the data set are consistent with the hypothesis that statistical errors place a significant limitation on the accuracy of predictions which incorporate pairwise interactions between neighboring residues. Since a large increase in the size of the data set will be required to reduce the statistical errors significantly, the use of homologous sequences appears to be the most promising way to improve the predictions.

In a previous paper (Maxfield & Scheraga, 1976), which will be referred to as paper 1, we described and evaluated a new method for the prediction of the backbone conformational states of proteins. That method was used to assign each residue in a protein to one of five conformational states, based on intraresidue interactions and on the interactions with the four nearest neighbors on either side. The data used to estimate the effects of these intraresidue and medium-range interactions[1] were obtained from an analysis of 3681 residues in 20 proteins. By use of these data, residues were predicted to occur in one of the five conformational states with an accuracy of 56%. A two-state prediction (α helical or non-

† From the Department of Chemistry, Cornell University, Ithaca, New York 14853. *Received June 28, 1978; revised manuscript received October 2, 1978.* This work was supported by research grants from the National Science Foundation (PCM75-08691) and from the National Institute of General Medical Sciences of the National Institutes of Health, U.S. Public Health Service (GM-14312).

[1] In this paper, medium-range interactions refer to the effects of neighboring residues, out to the fourth nearest neighbors, on the backbone conformation of a residue. Némethy & Scheraga (1977) have suggested another definition for medium-range interactions which is not adopted here in order to maintain consistency with our earlier paper (Maxfield & Scheraga, 1976).

0006-2960/79/0418-0697$01.00/0