Efficient Coupling of Glycopeptides to Proteins with a Heterobifunctional Reagent†

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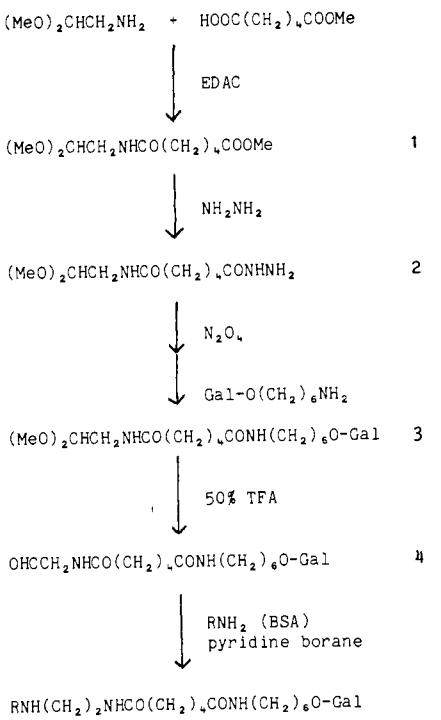
ABSTRACT: A heterobifunctional linking reagent containing a masked aldehydo group and acyl hydrazide was synthesized for coupling of glycopeptides and other amino-containing compounds to proteins. After conversion to acyl azide, the reagent reacts with the amino group of a glycopeptide, and the modified glycopeptide is deacetalized with a weak acid to unmask the aldehydo group, which is then conjugated to bovine serum albumin (BSA) by reductive alkylation with pyridine–borane. The overall reaction scheme proceeds under relatively mild conditions. When the protein amino group was in a large excess (>6-fold) of the aldehyde reagent, the efficiency of conjugation was as high as 88% even at submicromole levels. As a test case for application of this reagent, 6-aminohexyl β-D-galactopyranoside (Gal-AH) was attached to the linking reagent and conjugated to BSA at various aldehyde-to-protein molar ratios ranging from 25 to 200. The level of *O*-galactosyl residue incorporated into BSA by this reagent far exceeded that observed in a similar reductive alkylation involving *S*-galactoside reagents [Lee, R. T., & Lee, Y. C. (1980) *Biochemistry 19*, 156–163]. By use of the present conjugating procedure, as many as 112 mol of Gal-AH residues were incorporated per mole of BSA, which represents near total modification of the amino groups. Some binding characteristics of the new BSA derivatives were studied in the mammalian hepatic galactose/*N*-acetylgalactosamine specific lectin system along with other types of BSA derivatives (containing *S*-galactosyl residues). In general, the behavior of the new derivatives was similar to that of other types. For instance, the affinity increased exponentially at low sugar substitution levels (up to 30 mol of galactosyl residues/mol of BSA), and the slope of exponential increase and affinity at a given sugar substitution level was similar to those of other types.

Studies of carbohydrate binding in biological systems require ligands of well-defined structure. In a number of cases, proteins modified with simple sugar derivatives (Stowell & Lee, 1980) serve as useful ligands and were instrumental in deciphering sugar binding specificity of many animal lectins (Stowell et al., 1980; Shephard et al., 1981; Lee, 1982; Kuhlenshmidt & Lee, 1984). In some cases, however, neoglycoproteins bearing more complicated structure than simple sugars are required. Since chemical synthesis of more complex carbohydrates analogous to natural glycoconjugates is still a formidable task, readily available, naturally derived carbohydrate chains or glycopeptides become an attractive alternative source. Recent development in chromatography of glycopeptides (Hardy & Townsend, 1988; Chen et al., 1988) makes this approach even more attractive. Glycopeptides have been coupled to proteins by means of a bifunctional reagent (Rogers & Kornfeld, 1971) or by first oxidizing an Asn glycopeptide to an aldehydo derivative followed by its coupling to protein via reductive alkylation (Mencke & Wold, 1982). Recently, Wold and co-workers reported some ingenious ways of preparing neoglycoproteins by utilizing enzymic action of a transglutaminase (Yan & Wold, 1984) and by utilizing high-affinity, noncovalent complex formation, such as between a glycopeptide-modified biotin and avidin (Chen & Wold, 1984, 1986). The advantage of these methods is not in high coupling efficiency or universal applicability but in the fact that one has some control over the number and location of the glycopeptide on the protein matrices.

We sought to establish a more widely applicable method that accomplishes efficient coupling of glycopeptide under mild conditions. The existing chemical methods do not fully satisfy such conditions. To this end, we synthesized a heterobifunctional linking compound (see Scheme I) that can be stored indefinitely at room temperature. The linking compound is first attached to a glycopeptide via acyl azide coupling, the acetal is converted to an aldehydo group, and then the aldehydo group is coupled to proteins by reductive alkylation. All three steps can be carried out under mild conditions, and actual conjugation of a triantennary glycopeptide to BSA[1]

Scheme I



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showed that the coupling is very efficient (~85%) even with a submicromole quantity of the glycopeptide.

The earlier binding specificity studies of Gal-bearing BSA derivatives by the Gal/GalNAc-specific mammalian hepatic lectins rarely included the solubilized rat lectin or isolated rat hepatocytes. Therefore, we compared the binidng of various types of neoglycoproteins by the solubilized rabbit and rat lectins as well as by the isolated rat hepatocytes. Some differences in the binding characteristics of rabbit vs rat lectin, soluble lectin vs lectin on the hepatocyte surface, and *O*- vs *S*-galactoside-containing BSA derivatives are presented.

Materials and Methods

Aminoacetaldehyde dimethyl (or diethyl) acetal, methyl monoester of adipic acid, anhydrous hydrazine, and pyridine–borane were from Aldrich Chemical Co. Trinitrobenzenesulfonic acid (TNBS) and 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDAC), trifluoroacetic acid (TFA), and 2-thiobarbituric acid were from Sigma Chemical Co. BSA was from Armour Pharmaceutical Co. Carrier-free $Na^{125}I$ was obtained from Amersham (Arlington Heights, IL). Bicinchoninic acid assay kit for protein was obtained from Pierce and Accel QMA anion-exchange resin from Waters Associates. Desialylated glycopeptide preparation that contained mainly triantennary glycopeptides was prepared by Pronase digestion of bovine fetuin followed by Sephadex G-50 fractionation (Townsend et al., 1986). The following compounds were prepared according to methods described in the literature: 6-aminohexyl β-D-galactopyranoside (Weigel et al., 1979); (β-D-galactopyranosylthioglycolyl)aminoacetaldehyde [(Gal-AL) Lee & Lee, 1979]; Gal-AI-BSA (Lee et al., 1976); Gal-AD- and Gal-HD-BSA (Lee & Lee, 1980). Rabbit and rat liver lectins specific for Gal/GalNAc were isolated and purified as described (Hudgin et al., 1974), except that asialofetuin–Sepharose was used as affinant (Connolly et al., 1981). Rat hepatocytes were isolated by a collagenase perfusion method (Seglen, 1976). Orosomucoid (a gift of Dr. M. Wickenhauser, the American Red Cross, Bethesda, MD) was desialylated (Connolly et al., 1981) and radioiodinated by the Chloramine T method (Greenwood et al., 1963).

Silica gel 60 F_{254} (Merck) precoated on aluminum plate was used for TLC. The solvent systems used are (A) 4:1 (v/v) ethyl acetate:acetone, (B) 3:1 (v/v) toluene:methanol, and (C) 3:2:1 (v/v/v) ethyl acetate:2-propanol:water. Carbohydrates are visualized by charring (at ~140 °C) the plate after it has been sprayed with 15% sulfuric acid in 50% ethanol; the amino group is visualized by heating plates after they have been sprayed with 0.2% ninhydrin in 95% ethanol, the hydrazide group by spraying with 0.2% TNBS solution, and the aldehyde group by spraying with 0.4% 2,4-dinitrophenylhydrazine in 2 M HCl. The acetal group was detected similarly, except slight heat was applied after spraying.

Protein concentration was determined by the bicinchoninic acid method (Smith et al., 1985) with BSA as standard, carbohydrate concentration by the phenol–sulfuric acid method (McKelvy & Lee, 1969), and the aldehyde (or acetal) concentration by the neocuproine method (Dygert et al., 1965). *N*-Acetylneuraminic acid was assayed by using thiobarbituric acid method (Uchida et al., 1977). Melting points were determined with a Fisher-Johns apparatus, and elemental analyses were done by Galbraith Laboratories, Inc. The NMR spectra were obtained with a Varian XL-400 spectrophotometer. Microanalysis of monosaccharide mixture was done as described (Hardy et al., 1988) by using a Dionex BioLC equipped with a pulsed amperometric detector (PAD2).

Affinity of various neoglycoproteins and simple galactosides to the isolated lectins and to the lectin on the hepatocyte surface was estimated with an inhibition assay using ^{125}I-ASOR as the reference ligand (Connolly et al., 1982). Briefly, a test compound at various concentrations was incubated with fixed concentrations of ^{125}I-ASOR and lectin. The bound ASOR was separated and counted. For the binding by soluble lectins, the incubation was at 25 °C for $^1/_2$ h, and the bound ligand was separated by ammonium sulfate precipitation/filtration. For the binding by isolated hepatocytes, the incubation was at 2 °C for 2 h, and the bound ligand was separated by microfuge centrifugation. The percent inhibition was plotted against the logarithm of inhibitor concentration to obtain the concentration at which 50% inhibition occurred (I_{50}).

Results and Discussion

The reaction scheme for the preparation of a heterobifunctional reagent having a hydrazide and an acetal group at each end is shown in Scheme I. The scheme works equally well for dimethyl acetal (shown in the scheme) or diethyl acetal of aminoacetaldehyde as the starting material. The compounds **1′**, **2′**, etc. designate derivatives containing diethyl acetal that correspond to the compounds **1**, **2**, etc., respectively.

Preparation of N-(2,2-Dimethoxyethyl)-5-(methoxycarbonyl)pentanamide (**1**). To a mixture of aminoacetaldehyde dimethyl acetal (2.18 mL, 20 mmol) and adipic acid methyl monoester (2.96 mL, 20 mmol) in 75 mL of dichloromethane was added EDAC (4.98 g, 26 mmol), and the mixture was stirred until a clear solution resulted. After 24 h at room temperature, the solution was extracted successively with 50 mL each of cold solutions of 0.75 M sulfuric acid, 1 M NaCl, saturated sodium bicarbonate, and 1 M NaCl. The organic solution was dried with sodium sulfate and filtered, and the filtrate was evaporated to a syrup, which showed a single spot by charring upon TLC in solvent A (R_f 0.71) and solvent B (R_f 0.19). The apparent yield of **1** was 70%: ^1H NMR in $CDCl_3$ δ 5.757 (bs, 1 H, N*H*), 4.337 [t, 1 H, C*H*-$(OMe)_2$], 3.668 (s, 3 H, $COOCH_3$), 3.416–3.339 (s and t, 8 H, CH_3OC and $CHCH_2NH$), 2.358–2.195 (m, 4 H, CH_2CO), 1.685–1.642 [m, 4 H, $CH_2(CH_2)_2CH_2$].

Formation of Acyl Hydrazide **2** *from* **1**. To a solution of **1** (1.46 g, 5.9 mmol) in 4.5 mL of dry methanol was added 0.28 mL of anhydrous hydrazine (5.9 mmol). The mixture was left at room temperature overnight and evaporated to a solid residue, which was dissolved in 5 mL of water and extracted three times with an equal volume of dichloromethane. The aqeuous solution was evaporated to dryness and then further dried overnight in vacuo. The crystalline solid (1.04 g, 71% yield; mp 94–95 °C) was homogeneous on TLC in solvent A (R_f 0.10) when visualized by charring and by the TNBS spray. The ^1H NMR spectrum indicated the loss of the ester methoxy group in **1**.

Anal. Calcd for $C_{10}H_{21}N_3O_4$ (247.79): C, 48.57; H, 8.56; N, 16.99. Found: C, 48.38; H, 8.59; N, 17.13.

Coupling of **2′** *with 6-Aminohexyl β-D-Galactopyranoside* (*Gal-AH*). The acyl hydrazide was converted to acyl azide by means of dinitrogen tetraoxide (Pinto & Bundle, 1983). To a chilled (−40 °C, acetone–dry ice) solution of the hydrazide **2′** (275 mg, 1 mmol) in 10 mL of dimethylformamide

[1] Abbreviations: BSA, bovine serum albumin; EDAC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; DMF, dimethylformamide; TNBS, trinitrobenzenesulfonic acid; TFA, trifluoroacetic acid; AsFetGP, desialylated triantennary glycopeptide preparation from fetuin; Gal-AH, 6-aminohexyl β-D-galactopyranoside; Gal-AL, (β-D-galactopyranosylthioglycolyl)aminoacetaldehyde. For Gal-AI-, Gal-AD-, and Gal-HD-BSA, see Chart I.

Table I: Coupling of 3′ and AsFetGP-ac to BSA

acetal compounds	ratio[a] (mol/mol of BSA)	BSA (mg)	pyridine–borane		total volume (mL)	incorporation	
			mol/mol of acetal	mM[b]		mol of Gal/mol of BSA	efficiency[c] (%)
AsFetGP-ac	2	13	7.5	5	0.61	1.76	88
	5	13	3	5	0.67	4.20	84
	10	1o	3	10	0.72	8.34	83
3′	25	10	2	12	0.62	19.8	79
	50	10	2	24	0.61	25	50
	100	10	1.66	30	0.82	59 51[d]	60
	200	10	1.24	36	1.03	112 103[d]	56

[a] Molar ratio of aldehyde (as acetal) to BSA. [b] Concentration of pyridine–borane in the reaction mixture. [c] Based on the acetal compound used. [d] These samples were dialyzed against 5 M guanidine hydrochloride and reanalyzed (see text).

(DMF) was added 4 mL of 0.4 M N_2O_4 in dichloromethane. After 15 min at −20 to −10 °C, a solution of Gal-AH (307 mg, 1.1 mmol) in 4 mL of dry methanol–DMF (1:1 mixture) and triethylamine (153 μL, 1.1 mmol) were added, and the mixture was kept overnight in the cold. The mixture was evaporated and dissolved in 1 mL of water. TLC in solvent C showed that this solution contained in addition to Gal-AH (R_f 0.04, ninhydrin positive) a single spot (R_f 0.57), **3′**, that charred strongly. The mixture was fractionated on a column (2.5 × 136 cm) of Sephadex G-15 using 1 mM acetic acid as eluant. The product **3′** was eluted ahead of Gal-AH with complete separation. The fractions containing **3′** were combined and evaporated to yield 0.27 g (0.5 mmol), which was crystallized from absolute ethanol–diethyl ether: mp 115–117 °C. An ethanolic solution of **3′** was analyzed for galactose content with the phenol–sulfuric acid method using galactose and Gal-AH as standards and for the aldehyde content with the neocuproine assay after the acetal group was hydrolyzed with 50% TFA (see below) using Gal-AL (Lee & Lee, 1980) as standard. The galactose content was 100% of the expected value, and the potential aldehydo group was 94.5% of the expected value.

Anal. Calcd for $C_{24}H_{46}N_2O_{10}$ (522.63): C, 55.15; H, 8.87; N, 5.35. Found: C, 55.13; H, 8.97; N, 5.36.

Coupling of **2** *with Desialylated Fetuin Glycopeptide.* The coupling was done under similar conditions used for the preparation of **3′**. After **2** (16 mg, 65 μmol in 0.5 mL of DMF) was converted to azide by addition of 0.46 M N_2O_4 (0.18 mL, 83 μmol) in dichloromethane at −40 °C, the mixture was added to a solution of desialylated fetuin glycopeptide [(AsFetGP) 35 mg, 11 μmol] in 0.5 mL of 0.2 M sodium borate buffer, pH 8.5. Triethylamine (8 μL, 57 μmol) was added, and the mixture was kept in the cold overnight. The mixture was extracted twice with equal volumes of chloroform, and the aqueous solution was fractionated on a column (1.5 × 70 cm) of Sephadex G-25 in 10 mM pyridine acetate buffer, pH 4.5. The void-volume fractions containing carbohydrate (phenol–sulfuric acid assay) were combined and lyophilized to yield 33 mg (88% yield) of acetal-containing AsFetGP (AsFetGP-ac). ^{1}H NMR spectrum of this material showed a successful modification of AsFetGP with **2**, and hydrolysis with 50% TFA (see below) showed the expected content of the acetal group.

Acid Hydrolysis of the Acetal Group. To establish a suitable condition for hydrolysis of the acetal group, an accurately weighed amount of **2′** or **3′** was dissolved in 50% TFA (to make ~10 mM solution), and aliquots taken at various times were evaporated after addition of a 20-fold volume of absolute ethanol. The residue was dissolved in 50% ethanol, and aliquots thereof were analyzed for aldehyde content. Deacetalization was 80% complete in 1 h and 100% in 2 h. There was no further change in the aldehyde content upon overnight standing at room temperature. The TLC (solvent C) examination of the 2-h hydrolysate of **3′** showed that there was no release of monosaccharides. In addition, methyl glycosides of mannose, galactose, and *N*-acetylglucosamine were treated under the same conditions, and the samples were analyzed for the free monosaccharide by using Dionex BioLC (Hardy et al., 1988). The results indicated that no more than 0.5% of the glycoside was hydrolyzed under the conditions employed. A similar treatment of the sialylated FetGP released 7% of *N*-acetylneuraminic acid in 2 h.

AsFetGP-ac (10 mg) was similarly hydrolyzed in 50% TFA (5 mL). After 2 h at room temperature, the mixture was either evaporated with 20 volumes of absolute ethanol or freeze-dried. ^{1}H NMR analysis showed a loss of the acetal methyl signal, indicating that deacetalization was complete.

Preparation of Neoglycoproteins. AsFetGP-ac and **3′** were first deacetalized and then coupled to BSA by reductive alkylation. Since, in a large number of cases, pure glycopeptide of known structure is more precious than the proteins being coupled to, excess of protein amino group over aldehyde was used for the coupling of AsFetGP-ac. Conversely, excess amounts of **3′** over BSA were used to study the efficiency and the highest extent of coupling at higher concentrations of the acetal reagent. Deacetalization was carried out as described above, and after the removal of TFA, the aldehydo derivative was used immediately without isolation. Reductive alkylation was carried out as before (Lee & Lee, 1980) in 0.2 M sodium phosphate buffer, pH 7, at room temperature for 24–40 h. However, instead of $NaCNBH_3$, pyridine–borane was used as reducing agent, since the latter was reported to be a less toxic and better reducing agent for reductive alkylation (Cabacungan et al., 1982). Details of the coupling conditions are shown in Table I.

The time requirement for the coupling of AsFetGP-ac to BSA was studied by fractionating an aliquot of the reaction mixture at various times on a column (0.6 × 7 cm) of Accel QMA. Unreacted AsFetGP derivative was first eluted with 10 mM sodium phosphate buffer, pH 7, and then BSA derivatives were eluted with the same buffer containing 0.5 M sodium sulfate. The eluate was continuously monitored by the orcinol–sulfuric acid reagent (Lee, 1972) to determine the carbohydrate content in each peak. Coupling was essentially complete in 24 h at room temperature.

In the preparative experiments, the carbohydrate-derivatized BSA was dialyzed exhaustively against distilled water in the cold, and the dialyzed material was analyzed for protein and for the monosaccharide content. AsFetGP content was expressed on the basis of 3 mol of Gal/mol of AsFetGP. As

shown in Table I, coupling efficiency of AsFetGP to BSA was very high (83–88%) when the amino group (BSA) was in a large excess over the acetal reagent. Interestingly, at high acetal-to-BSA ratios, the reductive alkylation using **4** (deacetalized **3'**) incorporated much higher levels of sugar into BSA than previously attained with Gal-AL (Lee & Lee, 1980). For example, at the acetal-to-BSA ratio of 100, reductive alkylation using Gal-AL and **4** incorporated 20 and 59 mol of Gal/mol of BSA, respectively; at the ratio of 200, the incorporation was 28 and 112 mol/mol.

We observed in the past that about 30–35 residues of amino groups (of 60 total) of BSA are readily modified by amidination with an imidate-containing thioglycoside (Lee et al., 1976) or by the reductive alkylation with Gal-AL (Lee & Lee, 1980). The remaining amino groups of BSA are apparently less accessible to the reagents, since the modification rarely goes beyond 40 amino groups even at very high reagent-to-BSA ratios. In the case of the reductive alkylation, a second molecule of Gal-AL can react with a previously modified lysine residue to form a tertiary amino derivative, so that as high as 53 mol of sugar has been incorporated per mole of BSA. Incorporation of 112 mol of Gal/mol of BSA attained by **4** would represent a near total modification of all the amino groups in BSA. Since **4** is considerably more hydrophobic than Gal-AL, in order to eliminate any possibility of hydrophobic, noncovalent trapping of the reagent in BSA, the following experiment was carried out. Solutions of the highly coupled BSA derivatives (the last two samples in Table I) were made 5 M in guanidine hydrochloride and dialyzed overnight against 5 M guanidine hydrochloride solution (150 times the sample volume). They were then serially dialyzed against 0.5 M, 50 mM, and finally 1 mM guanidine hydrochloride. The dialyzed samples were reanalyzed for protein and galactose. Results are also shown in Table I. The guanidine treatment caused decreases in the Gal content of 8 and 13%. Therefore, the majority of Gal residues must have been incorporated via a covalent bond.

This vastly higher sugar incorporation attained with **4** may be explained by the following three possibilities. (i) A long (16-atom) nonhydrophilic arm of **4** allows it to react with those lysine residues that may be located in the interior of BSA and are not easily accessible to a reagent such as Gal-AL. (ii) Pyridine–borane used in these experiments is much superior to $NaCNBH_3$ in producing reductively alkylated product. (iii) The less bulky and more flexible aglycon of the *O*-galactoside (compared to the aglycon of the *S*-galactoside) allowed coupling to proceed much further. Possibility ii was discarded, because a comparative study of reductively alkylating BSA with Gal-AL using either pyridine–borane or sodium cyanoborohydride as reducing agent showed that there was no significant difference in Gal incorporation (data not shown). On the other hand, the *O*-galactosyl analogue of Gal-AL (R. T. Lee et al., unpublished results) incorporated slightly more sugar residues into BSA by reductive alkylation than Gal-AL, a thioglycoside. However, the incorporation level was still very much lower than that obtained with **4**. We speculate, therefore, that the very high level of Gal incorporation attained with **4** is due mainly to the long, nonhydrophilic arm.

Binding Studies with Various Neoglycoproteins. Inhibition assays were carried out with the Triton-solubilized, purified rat and rabbit lectins and the isolated rat hepatocytes to compare the binding affinity of the new type of BSA derivatives with the old types. The new type of BSA derivative obtained by coupling **4** (designated Gal-AH-EA-BSA) contains *O*-galactoside, while all the old types (Gal-AI-, -AD-, and -HD-BSA; see Chart I) contain *S*-galactoside. Results of the inhibition assay using the soluble lectins are shown graphically in Figure 1. In this figure, the I_{50} (*y* axis) was logarithmically plotted against the Gal content of the BSA derivatives. A curve is obtained for each set of ligand type (e.g., Gal-AI-BSA) and lectin, but only a few are shown in each figure for clarity. We have reported in the past similar inhibition studies using the crude plasma membrane and the purified soluble lectin of the rabbit liver (Lee & Lee, 1982; Lee, 1982). The present studies show that the overall binding behavior of the rat lectin is similar to that of the rabbit. However, the rat lectin bound all the Gal-containing BSA derivatives 2–5-fold tighter than the rabbit lectin.

The affinity of all Gal-BSA's increased exponentially at lower Gal density up to about 30 mol/mol of BSA for both lectins. Beyond this level, there is a distinct difference between Gal-AH-EA-BSA and all the thiogalactoside-containing BSA

Chart I

Gal-AI-BSA	β-Gal-$SCH_2\overset{NH}{C}$-NH-BSA
Gal-AD-BSA	β-Gal-$SCH_2CONH(CH_2)_2NH$-BSA
Gal-HD-BSA	β-Gal-$S(CH_2)_5CONH(CH_2)_2NH$-BSA
Gal-AH-EA-BSA	β-Gal-$O(CH_2)_6NHCO(CH_2)_4CONH(CH_2)_2NH$-BSA

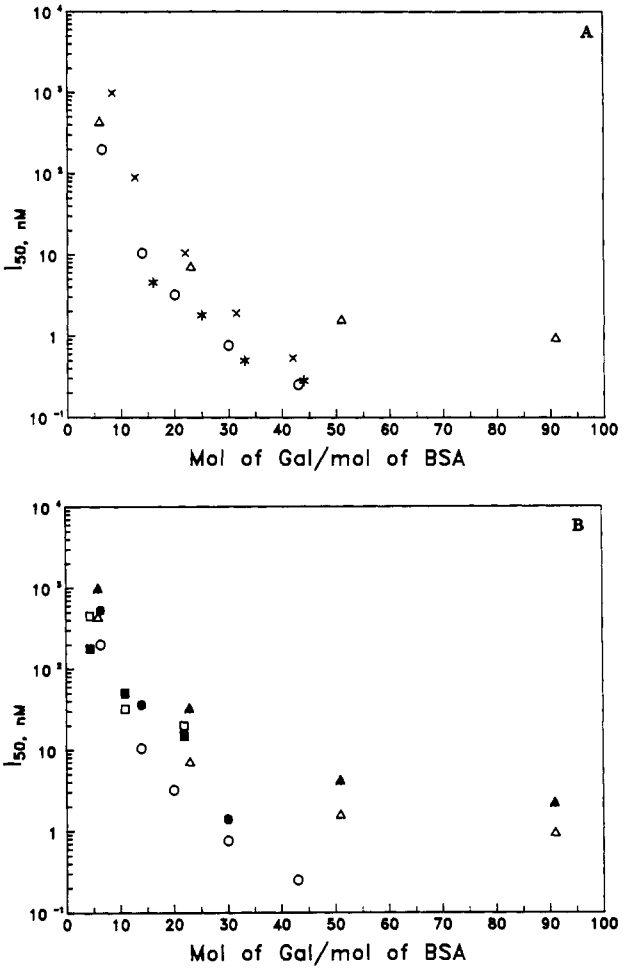

FIGURE 1: (A) Inhibitory potency (I_{50}) of various Gal-containing BSA derivatives toward the soluble rat hepatic lectin. (*) Gal-AI-BSA; (×) Gal-AD-BSA; (○) Gal-HD-BSA; (△) Gal-AH-EA-BSA. (B) Inhibitory potency (I_{50}) of some Gal-containing BSA derivatives. Comparison of the rabbit and rat soluble lectins. (○) Gal-HD-BSA, rat; (●) Gal-HD-BSA, rabbit; (△) Gal-AH-EA-BSA, rat; (▲) Gal-AH-EA-BSA, rabbit; (□) AsFetGP-EA-BSA, rat; (■) AsFetGP-EA-BSA, rabbit.

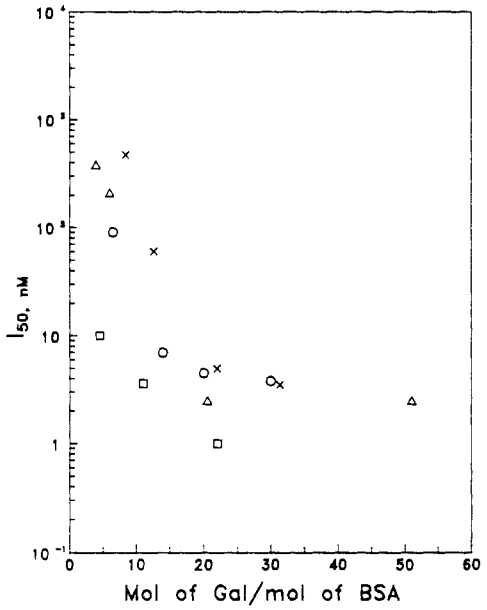

FIGURE 2: Inhibitory potency (I_{50}) of various Gal-containing BSA derivatives toward the lectin on the rat hepatocyte surface. (×) Gal-AD-BSA; (○) Gal-HD-BSA; (△) Gal-AH-EA-BSA; (□) AsFetGP-EA-BSA.

derivatives: the affinity of the former leveled off abruptly at about 30 mol/mol of BSA, while the affinity of the latter continued to increase. We prepared another *O*-galactoside-containing BSA that is the direct counterpart of Gal-AD-BSA. Though this new *O*-galactoside-containing BSA is structurally related to Gal-AD-BSA, its affinity curve did not resemble that of Gal-AD-BSA; instead the curve paralleled that of Gal-AH-EA-BSA. For this reason, we believe that the different shape of the affinity curve is directly related to the galactosidic linkage (*O*- or *S*-). Biochemical and other studies involving these *S*- and *O*-galactoside-containing BSA derivatives, as well as simple *S*- and *O*-galactosides, are currently under way and will be presented elsewhere.

The results of similar inhibition studies using the isolated rat hepatocytes are shown in Figure 2. The exponential increase in inhibitory potency of all the Gal-BSA's leveled off sharply around the Gal density of 15–20 mol/mol. A similar affinity–Gal density plot was observed with the isolated rabbit hepatocytes also (Connolly, 1981). The exponential increase in affinity at lower Gal density seen in both the soluble lectin and the isolated hepatocytes is presumably due to the ability of a polyvalent Gal-BSA to bind simultaneously to several Gal-combining sites and cross-link subunits of the lectin. Perhaps beyond the substitution level of 30–40 mol/mol in the soluble lectin, the additional Gal residues can no longer bind effectively to the combining sites because of physical constraint, resulting in leveling off of the affinity. The fact that this leveling off occurs much earlier (15–20 mol/mol) for the lectins on the isolated hepatocytes suggests that these lectins experience more severe physical constraint than the soluble lectins. One striking phenomenon observed in the hepatocyte system is that BSA derivatives bearing AsFetGP are much more potent inhibitors than any of the neoglycoproteins containing simple Gal derivatives at comparable Gal density (Figure 2). However, as seen in Figure 1B, the inhibitory potency of the same AsFetGP-EA-BSA derivatives was no better than Gal-containing BSA derivatives for the soluble lectins. It is known that a Gal-terminated triantennary structure found in desialylated α_1-protease inhibitor and fetuin possesses binding affinity nearly a millionfold stronger than monovalent galactosides toward this lectin on the hepatocyte surface (Lee et al., 1983). Once the lectin is solubilized in Triton, the same triantennary structure manifests only a thousandfold higher affinity than simple galactosides (Lee, R. T., et al., 1984). It has been suggested that this tremendously enhanced binding of the triantennary oligosaccharide results from the fact that the relative orientation of three Gal residues as they exist in the preferred conformation of this oligosaccharide is complementary to three adjacent Gal-combining sites of the lectin on the hepatocyte surface (Lee, Y. C., et al., 1984). Obviously, in the hepatocyte system, BSA conjugate of the triantennary structure also possessed much stronger binding affinity than the Gal-BSA of a comparable sugar density.

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Registry No. **1**, 117941-35-2; **2**, 117941-36-3; **2′**, 117941-38-5; **3**, 117958-79-9; **3′**, 117941-39-6; **4**, 117941-37-4; Gal-AH, 64894-75-3; $(MeO)_2CHCH_2NH_2$, 22483-09-6; $HO_2C(CH_2)_4COOMe$, 627-91-8; aminoacetaldehyde diethyl acetal, 645-36-3.

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Oxidoreduction Reactions Involving the Electrostatic and the Covalent Complex of Cytochrome *c* and Plastocyanin: Importance of the Protein Rearrangement for the Intracomplex Electron-Transfer Reaction†

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ABSTRACT: Horse heart cytochrome *c* and French bean plastocyanin are cross-linked one-to-one by a carbodiimide [Geren, L. M., Stonehuerner, J., Davis, D. J., & Millett, F. (1983) *Biochim. Biophys. Acta 724*, 62] in the same general orientation in which they associate electrostatically [King, G. C., Binstead, R. A., & Wright, P. E. (1985) *Biochim. Biophys. Acta 806*, 262]. The reduction potentials of the Fe and Cu atoms in the covalent diprotein complex are respectively 245 and 385 mV vs NHE; the EPR spectra of the two metals are not perturbed by cross-linking. Four isomers of the covalent diprotein complex, which probably differ slightly from one another in the manner of cross-linking, are separated efficiently by cation-exchange chromatography. Stopped-flow spectrophotometric experiments with the covalent diprotein complex show that the presence of plastocyanin somewhat inhibits oxidation of ferrocytochrome *c* by $[Fe(CN)_6]^{3-}$ and somewhat promotes oxidation of this protein by $[Fe(C_5H_5)_2]^+$. These changes in reactivity are explained in terms of electrostatic and steric effects. Pulse-radiolysis experiments with the electrostatic diprotein complex yield association constants of $\geq 5 \times 10^6$ and 1×10^5 M^{-1} at ionic strengths of 1 and 40 mM, respectively, and the rate constant of 1.05×10^3 s^{-1}, regardless of the ionic strength, for the intracomplex electron-transfer reaction. Analogous pulse-radiolysis experiments with each of the four isomers of the covalent diprotein complex, at ionic strengths of both 2 and 200 mM, show an absence of the intracomplex electron-transfer reaction. A rearrangement of the proteins for this reaction seems to be possible (or unnecessary) in the electrostatic complex but impossible in the covalent complex.

Pairwise associations between various redox proteins have been simulated by computer graphics and examined by chromatographic, spectroscopic, and other methods. These complexes owe their stability to electrostatic and hydrophobic interactions between the protein molecules involved (Tam & Williams, 1985). Most of the studies to date have dealt with complexes containing cytochrome *c* as one component and any of the following metalloproteins as the other: cytochrome *c* peroxidase (Poulos & Finzel, 1984; Waldmeyer & Bosshard, 1985), cytochrome b_5 (Salemme, 1976; Mauk et al., 1986), cytochrome *c* reductase (Bosshard et al., 1979), cytochrome *c* oxidase (Michel & Bosshard, 1984), flavodoxin (Hazzard et al., 1986), and plastocyanin (Augustin et al., 1983; Chapman et al., 1984; King et al., 1985). Although a true structure for none of the complexes is known, the first crystallographic success probably is not far in the future.

Diprotein complexes and hybrid hemoglobins are well suited to the study of biological electron-transfer reactions. The distance between the donor and the acceptor and their mutual orientation can be estimated reliably from the crystal structures and from the computed models. The driving force and the intervening medium can be varied purposefully by metal substitution and by site-specific mutagenesis. Although the

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