[Chem. Pharm. Bull. 33(7)2871—2876(1985)]

Water-Soluble Carrier Proteins Having Carboxyl Spacer Groups

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(Received October 31, 1984)

Three water-soluble bovine serum albumin derivatives with carboxyl spacer groups have been synthesized as carrier proteins for direct preparation of conjugates between proteins and low molecular haptens with amino groups. *N*-Glycylaminomethanesulfonic acid, a newly developed solubilizing reagent for proteins, was first introduced into bovine serum albumin through its carboxyl groups. After the methylation of carboxyl groups remaining unreacted in the modified albumin, *N*-succinylglycine and *N*-bromoacetyl-*β*-alanine, both carboxyl spacer reagents, were reacted with the amino or imidazole groups of the modified albumin, respectively, to give two water-soluble albumin derivatives. Another water-soluble albumin derivative was obtained by treating the modified albumin with *N*-bromoacetyl-*β*-alanine followed by amidination of the remaining amino groups of the albumin with methylacetimidate. Glycine ethyl ester, selected as a model hapten that has an amino group, was reacted with these albumin derivatives to form conjugates, and 4—15 ester moieties can be incorporated into one molecule of the albumin derivatives without loss of their water-solubility.

Keywords——*N*-glycylaminomethanesulfonic acid; *N*-succinylglycine; *N*-bromoacetyl-*β*-alanine; methylacetimidate; glycine ethyl ester; bovine serum albumin; water-soluble carrier protein; carboxyl spacer

Preparation of a protein–hapten conjugate is a key step in the immunoassay of a low molecular hapten.[1] In order to produce specific antibody, haptenic antigen cross-linked to a carrier protein through a spacer group of appropriate length should be used for immunization.[2,3] Usually, a spacer is first introduced into a hapten molecule, and then the modified hapten is connected with a carrier protein such as bovine serum albumin (BSA) through the spacer.[4-7] In this regard, we have recently developed two heterobifunctional reagents as reactive spacers.[8] However, a carrier protein having spacer groups, if available, should be preferable for the preparation of protein–hapten conjugates. Carrier proteins often become insoluble in water during the preparation of hapten–carrier protein conjugates. Such insoluble conjugates can be used for immunization, though this makes the characterization of the resulting antibody difficult.[2] We have prepared three water-soluble BSA derivatives with carboxyl spacer groups as new carrier proteins. *N*-Glycylaminomethanesulfonic acid (I, Chart 1), newly synthesized as a solubilizing reagent for proteins, was first introduced into BSA through its carboxyl groups, and the remaining carboxyl groups of the BSA were methylated. Newly developed *N*-succinylglycine (II, Chart 1) and *N*-bromoacetyl-*β*-alanine, both carboxyl spacer reagents, are incorporated into the product through the amino or imidazole groups of the BSA, respectively, to give two carrier proteins. *N*-Bromoacetyl-*β*-alanine was also incorporated directly into I-containing BSA and then the remaining amino groups of the product were amidinated to obtain a carrier protein. The carboxyl groups in these carrier proteins can be coupled with haptenic antigens with primary amino group(s) under mild

reaction conditions in the presence of 1-ethyl-3-[3-(dimethylamino)propyl]-carbodiimide (EDC). Such reactions of these proteins have been investigated by using glycine ethyl ester (GlyOEt) as a simple model compound with a primary amino group.

$NH_2CH_2CONHCH_2SO_3H$ (I) $\qquad$ $HO_2C(CH_2)_2CONHCH_2CO_2H$ (II)

Chart 1

Experimental

Materials and Apparatus——EDC and BSA (fraction V) were purchased from the Protein Research Foundation (Osaka, Japan) and Sigma (St. Louis, U.S.A.), respectively. Aminomethanesulfonic acid was obtained from Tokyo Kasei Kogyo (Tokyo, Japan). *N*-Bromoacetyl-*β*-alanine and methylacetimidate·HCl were synthesized according to the literature.[9,10] Other chemicals were of reagent grade. Infrared (IR) spectra were measured in KBr pellets with a Hitachi 215 IR spectrophotometer. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were taken with a JEOL PS-100 spectrometer at 100 MHz, using approximately 10% D_2O solutions of samples containing approximately 1% sodium 2,2-dimethyl-2-silapentane-5-sulfonate as an internal standard. Amino acid analyses were performed on a Hitachi 835 amino acid analyzer after hydrolysis of protein samples in 6 M HCl *in vacuo* at 110 °C for 24 h, using a column (15 × 0.4 cm i.d.) packed with a Hitachi 2619 custom ion-exchanger resin with sodium citrate buffer system as a mobile phase. The column temperature was 53 °C.

Synthesis of I——Aminomethanesulfonic acid (5 g, 44.96 mmol) was dissolved in 50 ml of 2.5 M NaOH. Bromoacetyl bromide (5.88 ml, 67.45 mmol) was added in small portions to the above solution in an ice bath with vigorous stirring. The mixture was stirred at approximately 23 °C for another 30 min. Then the pH was adjusted to 4.0 with 47% HBr and the mixture was concentrated *in vacuo*. The residue, *N*-bromoacetylaminomethanesulfonic acid, was dissolved in 20 ml of 28% NH_4OH. The solution was allowed to stand for 1 h in a water bath at approximately 15 °C with stirring, then concentrated and chromatographed on a Dowex 50 W × 2 column (25 × 3.5 cm i.d., H^+ form) with H_2O. The ninhydrin positive fraction was concentrated and recrystallized from H_2O–EtOH to give I as colorless needles (1.83 g, 16.3%). mp above 290 °C. ^{1}H-NMR δ: 3.70 (2H, s), 4.20 (2H, s). IR: 3450 (NH), 1700 (C=O), 1200 cm^{-1} (SO_3). *Anal.* Calcd for $C_3H_8N_2O_4$: C, 21.43; H, 4.79; N, 16.66. Found: C, 21.29; H, 4.71; N, 16.55.

Synthesis of II——Glycine (3.75 g, 49.46 mmol) and powdered succinic anhydride (5 g, 49.46 mmol) were mixed and the mixture was heated in an oil bath at 140 °C for 30 min. The resulting *N*-carboxymethylsuccinimide was dissolved in 40 ml of 2.55 M KOH and heated at 100 °C for 1 h to hydrolyze it. After cooling, the solution was neutralized with concentrated HCl, then concentrated and chromatographed on a Dowex 50 W × 2 column (25 × 3.5 cm i.d., H^+ form) with H_2O. The acidic fraction was concentrated and recrystallized from H_2O–tetrahydrofuran to give II as a colorless solid (1.44 g, 16.5%). mp 119.5—120.5 °C. ^{1}H-NMR δ: 3.70 (2H, s), 2.40 (4H, s). IR: 3350 (NH), 1690 cm^{-1} (C=O). Field desorption mass spectrum (FDMS) *m/z*: 176 ($M^+ + 1$). *Anal.* Calcd for $C_6H_9NO_5$: C, 41.15; H, 5.18; N, 8.00. Found: C, 41.34; H, 5.25; N, 7.62.

Reaction of BSA with I (Chart 2)——BSA (1 g, 15.2 *μ*mol) and I (764 mg, 4.55 mmol) were dissolved in 20 ml of H_2O, and the pH of the solution was adjusted to 4.7 with 1 M HCl. EDC (8.35 g, 43.56 mmol) was added to the solution and the mixture was stirred at approximately 23 °C for 2—3 h, the pH of the mixture being kept at 4.7 with 1 M HCl during the reaction. The resulting mixture was dialyzed exhaustively against H_2O and lyophilized to give I-containing BSA (III, 1.0 g, 88.5%). The amount of I introduced into BSA was estimated from the increase in the number of glycine residues in the amino acid composition of III as compared with that of intact BSA.

Reaction of III with GlyOEt——III (approximately 15 mg) and GlyOEt·HCl (6.05 mg) were dissolved in 2 ml of H_2O, and the pH of the solution was adjusted to 4.7 with 1 M HCl. EDC (830 mg) was added to the solution and the mixture was stirred at approximately 23 °C for 1 h, keeping the pH at 4.7 with 1 M HCl. The amount of GlyOEt introduced into III was estimated from the amino acid composition in the same way as described above.

Methylation of Carboxyl Groups in III (Chart 2)——Concentrated HCl (0.9 ml) was added to 1 g of III suspended in 100 ml of dry methanol, and the mixture was allowed to stand for 3 d at 23 °C in the dark with occasional shaking. The resulting precipitates were collected by centrifugation, then washed twice with 100 ml of dry methanol and twice with 100 ml of dry Et_2O to give methylated III as a colorless powder (IV, 849 mg).

Introduction of II into IV (Chart 2)——IV (86 mg) and II (60.2 mg, 0.34 mmol) were dissolved in 3 ml of H_2O and the pH of the solution was adjusted to 4.7 with 1 M HCl. EDC (286 mg) was added, and the mixture was stirred at 23 °C for 1 h, the pH being kept at 4.7 with 1 M HCl. The resulting solution was dialyzed against 10% (v/v) AcOH and lyophilized to give II-modified IV (V, 92 mg). The amount of II introduced into IV was estimated in the same way as described in the reaction of BSA with I.

Introduction of *N*-Bromoacetyl-*β*-alanine into IV (Chart 2)——IV (100 mg) and *N*-bromoacetyl-*β*-alanine (14.0 mg, 67 *μ*mol) were dissolved in 5 ml of H_2O. The pH of the solution was adjusted to 8.3 with 1 M NaOH, and

then the mixture was allowed to stand overnight at 23 °C with stirring, the pH being maintained at 8.3 with the NaOH. The resulting mixture was dialyzed against 10% (v/v) AcOH and lyophilized to give IV modified with *N*-bromoacetyl-*β*-alanine (VI, 103 mg). The amount of *N*-bromoacetyl-*β*-alanine introduced into IV was estimated from the number of *β*-alanine residues in the amino acid composition of VI.

Introduction of *N*-Bromoacetyl-*β*-alanine into III and Subsequent Amidination (Chart 2)——The introduction of *N*-bromoacetyl-*β*-alanine into III was performed in the same way as described for the introduction of *N*-bromoacetyl-*β*-alanine into IV, except that different amounts of III (71 mg) and *N*-bromoacetyl-*β*-alanine (22.7 mg) were used. The resulting mixture that contained compound VII was subjected directly to amidination as follows. The reaction mixture was cooled in an ice bath and the pH was adjusted to 8.5 with 1 M NaOH (solution A). Methylacetimidate·HCl (557 mg) was dissolved in 1.5 ml of cold H_2O (0 °C) and the pH of the solution was adjusted to 8.0 with 3 M NaOH (solution B). The mixture of solutions A and B was stirred at 4 °C for 1.5 h, the pH being maintained at 8.5—9.0 with 1 M HCl. The resulting mixture was dialyzed exhaustively against H_2O, then again against 10% (v/v) AcOH, and lyophilized to give amidinated VII (VIII, 82 mg). The estimation of the amount of *N*-bromoacetyl-*β*-alanine incorporated into VIII was performed in the same way as described for the introduction of *N*-bromoacetyl-*β*-alanine into IV. The extent of the amidination was determined according to the method of Ludwig and Hunter.[11]

Conjugation of V with GlyOEt (Chart 3)——EDC (100 mg) and GlyOEt·HCl (4.4 mg) were added to V (5 mg) dissolved in 1 ml of 5 M guanidine·HCl or 6 M urea, and the mixture was stirred at 23 °C for 1 h, the pH being maintained at 4.7 with 1 M HCl. The resulting mixture was chromatographed on a Sephadex G-25 column

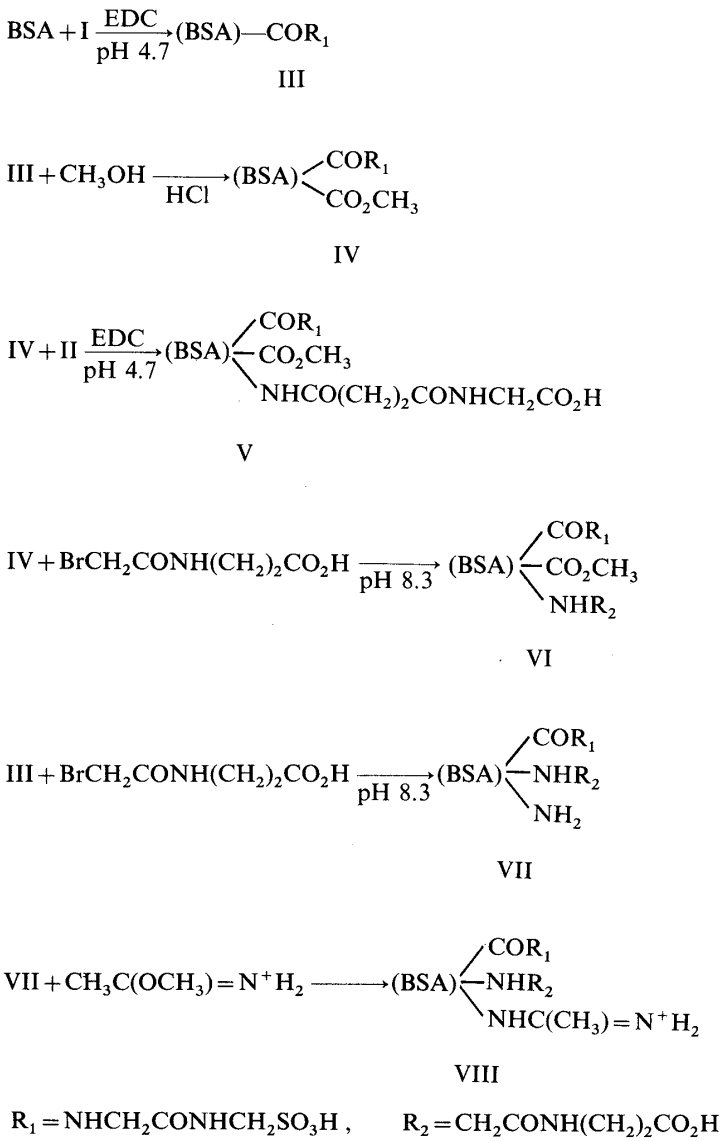

Chart 2

(70 × 1.5 cm i.d.) with 10% (v/v) AcOH and lyophilized to give V modified with GlyOEt. The amount of GlyOEt introduced into V was estimated in the same way as described in the reaction of BSA with I.

Conjugation of VI or VIII with GlyOEt——EDC (150 mg) and GlyOEt·HCl (about 50—500 times the molar quantity of VI or VIII) were added with stirring to a solution of VI or VIII (0.5%, w/v) in 1 ml of H_2O at 23 °C for 1 h. The subsequent procedure was the same as described for the conjugation of V with GlyOEt.

$$\text{V (or VI or VIII)} + \text{GlyOEt} \xrightarrow[\text{pH 4.7}]{\text{EDC}} \text{V (or VI or VIII)—COR}$$

$$\text{R} = \text{NHCH}_2\text{CO}_2\text{C}_2\text{H}_5$$

Chart 3

Results and Discussion

Carrier proteins usable as starting materials for the preparation of soluble hapten–protein conjugates should have spacer groups which can be coupled with haptenic antigens and should not lose their water-solubility when they are subjected to conjugation. EDC has been widely used to form amide bonds from carboxyl groups of proteins and low molecular compounds with primary amines under very mild conditions.[12-14] This reaction is very useful to prepare hapten–carrier protein conjugates provided that the haptenic molecule of interest has a primary amino group. In order to allow the use of this reaction, we planned to introduce carboxylic acids with bridges of suitable length into BSA as carboxyl spacer groups. In this case, the intact carboxyl groups in BSA should be protected without loss of solubility, otherwise haptenic molecules may react with the intact carboxyl groups as well as carboxyl groups in the spacer groups. Aminomethanesulfonic acid may be a suitable compound to protect the intact carboxyl groups in BSA without reducing the solubility because it can react with the intact carboxyl groups without changing the net charge of BSA and also the sulfonic acid moiety is not activated by EDC.[13] However, aminomethanesulfonic acid shows a very weak coloration in the ninhydrin reaction and so it is difficult to determine the number of aminomethanesulfonic acid moieties introduced into BSA by means of the conventional amino acid analysis. Therefore, we prepared *N*-glycylaminomethanesulfonic acid (I).

As a spacer reagent having carboxyl groups, we prepared *N*-succinylglycine (II). Compound II has two carboxyl groups and whichever reacts with an amino group of BSA, the remaining carboxyl group at the other end of the reagent is free to be utilized for the coupling of a haptenic molecule. Moreover, the number of II moieties incorporated into BSA can be easily determined from the increase of glycine in the amino acid composition. *N*-Bromoacetyl-*β*-alanine[9] was also used as a spacer reagent. The bromoacetyl group of the reagent can alkylate amino groups and histidine nitrogens in BSA[8] and the number of *N*-bromoacetyl-*β*-alanine incorporated can be determined from the amount of *β*-alanine measured by the amino acid analysis (the peak of *β*-alanine is detected between the peaks of tyrosine and phenylalanine in the chromatogram).

BSA contains 98 carboxyl groups per molecules (38 aspartic acids, 59 glutamic acids and an *α*-carboxyl,[15] and 57.1—59.6 of the carboxyl groups reacted with I under the reaction conditions described, to form III (Chart 2, Table I). In order to check the reactivity of the remaining carboxyl groups in III, III was further treated with GlyOEt in the presence of EDC. The increase of glycine was only 0.3 residue per molecule of the product, suggesting that the remaining intact carboxyl groups in III are essentially unreactive with GlyOEt. Methylation of III with methanol in the presence of hydrochloric acid according to the method of Mandell and Hershey[16] in order to protect completely the intact carboxyl groups gave derivative IV (Chart 2).

Table I. Numbers of I, II and/or *N*-Bromoacetyl-β-alanine and Methylacetimidate Moieties Introduced into V, VI and/or VIII[a]

BSA derivative	I	II	Spacer $BrCH_2CONH(CH_2)_2CO_2H$	Methylacetimidate
V	57.1	24.8		
VI	57.1		23.4	
VIII	59.6		36.1	3.7

a) Numbers per protein molecule.

Table II. Reactivity of V, VI and VIII with GlyOEt in the Presence of EDC

BSA derivative	Molar ratio of GlyOEt to protein	Reaction conditions[a] Solvent	EDC (mg)	GlyOEt incorporated (mol/mol)	GlyOEt/spacer (mol%)
V	500	5 M guanidine	100	4.9	19.8
V	500	6 M urea	100	4.1	16.5
VI	500	Water	150	12.7	54.3
VIII	50	Water	150	6.6	18.3
VIII	100	Water	150	11.3	31.3
VIII	300	Water	150	15.0	41.6

a) In every case, a solution of 5 mg of the protein in 1 ml of the indicated solvent was subjected to reaction at 23 °C and pH 4.7.

Since BSA has 59 lysine residues per molecule and ε-amino groups of the residues remain in derivatives III and IV, they can be utilized to introduce spacer groups possessing carboxyl groups at the end. Derivative V was prepared by treating IV with a large excess of II in the presence of EDC at pH 4.7, and 24.8 moieties of II were incorporated into one molecule of the protein (Table I). When the alkylation of IV and III with *N*-bromoacetyl-β-alanine was performed at pH 8.3, 23.4 (derivative VI) and 36.1 (derivative VIII) moieties of the reagent were introduced into each molecule of the proteins, respectively (Chart 2, Table I). Derivative VII was not isolated, and it was allowed to react further with methylacetimidate; only a small number of lysine residues (3.7 mol) was amidinated (derivative VIII, Chart 2, Table I). It is known that 90% of the lysine residues in intact BSA can be amidinated under similar conditions,[11] and thus the remaining lysine residues in VIII are considered to be unreactive. These derivatives are all soluble in water. The introduction of II or *N*-bromoacetyl-β-alanine into methylated BSA[16] resulted in precipitation of the protein. Therefore, it is concluded that the modification of the carboxyl groups in BSA with I plays two roles, *i.e.* maintenance of the solubility and protection of the intact carboxyl groups.

The reactivity of the spacer carboxyl groups in derivatives V, VI and VIII with GlyOEt was examined in the presence of EDC. Derivative V gave some precipitates in the reaction with GlyOEt. The precipitation could be avoided when the reaction was carried out in 5 M guanidine[17] or 6 M urea solution,[18] but only 4—5 residues of GlyOEt per molecule of V (17—20% of the carboxyl spacer groups in V) were incorporated (Table II). On the other hand, the reactions of VI and VIII with GlyOEt could be carried out in aqueous solution, and 13—15 residues of the ester could be introduced into VI and VIII (54 and 42% of the carboxyl spacer groups in VI and VIII, respectively) in the reactions at high molar ratios of the ester to the protein (Table II).

All of the protein–GlyOEt conjugates described are water-soluble. Compound V is hardly soluble in water in the absence of guanidine or urea, while VI and VIII are water-soluble, and the numbers of GlyOEt residues introduced into VI and VIII are greater than those introduced into V. These results suggest that VI and VIII at least should be useful as carrier proteins which have carboxyl spacer groups for the direct preparation of conjugates between proteins and low molecular haptens with primary amino groups.

Acknowledgements The authors are indebted to Mr. K. Kotoh, Wakunaga Pharmaceutical Co., Ltd., for the measurement of MS, to Mr. Y. Tanaka and Miss K. Soeda, Faculty of Pharmaceutical Sciences, Kyushu University, for the measurement of ^{1}H-NMR spectra, and to the staff of the Central Analysis Room of Kyushu University for elemental analyses.

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