Preparation of Protein Conjugates via Intermolecular Disulfide Bond Formation†

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ABSTRACT: Conjugates of two unlike proteins can be prepared via the intermolecular disulfide interchange reaction, namely, protein A containing thiol groups reacts with protein B containing 4-dithiopyridyl groups to yield a conjugate with the release of 4-thiopyridone. Thiol groups can be introduced into proteins upon amidination with methyl 3-mercaptopropionimidate ester or 2-iminothiolane, and 4-dithiopyridyl groups can be introduced into proteins with these same reagents in the presence of 4,4′-dithiodipyridine. 2-Iminothiolane is stable on storage in contrast to the known lability of imidate esters; therefore 2-iminothiolane is a more convenient reagent for the modification of proteins than are the imidate esters. All the reactions can be carried out easily under mild conditions in good yields. Conjugates of bovine plasma albumin with itself, ribonuclease, or a copolymer of D-glutamic acid and D-lysine and of sheep antibody and horseradish peroxidase were prepared with modified proteins containing an average of 1 to 5 thiol or dithiopyridyl groups per mol. These conjugates formed mainly dimers, trimers, and tetramers. The peroxidase labeled antibody retained more than 80% of its enzymatic and antigenic binding activities.

Conjugates of proteins or protein-peptide have many practical uses, as indicated by the following examples. Enzyme-labeled antibodies or antigens are useful reagents for immunoassay (Engvall et al., 1971) and for immunohistochemistry (cf. Avrameas et al., 1976). Coupling of a protein antigen to different protein carriers can be used to enhance or suppress the immunogenicity of an antigen (cf. Naor & Galili, 1977). Conjugates of peptide hormone with a protein carrier may be useful for elucidating hormone-receptor interactions (Eberle et al., 1977).

The common procedures for the preparation of protein conjugates utilize symmetrical bifunctional reagents such as glutaraldehyde and bisimidate esters (cf. Glazer, 1976). Less commonly, unsymmetrical bifunctional reagents have been used, e.g., *m*-maleimidobenzoyl-*N*-hydroxysuccinimide (Kitagawa & Aikawa, 1976) and its aliphatic analogues (Keller & Rudinger, 1975). With the symmetrical bifunctional reagents, inter- as well as intramolecular coupling reactions can give conjugates of varying molecular sizes containing like or unlike proteins. With the unsymmetrical reagents many of the undesired side reactions cannot take place so that only conjugates of unlike proteins can form.

Another approach is to use two proteins with different reactive groups so that proteins A and B can react with each other but not with themselves. For example, Eberle et al. (1977) prepared conjugates of human plasma albumin with melanotropin peptides on reaction of α-mercaptosuccinyl albumin with bromoacetyl melanotropin peptide.

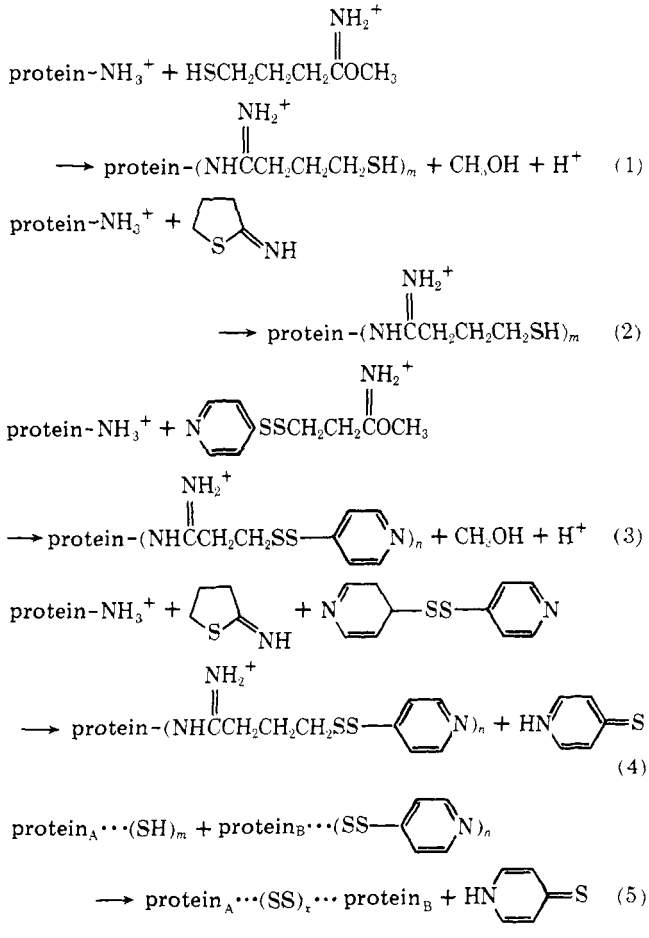
Intermolecular disulfide bond formation has been applied previously for the purification of thiol containing proteins on agarose gels containing reactive disulfide groups (Brocklehurst et al., 1974). It should be possible to apply this reaction for the preparation of conjugates of unlike proteins, namely, protein A containing thiol groups reacts with protein B containing 4-dithiopyridyl groups by a disulfide interchange reaction with the release of 4-thiopyridone. The required thiol and 4-dithiopyridyl groups can be introduced upon amidination of the lysyl side chains of proteins with the appropriate imidate ester, 2-iminothiolane, or a mixture of 2-iminothiolane and 4,4′-dithiopyridine. The chemical reactions involved are depicted in Scheme I. These studies will be reported in this paper.

Experimental Section

Materials. Bovine plasma albumin is from Armour Pharmaceutical Co., bovine pancreatic ribonuclease and horseradish peroxidase (Grade HPOFF) are from Worthington Biochemical Corp., and a copolymer of D-glutamic acid and D-lysine (60:40) with an average molecular weight of 34 000 is from Miles-Yeda Ltd. Human IgE myeloma serum (PS) and sheep anti-IgE (PS) sera are gifts of Drs. S. Kochwa and S. M. Fu, respectively.

Human IgE (PS) was isolated from serum by chromatog-

SCHEME I: Reactions for Preparation of Conjugates of Proteins.



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raphy on DE-32 cellulose column (0.9 × 23 cm) with a linear NaCl gradient (50 mM per 100 mL) in 25 mM Tris-HCl buffer (pH 7.95); IgE was eluted at about 25 mM NaCl concentration.

Immunosorbent containing IgE (PS) was prepared by coupling 6 mg of IgE with 1 g of CNBr activated Sepharose 4B (Pharmacia; dry weight before swollen in buffer) in 10 mL of 0.1 M $NaHCO_3$ and 0.5 M NaCl for 2 h at room temperature, as described in the manufacturer's pamphlet. Sheep antibody specific for IgE was obtained on passage of sheep anti-sera (3 mL) through a column (5 × 0.9 cm) of immunosorbent containing IgE (PS) at a flow rate of 30 mL per h. The column was first washed with 15 mL of 0.1 M Tris-HCl buffer (pH 7.95) followed with 15 mL of 0.05 M glycine hydrochloride buffer (pH 2.9) to elute the adsorbed antibody. Fractions of 2-mL volume were collected in tubes containing 0.5 mL of 0.2 M Tris-HCl buffer (pH 7.95) to neutralize the eluent.

4,4′-Dithiopyridine and 4-chlorobutyronitrile are from Aldrich Chemical Co. 3-Mercaptopropionitrile and methyl 3-mercaptopropionimidate ester hydrochloride were prepared as reported (Traut et al., 1973).

3-(4′-Dithiopyridyl)propionitrile. 3-Mercaptopropionitrile (548 mg, 6.13 mmol) in 10 mL of methanol was added dropwise to 4,4′-dithiopyridine (2.75 g, 12.5 mmol) in 10 mL of methanol over a period of 30 min with stirring at room temperature. After 15 min methanol was removed under reduced pressure to give 3.28 g of orange colored oil. The oil was extracted with three 10-mL portions of diethyl ether, leaving behind the insoluble 4-thiopyridone. The ether extract, after evaporation under reduced pressure, gave 2.06 g of yellow oil. The oil was next separated on a column (2.2 × 24 cm) of silica gel (E. Merck, Grade 60, 230–400 mesh). The column was eluted at a flow rate of about 70 mL per h, with ethyl acetate (300 mL) followed with ethanol (300 mL). The collected fractions (10-mL volume) were monitored by $A_{247\text{-nm}}$ and by TLC (E. Merck F-254 silica gel sheet; with ethyl acetate as solvent, the R_fs of 4-thiopyridone, 4,4′-dithiopyridine, and product were respectively 0, 0.11, and 0.31). The product and the excess 4,4′-dithiopyridine were eluted at 180–270 mL and 320–480 mL volumes, respectively. After rechromatography under the same conditions as described above, the product was obtained as a colorless liquid (385 mg; 32% yield): UV (CH_3OH) $\epsilon_{245\text{-nm}}$ 0.98×10^4; NMR (CH_2Cl_2) δ 1.93 (4 H, m), 6.53 (2 H, d), 7.57 (2 H, d). Anal. Calcd for $C_8H_8N_2S_2$: C, 48.95; H, 4.11; N, 14.27. Found: C, 48.80; H, 4.14; N, 14.08.

Methyl 3-(4′-Dithiopyridyl)propionimidate Ester Hydrochloride. Method A. Hydrogen chloride gas (3.25 g) was bubbled through methanol (4.0 mL) cooled in an ice bath. To 450 μL of this methanolic hydrogen chloride was added 3-(4′-dithiopyridyl)propionitrile (229 mg, 1.16 mmol). After being kept overnight in the ice bath, ethyl ether (1 mL) was added. The resulting crystals were collected, washed with methanol–ether (1:3), and dried for 1 h under reduced pressure at room temperature. The hygroscopic product (300 mg, 86% yield) had a mp of 79–82 °C with decomposition. It was stable on storage in the freezer for at least 2 months. Anal. Calcd for $C_9H_{14}Cl_2N_2OS_2$: Cl, 23.54. Found: Cl, 23.80.

Method B. Methyl 3-mercaptopropionimidate ester hydrochloride (18.1 mg, 0.116 mmol) in 0.5 mL of methanol was added dropwise to 4,4′-dithiodipyridine (124 mg, 0.562 mmol) in 0.5 mL of methanol. After 15 min at room temperature, the reaction mixture was used directly for modification of proteins.

4-Isothioureidobutyronitrile Hydrochloride. A solution of thiourea (38.1 g, 0.50 mol) and 4-chlorobutyronitrile (60 g, 0.55 mol) in water (50 mL) was refluxed for 3 h. Water was removed under pressure. On further drying at <1 Torr, the product (mp 126–128 °C) crystallized in quantitative yield. After recrystallization from methanol–acetone (1:3) mixture, it melted at 132–133 °C. Anal. Calcd for $C_5H_{10}ClN_3S$: C, 33.43; H, 5.61; N, 23.39. Found: C, 33.38; H, 5.54; N, 23.24.

4-Mercaptobutyronitrile. To 4-isothioureidobutyronitrile hydrochloride (37.7 g, 0.21 mol) in 50 mL of water was added a hot solution of glycine (33 g, 0.44 mol) in 70 mL of water at 80 °C. After refluxing for 30 min under N_2 and cooling to room temperature, the mixture was acidified to pH 1 with concentrated hydrochloric acid (about 33 mL); then it was extracted with three portions of ethyl ether (200, 100, and 100 mL). The combined ether extract, after washing once with saturated sodium chloride solution (100 mL), drying over sodium sulfate (20 g), and removal of solvent, gave 5.0 g of crude product. The product (3.3 g, 15%) was purified by distillation (bp 76–78 °C at 5 Torr): IR (neat) 2560 cm^{-1} (thiol, weak) and 2250 cm^{-1} (nitrile, strong).

Titration of the freshly prepared 4-mercaptobutyronitrile with 4,4′-dithiodipyridine (Grasetti & Murray, 1967) gave a sulfhydryl content of 0.92 residue per mol. After storage at −34 °C for 12 days, its sulfhydryl content decreased to 0.42 residue per mol.

The above method is a modification of that reported by Traut et al. (1973); these authors used sodium hydroxide for hydrolysis of the isothioureidobutyronitrile. The present method gives a slightly improved yield over that reported.

2-Iminothiolane. To 4-isothioureidobutyronitrile hydrochloride (71.8 g, 0.40 mol) in 25 mL of water and 100 mL of 4 N sodium hydroxide was added a solution of glycine (30 g, 0.40 mol) in 60 mL of water at 80 °C. The mixture was refluxed for 30 min under N_2; then it was worked up in the same manner as described for 4-mercaptobutyronitrile, to give 9.0 g (22% yield) of crude product. The product was purified by distillation (bp 71–72 °C at 6 Torr): IR (neat) 1700 cm^{-1} (lactone, strong); NMR (chloroform) δ 3.48 (2 H, t), 2.10–2.80 (4 H, m).

The thiol content of a freshly prepared solution of 2-iminothiolane at room temperature and pH 9.1 (0.025 M borax buffer) was less than 0.008 residue per mol, and it slowly increased to 0.015 and 0.064 residue per mol after 2 and 32 h, respectively.

The pH of a freshly prepared solution of 0.058 M 2-iminothiolane was 5.4, the same as that of distilled water used, and addition of 0.15 equiv of HCl reduced its pH to 2.0. Therefore the pK_a of 2-iminothiolane is much less than 2.

2-Iminothiolane Hydrochloride. 2-Iminothiolane (1.6 g, 14.8 mmol) was dissolved in CH_3OH (2.2 mL) containing 1.6 g of hydrogen chloride (44.3 mmol). On standing at 0 °C overnight followed by the addition of diethyl ether (5 mL), crystals (mp 187–192 °C) formed in a yield of 27% (559 mg). The crystals, after sublimation at about 180 °C and 0.2 Torr, melted at 202–203 °C; mp reported 190–195 °C (Addor, 1968): NMR (dimethyl-d_6 sulfoxide) δ 2.27 (2 H, t), 3.25 (2 H, t), 3.52 (2 H, t). Anal. Calcd for C_4H_8ClNS: C, 34.91; H, 5.86; N, 10.18. Found: C, 35.16; H, 5.90; N, 10.14.

Methyl 4-Mercaptobutyrimidate Ester Hydrochloride. Hydrogen chloride gas (1.6 g, 44.3 mmol) was bubbled through ice cold methanol (2.2 mL); then 4-mercaptobutyronitrile (1.45 g, 14.3 mmol) was added. After being kept overnight at 0 °C, 5 mL of diethyl ether was added. The crystals formed were collected after 1 h, washed with methanol–ether (1:3) mixture, and dried in vacuo. The hydroscopic product (1.4 g) was obtained in a yield of 58%. The product was unstable on

storage at −34 °C, and its thiol content was 0.4 residue per mol after 14 days at −34 °C. The melting point of the freshly isolated product varied with the initial temperature at which the sample was introduced. With the melting point block initially at 90 °C or higher, gas evolution of the sample was observed and the residue melted at 165–179 °C. With the melting point block initially at 70–87 °C, no gas evolution was observed and the sample melted at 185–190 °C. After storage at −34 °C for 40 days, no such difference in melting point behavior was seen, and the sample melted at 185–190 °C without prior gas evolution at 90 °C.

Methods. Amino acid analyses were made on a Beckman Spinco Model 120 amino acid analyzer, modified for increased sensitivity and single column analyses (Liao et al., 1973); protein samples (about 0.2 nmol) were hydrolyzed in 6 N HCl (50 μL) in sealed and evacuated tubes at 110 °C for 20 h.

Disc electrophoresis in polyacrylamide gel was carried out as described (Davis, 1964).

Peroxidase assays were made as described in the Worthington Enzyme Manual (1977).

Results

Reagents for Preparing Amidinated Proteins Containing Thiol or 4-Dithiopyridyl Groups. Methyl 3-mercaptopropionimidate and 4-mercaptobutyrimidate ester hydrochloride are two known reagents for preparing thiolated proteins upon amidination of the ε-amino groups of the lysyl residues (Traut et al., 1973). They can be synthesized on methanolysis of the appropriate mercaptonitrile, as shown for 4-mercaptobutyronitrile in reaction 3 of Scheme II, and they were reported to have melting points of 78–79 and 192–193 °C, respectively, both with decomposition. The large difference in melting points of these two homologous compounds is unexpected.

In the course of this work we found that 4-mercaptobutyronitrile can cyclize to form 2-iminothiolane under certain conditions. Reaction of 4-isothioureidobutyronitrile hydrochloride with glycine gave 4-mercaptobutyronitrile, but the same reaction carried out in the presence of 1 equiv of sodium hydroxide gave 2-iminothiolane (reaction 2 of Scheme II). The cyclized product differs from the open chain compound by the disappearance of the titrable thiol group and by a diminished mercaptan odor. Also, infrared spectroscopy showed that the cyclized product has a lactone band at 1700 cm^{-1} with concomitant loss of the nitrile band at 2250 cm^{-1} which is present in the open chain compound. 2-Iminothiolane has been synthesized previously on acid-catalyzed methanolysis of *S*-acetyl-4-mercaptobutyronitrile, and its hydrochloride was reported to have a mp of 190–195 °C (Addor, 1968).

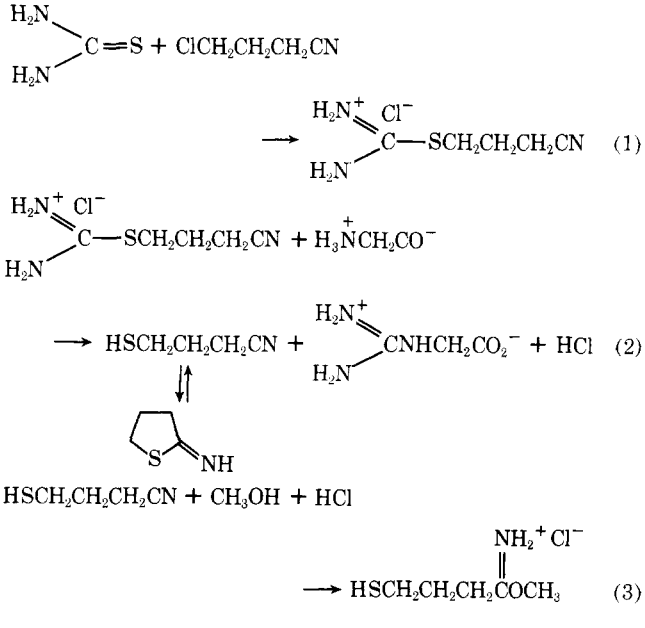
We found that freshly prepared methyl 4-mercaptobutyrimidate ester hydrochloride decomposed at 90 °C with gas evolution and the residue melted at 165–170 °C, and that on storage its thiol content slowly decreased. The findings indicate that the unstable methyl 4-mercaptobutyrimidate can slowly cyclize to give the stable and higher melting 2-iminothiolane hydrochloride. The cyclic structure of 2-iminothiolane suggests that it can be used for amidination of proteins, analogous to the acylation of proteins with *N*-acetylhomocysteine thiolactone (Benesch & Benesch, 1956). Indeed such use of 2-iminothiolane has been reported recently (Schramm & Dülffer, 1977).

4-Dithiopyridyl containing proteins can be prepared by treatment of proteins with methyl 3-(4′-dithiopyridyl)propionimidate ester, or with a mixture of 2-iminothiolane and 4,4′-dithiopyridine, reactions 3 and 4 of Scheme I, respectively. The required imidate ester was prepared on methanolysis of 3-(4′-dithiopyridyl)propionitrile which was obtained on reaction of 3-mercaptopropionitrile with 4,4′-dithiopyridine. Alternatively the required imidate ester can be prepared on treatment of methyl 3-mercaptopropionimidate ester with excess 4,4′-dithiodipyridine. These reactions are depicted in Scheme III.

Preparation and Stability of Amidinated Proteins Containing Thiol or 4-Dithiopyridyl Groups. Amidination of proteins with the substituted propionimidates and 2-iminothiolane was studied under different conditions, by measuring the incorporation of thiol or 4-dithiopyridyl groups into proteins. The results are summarized in Table I. The extent of amidination of proteins with the two propionimidates and 2-iminothiolane was higher at pH 9 than those at pH values of less than 9. This is in accord with the known reactivity of methyl acetimidate, the amidination reaction being optimal at pH 9–10 (Ludwig & Hunter, 1972; Browne & Kent, 1975).

Methyl 3-(4′-dithiopyridyl)propionimidate (10 mM) was used at a lower concentration than that of methyl 3-mercaptopropionimidate (50 mM). This is because methyl 3-(4′-dithiopyridyl)propionimidate has a lower solubility in water than methyl 3-mercaptopropionimidate. The results indicate that these two propionimidates are about equally active as amidinating agents when one takes into account the concentration difference of these two reagents. 2-Iminothiolane (180 mM)

SCHEME II: Synthesis of 4-Mercaptobutyronitrile, 2-Iminothiolane, and Methyl 4-Mercaptobutyrimidate Hydrochloride.



SCHEME III: Synthesis of Methyl 3-(4′-Dithiopyridyl)-mercaptopropionimidate.

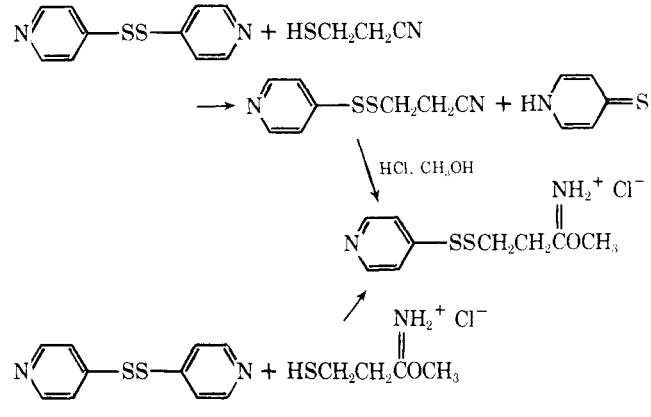


TABLE I: Amidination of Proteins with Imidate Esters or 2-Iminothiolane.[a,d]

A. Methyl 3-mercaptopropionimidate ester (50 mM)

Protein (0.15 mM)	pH	Reaction time (h)	Thiol groups/mol of protein[e]	% of Lys residues modified
Bovine plasma albumin	6.1	1	3	5
	6.6	1	6	9
	7.2	1	10	17
	8.1	1	11	19
	9.0	1	12	20
Ribonuclease	9.0	1	2	18

B. Methyl 3-(4′-dithiopyridyl)propionimidate ester (10 mM)

Protein (0.20 mM)	pH	Reaction time (h)	4-Dithiopyridyl groups/mol of protein[f]	% of Lys residues modified
Bovine plasma albumin	7.2	2	0.8	1.4
	8.1	2	2.2	3.7
	9.0	2	3	5.1

C. 2-Iminothiolane (180 mM)

Protein (0.18 mM)	pH	Reaction time (h)	Thiol groups/mol of protein[e]	% of Lys residues modified
Bovine plasma albumin	8.0	1	4.5	7.6
	9.0	1	6.5	11
Ribonuclease	8.0	1	1	9.1
	9.0	1	1	9.1
Sheep antibody	8.0	2	3.9	
	8.5	2	4.8	

D. 2-Iminothiolane (200 mM) and 4,4′-dithiodipyridine (10 mM)

Protein (0.18 mM)	pH	Reaction time (h)	4-Dithiopyridyl groups/mol of protein[f]	% of Lys residues modified
Bovine plasma albumin	9.0	1	4.2	7.1
D-GL	9.0	2	3.1	2.9
Horseradish peroxidase	9.0	2	1.1	18

[a] Reactions were carried out at room temperature using 25 mM sodium borax for pH 8.5 and 9.0 buffers and 100 mM sodium phosphate for all other buffers. [b] For reactions A and D, the amidinating reagent in methanol was added at zero time, and the final reaction mixture contained 10% methanol. For reaction B, the reagent in methanol was added in four equal aliquots at 30-min intervals to reach the final concentration of 10 mM. For reaction C, the reagent was added directly as methanol was not required to dissolve the reagent. [c] Protein concentrations were determined by spectrophotometry using the following E_{1cm} values (c = 1 mg per mL) at 280 nm: 0.64, 0.60, and 1.4 for albumin, ribonuclease, and sheep antibody and E_{1cm} value (c = 1 mg per mL) at 403 nm of 2.5 for horseradish peroxidase. Concentration of D-GL was determined by amino acid analysis after acid hydrolysis. The molecular weights of these proteins were taken to be 66 000, 13 000, 160 000, 40 000, and 34 000, respectively. The number of lysine residues per mole of protein was taken to be 58, 11, 6, and 106, respectively; for albumin (Brown, 1976), ribonuclease (Smyth et al., 1963), peroxidase (Welinder et al., 1972), and D-GL. [d] The amidinated protein was freed of reagents by passage of the reaction mixture (2.2 mL) through a Sephadex G-25 column (25 × 0.9 cm) equilibrated with 100 mM phosphate buffer (pH 6.6) containing 1 mM Na_2EDTA. There was an exception for amidinated D-GL. Its turbid mixture was first acidified with glacial acetic acid (40 μL) and then separated on a Sephadex G-25 column equilibrated with 0.2 N acetic acid. The product was lyophilized. Slight turbidity developed for the amidination of peroxidase. It was clarified by centrifugation before being applied to Sephadex column. Greater than 95% of the enzyme units was recovered in the modified protein. [e] The number of thiol groups present was determined by spectrophotometric titration of the amidinated protein (0.01 mM or more) with 4,4′-dithiodipyridine (1 mM) in pH 6.6 phosphate buffer (Grassetti & Murray, 1967). [f] The number of 4-dithiopyridyl groups was determined by following the release of 4-thiopyridine at 324 nm on addition of mercaptoethanol (6 mM) to amidinated protein (0.01 mM or more) in pH 6.6 phosphate buffer.

was used at a higher concentration than that of methyl 3-mercaptopropionimidate; yet its extent of amidination of proteins is less than that obtained with methyl 3-mercaptopropionimidate. This indicates that 2-iminothiolane is a less reactive reagent than the open chain imidate ester. This reduced activity is in fact desirable as it can permit better control of the extent of modification.

The thiol groups of the amidinated proteins are moderately stable to air oxidation in 0.1 M phosphate buffer (pH 6.6) containing 1 mM EDTA. For example, the thiol content of a solution of 3-mercaptopropionimidinyl bovine plasma albumin (11 thiol groups per mol) in pH 6.6 buffer decreased by 9% and 45%, respectively, after 1- and 5-day storage at room temperature.

The stability of the 4-dithiopyridyl groups of the amidinated proteins in solutions at pH 3.6, 6.6, and 8.0 was studied. The results in Table II indicate that the dithiopyridyl groups are fairly stable at pH values more acidic than 6.6, but they are labile at pH values more alkaline than 6.6. 4-(4′-Dithiopyridyl)butyriminidyl group appears to be more stable than 3-(4′-dithiopyridyl)propionimidinyl group.

Coupling of Amidinated Proteins Containing Thiol Groups and 4-Dithiopyridyl Groups. The thiol–disulfide interchange reaction (reaction 5 of Scheme I) is a relatively fast reaction

TABLE II: Stability of the Mixed Disulfide, 4-Dithiopyridyl Group of Amidinated Bovine Plasma Albumin at Different pH Values.[a]

pH	3-(4′-Dithiopyridyl)propionimidinyl groups per mol of albumin at days				4-(4′-Dithiopyridyl)butyriminidyl groups per mol of albumin at days		
	0	1	16	45	0	1	6
pH 3.8, 0.06 N HoAc	3.7; 100%			3.4; 92%	4.2; 100%	3.4; 81%	2.8; 67%
pH 6.6, 0.1 M phosphate + 1 mM EDTA	3.8; 100%	2.4; 86%	2.4; 86%		3.7; 100%	4.0; 108%	
pH 8.0, 0.1 M phosphate + 1 mM EDTA	2.0; 100%	0.9; 45%			4.2; 100%	3.4; 81%	2.5; 60%

[a] After storage at room temperature in the indicated buffer, the amidinated protein was freed of impurities after passage through a Sephadex G-100 column equilibrated with the same buffer, and the number of 4-dithiopyridyl groups remaining in the monomeric albumin (>95% recovery) was determined as indicated in the footnote to Table I.

TABLE III: Rate of Reaction of 4-(4′-Dithiopyridyl)-Butyrimidinated Bovine Plasma Albumin (37.3 μM) with 2-Mercaptoethanol (89 μM) at Different pHs.[a]

Time (h)	% reaction at indicated pH		
	6.53	7.23	7.83
0.08	42	54	68
0.17	55	66	76
0.50	71	78	84
1.0	78	84	88
4.0	89	92	93
7.0	92	94	95

[a] The sample of amidinated albumin used contained 4.3 residues of dithiopyridyl group per mol of protein. The reaction was followed by measuring the release of 4-thiopyridone at 324 nm.

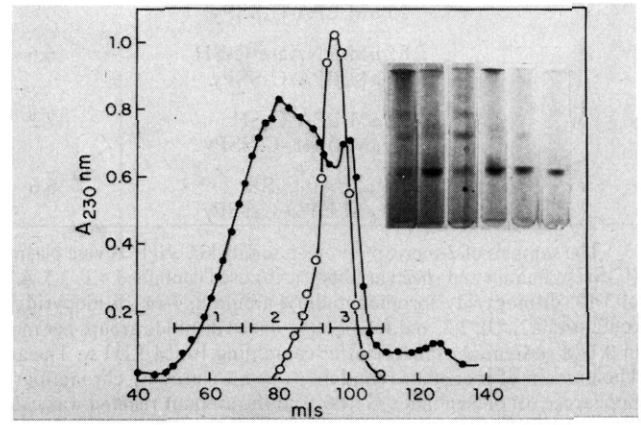


FIGURE 1: Separation of a coupling mixture of 3-mercaptopropionimidinyl albumin and 3-(4′-dithiopyridyl)propionimidinyl albumin on a column (200 × 0.9 cm) of Sepharose 6B. The reaction mixture (0.8 mL) from experiment 1 of Table IV was used. The column was eluted with 0.1 M NH_4HCO_3 at a flow rate of 6 mL per h. The curve with open circles stands for a sample of 3-(4′-dithiopyridyl)propionimidinyl albumin chromatographed under the same conditions. Inset shows the disc electrophoretic patterns of the indicated cuts 1, 2, and 3 before and after mercaptoethanol reduction from left to right.

and its rate is dependent on the pH of the medium. This is shown in Table III for the model reaction of 2-mercaptoethanol with 4-(4′-dithiopyridyl)butyrimidinyl albumin. With the reactants at 50–100 μM concentration range and the pH of the medium in the range of 6.6 to 8.0, the reaction was near completion after 7 h room temperature. Therefore, the coupling reaction in this pH range is a much faster reaction than the decomposition of 4-dithiopyridyl group, as shown by comparing the data of Tables II and III.

The thiol–disulfide reaction was next studied for the coupling of proteins containing thiol and 4-dithiopyridyl groups. The results obtained on the coupling of albumin with itself, of albumin with ribonuclease, of albumin with a copolymer of D-glutamic acid and D-lysine and of sheep antibody specific for human IgE with horseradish peroxidase are summarized in Table IV. The extent of coupling was followed spectrophotometrically for the release of 4-thiopyridone, and chromatographically for the formation of oligomeric protein conjugates. In all cases studied, coupling of proteins occurred readily in the concentration range of 20–80 μM at room temperature and neutral pH to give 60–90% yields of conjugates after a reaction time of 3 h or more.

The preparation of conjugates of albumin with itself under different conditions is given in experiments 1 to 4 of Table IV. A comparison of experiments 1 and 2 shows that 3-mercaptopropionimidinyl and 4-mercaptobutyrimidinyl albumins are about equally reactive for coupling with 3-(4′-dithiopyridyl)-propionimidinyl albumin. A comparison of experiments 1 and 3 shows that 3-(4′-dithiopyridyl)propionimidinyl and 4-(4′-dithiopyridyl)butyrimidinyl albumins are about equally reactive for coupling with 3-mercaptopropionimidinyl albumin. The coupling reaction is faster at pH 8 than that of pH 6.6 as shown in experiments 3 and 4 for the coupling of 3-mercaptopropionimidinyl albumin with 4-(4′-dithiopyridyl)butyrimidinyl albumin.

The molecular size and composition of the conjugates formed were investigated with the coupling products from experiments 1, 5, 6, and 7 of Table IV. The elution profile of the conjugate of albumin with itself from a column of Sepharose 6B indicates that it contains mainly dimers, trimers, and tetramers, and this is seen by comparing its elution profile with that of a sample of albumin containing small amounts of dimer (Figure 1). This conclusion is also supported by the disc electrophoretic results of cuts 1, 2, and 3 of Figure 1, as shown in the inset. Cuts 1 and 2 contained at least three more components than did cut 3, and the relative mobilities of these slower components as compared with that of the major component in cut 3 are suggestive of their dimer, trimer, and tetramer nature. On treatment with 0.11 M mercaptoethanol at 25 °C for 10 min, the slower migrating components of cuts 1 and 2 were converted to the fast migrating major component present in cut 3.

The coupling mixture of albumin with ribonuclease was first separated on a column of Sephadex G-100. The chromatogram in Figure 2A indicates that the conjugates are well resolved from ribonuclease but not from albumin. The indicated cut in Figure 2A, after concentration, was rechromatographed on a column of Sepharose 6B (Figure 2B). The conjugates were still not well resolved from albumin, but the results do show that the conjugates formed are relatively small in size, mainly dimers, trimers, and tetramers.

The coupling mixture of albumin with D-GL was separated on a column of Sepharose CL-6B (Figure 3). The conjugates were eluted as a broad zone extending from the column breakthrough volume to that of albumin. The broadness of the elution pattern reflects not only size heterogeneity of conjugates but also that of D-GL used, as the elution peak of D-GL is about twice as broad as that of albumin. The elution position

TABLE IV: Coupling of Proteins Containing 4-Dithiopyridyl and Thiol Groups.[a]

Expt	Reaction conditions: Reactants	pH	Time (h)	4-Thiopyridone released (%)	Monomer reacted (%)
1	33 μM BPA-C_3SH 43 μM BPA-C_3SSPy	6.6	3.0	86	80
2	43 μM BPA-C_4SH 29 μM BPA-C_3SSPy	6.6	7.0	73	58
3	37 μM BPA-C_3SH 21 μM BPA-C_4SSPy	6.6	7.0	59	63
4	42 μM BPA-C_3SH 25 μM BPA-C_4SSPy	8.0	2.5	64	76
5	85 μM RNAase-C_3SH 52 μM BPA-C_3SSPy	6.6	3.0	100	90
6	92 μM BPA-C_4SH 136 μM D-GL-C_4SSPy	7.2	2.0	75	86
7	4.4 μM Ab-C_4SH 9.0 μM HPO-C_4SSPy	6.6	17	nd	95

[a] The samples of 3-mercaptopropionimidinyl (C_3SH) bovine plasma albumin (BPA) and ribonuclease (RNase), and 4-mercaptobutyrimidinyl (C_4SH) albumin and sheep antibody (Ab) used contained 4.1, 3.5, 4.7, and 4.9 (±15%) thiol groups per mol of protein, respectively. The samples of 3-(4′-dithiopyridyl)propionimidinyl albumin, 4-(4′-dithiopyridyl)butyrimidinyl albumin, D-GL, and horseradish peroxidase (HPO) used contained 3.2, 4.0, 2.5, and 1.1 (±15%) mixed disulfide groups per mol of protein, respectively. All reactions were carried out at room temperature in 0.1 M sodium phosphate buffer containing 1 mM EDTA. The amount of 4-thiopyridone released was measured by absorption at 324 nm. The amount of monomer remaining was measured by chromatographic separation of the reaction mixture on a column (200 × 0.9 cm) of Sepharose 6B or Sephadex G-100, and the amount reacted was calculated on the basis of the protein used in limiting amount.

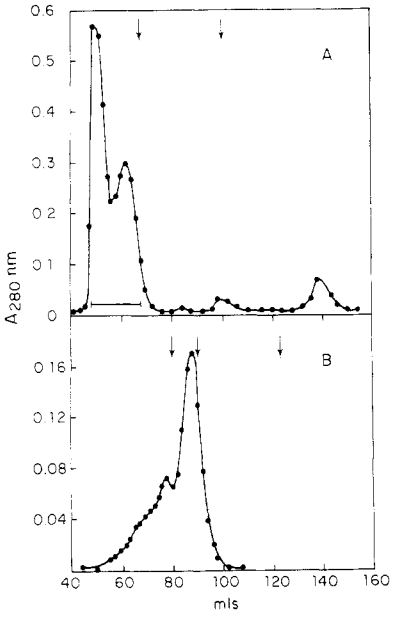


FIGURE 2: (A) Separation of a coupling mixture of 3-mercaptopropionimidinyl albumin and 3-(4′-dithiopyridyl)propionimidinyl ribonuclease on a column (200 × 0.9 cm) of Sephadex G-100. The reaction mixture (1.8 mL) from experiment 5 of Table IV was used. The column was eluted with 0.1 M phosphate buffer (pH 6.6) containing 1 mM EDTA at a flow rate of 10 mL per h. The elution positions of monomeric albumin and ribonuclease are indicated by arrows at 68 and 100 mL, respectively. (B) The indicated cut from Figure A was rechromatographed on a column (200 × 0.9 cm) of Sepharose 6B. The column was eluted with 0.1 M NH_4HCO_3 at a flow rate of 6 mL per h. The elution positions of monomeric and dimeric albumins and sodium chloride are indicated by arrows at 80, 90, and 126 mL, respectively.

of D-GL is ahead of that of albumin even though its molecular weight of 34 000 is smaller than that of albumin of 66 000; this difference is probably related to the shape factor of the two solutes. Amino acid analyses of the indicated cuts of Figure 3 showed that cuts 1, 2, 3, and 4 contained 14, 37, 36, and 14%, respectively, of the albumin used, and their molecular ratios of D-GL to albumin were respectively 1.30, 1.45, 1.05, and 0.65.

The coupling mixture of sheep antibody specific for human IgE with horseradish peroxidase was separated on a column of Sepharose CL-6B (Figure 4). About 75% of the enzyme activity used in the reaction mixture was recovered in the chromatogram. The first peak of the chromatogram contained the enzyme–antibody conjugate as indicated by its elution position and enzyme activity. Its elution position indicates the conjugates to be comprised of mainly dimers and trimers. The indicated cut, comprising 75% of the first peak, was pooled and concentrated by ultrafiltration. From its absorbances at 280 and 403 nm, the molar ratio of antibody to peroxidase of the conjugate was estimated to be 1.5. The specific activity of peroxidase in the conjugate was found to be 530 units per mg, while that of the native enzyme was 710 units per mg. The antigen binding activity of the conjugate was tested by affinity chromatography on immunosorbent containing IgE; 95% of the enzyme activity and 70% of the absorbance units of the sample were adsorbed. These values indicate that at least 80% of the conjugate can still combine with the antigen. The conjugate absorbed to the immunosorbent could be eluted with pH 2.9 glycine hydrochloride buffer with 50% recovery of enzyme activity.

The present method of preparation of antibody–peroxidase conjugates compares very well with other reported methods in terms of yields and retention of biological activities (Nakane & Kawaoi, 1974; Boorsma & Streefkerk, 1976).

Discussion

The above results show that conjugates of two unlike proteins can be prepared via the intermolecular disulfide bond exchange reaction. The proteins which are to be coupled are first modified so that they contain an average of 1 to 5 thiol or 4-dithiopyridyl groups per mol of protein. All the reactions are carried out easily under gentle conditions. When the conjugates can be isolated by affinity chromatography, e.g., conjugates of antibody with horseradish peroxidase, the entire sequence

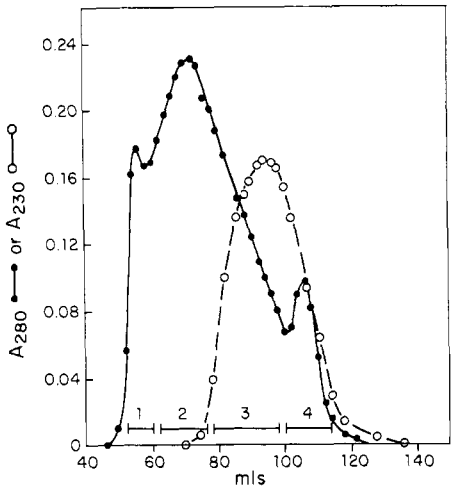


FIGURE 3: Separation of a coupling mixture of 4-mercaptobutyrimidinyl albumin and 4-(4′-dithiopyridyl)butyrimidinyl-D-GL on a column (200 × 0.9 cm) of Sepharose CL-6B. The reaction mixture (2.0 mL) from experiment 6 of Table IV was used. The column was eluted with 0.1 M phosphate buffer (pH 7.2) containing 1 mM EDTA at a flow rate of 6 mL per h. The dashed curve stands for a 3.6-mg sample of 4-(4′-dithiopyridyl)butyrimidinyl-D-GL chromatographed under the same conditions as described above; an identical pattern was obtained with unmodified D-GL

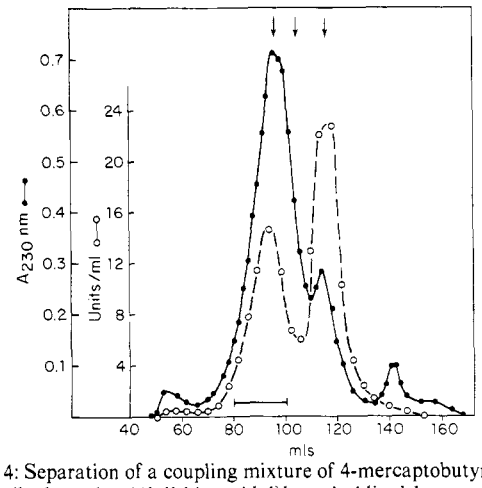


FIGURE 4: Separation of a coupling mixture of 4-mercaptobutyrimidinyl sheep antibody and 4-(4′-dithiopyridyl)butyrimidinyl horseradish peroxidase on a column (200 × 0.9 cm) of Sepharose CL-6B. The reaction mixture (2.7 mL) from experiment 7 on Table IV was used. The column was eluted with 0.1 M phosphate buffer (pH 6.6) containing 1 mM EDTA at a flow rate of 6 mL per h. The elution positions of three marker proteins, monomeric and dimeric albumins and ragweed antigen E (King et al., 1964), are indicated by arrows. These three proteins with molecular weights of 132 000, 66 000, and 38 000 were eluted at 96, 105, and 126 mL, respectively.

of reactions and isolation can be carried out in about a day and a half.

Thiol groups can be introduced into proteins upon acylation or amidination of the lysyl residues with the appropriate reagents (Glazer, 1976). Amidination was chosen in this work, as the amidinated lysyl residues retain the cationic charges of the native molecule which can be of importance for its conformational stability. Two known reagents which can be used for our purpose are methyl 3-mercaptopropionimidate and 4-mercaptobutyrimidate esters (Traut et al., 1973). In the course of our studies we found that 4-mercaptobutyronitrile, the precursor compound of methyl 4-mercaptobutyrimidate, readily cyclizes to form 2-iminothiolane. On storage, methyl 4-mercaptobutyrimidate probably also cyclizes to 2-iminothiolane.

2-Iminothiolane was found to be useful as an amidinating agent; its use has also been reported by others (Schramm & Düffler, 1977). 2-Iminothiolane is less active than the imidate esters, as shown by the results in Table I. For the purpose of limited amidination of proteins, 2-iminothiolane is a more convenient reagent than the imidate esters. It is stable on storage at room temperature in contrast to the lability of imidate esters, and it has good solubility in water in contrast to the limited solubility of the corresponding imidate ester.

4-Dithiopyridyl groups cannot be introduced into proteins by reaction of thiolated proteins with excess 4,4′-dithiopyridine. We found that rapid intramolecular disulfide bond formation is an important side reaction (results not shown). 4-Dithiopyridyl groups can be introduced into proteins upon amidination with methyl 3-(4′-dithiopyridyl)propionimidate or with a mixture of 2-iminothiolane and 4,4′-dithiopyridine. In the latter case, the 4-mercaptobutyrimidinyl groups formed upon amidination of the proteins are converted immediately in situ to 4-(4′-dithiopyridyl)butyrimidinyl groups and there is no inter- or intramolecular disulfide bond formation. This is shown by these two findings. The dithiopyridyl content of albumin modified with a mixture of 2-iminothiolane and 4,4′-dithiopyridine is the same as the thiol content of albumin modified with 2-iminothiolane (Table I). 4-Dithiopyridyl containing albumin or D-GL had the same elution profiles as the unmodified proteins.

There is no difference between 3-mercaptopropionimidinyl and 4-mercaptobutyrimidinyl groups in terms of their stability on storage and their reactivity in the disulfide interchange reaction (Table IV). 4-(4′-Dithiopyridyl)butyrimidinyl group appears to be slightly more stable than 3-(4′-dithiopyridyl)-propionimidinyl group (Table II) but there is no difference in their reactivity in the disulfide exchange reaction (Table IV). Therefore, there is no chemical preference for the use of the substituted propionimidinyl groups over that of the substituted butyrimidinyl groups.

For the preparation of protein conjugates with well-defined molecular sizes, it is preferable to have proteins uniformly modified with a defined number of thiol or 4-dithiopyridyl groups. This is difficult to attain. The modified proteins used in this work contain an average of 1 to 5 reactive groups per mol of protein. Assuming random modification of a protein to give an average of 3 groups per mol, the distribution of proteins having 0 to 5 reactive groups per mol is calculated with Poisson's equation to be 5, 15, 22, 17, and 10% (Gennis & Cantor, 1972). If all the groups are equally reactive in the thiol–disulfide interchange reaction, the conjugates formed will be complex in their size distribution.

Our results show that conjugates formed with proteins having an average of 1 to 5 reactive groups per mol are mainly in the size range of dimers, trimers, and tetramers. Two possible explanations may be considered for the observed size distribution of conjugates. First, steric hindrance present in the conjugates may decrease the accessibility of the remaining groups for further reactions to give conjugates with sizes larger than those observed. Second, the disulfide bond interchange reaction was carried out in 10–100 μM concentration, and the low concentration may reduce the probability of dimers, trimers, and higher oligomers for further reaction to give complex polymers. The first explanation is supported by the following findings. In the disulfide interchange reaction of the 4-dithiopyridyl group of albumin with mercaptoethanol, more than 95% of the expected 4-thiopyridone was released (Table III), but, in the reaction of 4-dithiopyridyl and thiol groups of proteins, about 70% of the expected 4-thiopyridone was released (Table IV). The second explanation of concentration dependence is probably less important, as the conjugates after

storage for 1 to 2 weeks at 4 °C gave a chromatographic pattern from Sepharose 6B identical with that of a freshly prepared sample.

In addition to the size heterogeneity, the conjugates formed are heterogeneous in their sites and numbers of disulfide bond linkages. This is inevitable as the conversion of lysyl residues of proteins A or B into reactive thiol or mixed disulfide groups occurs randomly to give a mixture of protein derivatives differing in their sites and numbers of lysyl residues modified. Therefore, on coupling such mixtures of proteins A and B, different pairings of disulfide bonds will result. This type of heterogeneity can be avoided if specific modifications of a single site of proteins A or B can be carried out, but this is difficult to attain.

The protein conjugates prepared in this work have molar ratios of the two proteins of about 1.5 as compared with the expected value of one. This is shown for the conjugate of albumin with D-GL on amino acid analysis and for the conjugate of specific sheep antibody and horseradish peroxidase on enzyme assay and antigen binding activity. The retention of biological activities of the conjugates, their good yields, and ease of preparation all indicate the usefulness of the procedure described in this paper.

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Pressure-Induced Changes in the Nuclear Magnetic Resonance Spectra of a Biopolymer in Aqueous Solution†

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ABSTRACT: High resolution proton nuclear magnetic resonance spectra of the poly(amino acid) poly[N^5-(3-hydroxypropyl)-L-glutamine], of degrees of polymerization 685 and 137, were measured in a mixed D_2O and H_2O solvent, at pressures from 1.03 to 1968.5 kg/cm^2, and at temperatures of 2 and 10 °C. Increasing the pressure appeared to cause an increased mobility of the side chain hydrocarbon residues, and also of the α-hydrocarbon residue of the polymer chain. This is interpreted to imply the occurrence of a volume decrease on unfolding of the polymer from a helix to a random coil, with subsequent exposure of hydrophobic groups to the solvent.

The study of the denaturation of proteins by pressure has been the source of considerable recent interest due to the difficulties which have occurred in interpreting the results (Brandts et al., 1970; Zipp & Kauzmann, 1973; Li et al., 1976; Williams & Shen, 1972). Attempts to explain these results in terms of model compound studies have not been completely successful (Kliman, 1969).

In order to bridge this gap, we have studied the effect of pressure on molecules of high molecular weight but relatively simple structure, namely poly(amino acids) (Suzuki & Tani-

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