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CROSS-LINKING OF ERYTHROCYTE MEMBRANES WITH DIMETHYL ADIPIMIDATE

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SUMMARY

Dimethyl adipimidate reacts with lysine residues of proteins to form covalent cross-links. When human erythrocyte membranes are treated with dimethyl adipimidate the percentage of protein which can subsequently be solubilized by treatment with aqueous pyridine is reduced. Evidence is presented that this is due to the formation of cross-links between soluble and insoluble protein molecules. The solubility distribution of protein-bound sialic acid, hexose, and hexosamine is altered in a parallel manner. This effect is not produced by treatment of the membranes with methyl butyroimidate, a monofunctional analogue.

INTRODUCTION

Bifunctional reagents have been useful tools in the elucidation of protein structure[1,2]. It is possible that such cross-linking reagents could be used to study the configuration and spatial relationships of the protein components of biological membranes. Intact red blood cells after treatment with imidoester[3] or difluorodinitrobenzene[4] cross-linking reagents are resistant to hemolysis. It has been suggested[4] that this is due to a strengthening of the cell membrane by the cross-links formed.

A number of methods of fractionation of erythrocyte membrane proteins have recently been published[5–7]. The method employing aqueous pyridine[7] affords a rapid separation of the membrane proteins into two fractions. We have studied the effect of a cross-linking reagent, dimethyl adipimidate, on the subsequent separation of human erythrocyte membranes into these two fractions. These studies are of an exploratory nature to investigate the applicability of the cross-linking technique to a study of membrane structure.

MATERIALS AND METHODS

Human blood was collected using EDTA as anticoagulant and was used within 24 h. Membrane ghosts were prepared by the method of DODGE *et al.*[8] and were stored at 4° under a N_2 atmosphere until used, which was always within 3 days of preparation.

Dimethyl adipimidate was synthesized from adiponitrile (Aldrich), methanol, and anhydrous HCl (ref. 2). Methyl butyroimidate was similarly synthesized from butyronitrile (Aldrich). These reagents were used without recrystallization.

Sialic acid was assayed using *N*,*N*-dimethylaminobenzaldehyde[9]. In contrast to the method of WARREN[10], this method does not give a positive reaction with malonaldehyde, which may arise from peroxidation of the polyunsaturated fatty acids of the membrane phospholipids. Glucosamine and galactosamine were quantitated on the Spinco 120C amino acid analyzer after hydrolysis of the membrane fractions in 3 M HCl at 100° for 21 h. Hexose was quantitated with orcinol–H_2SO_4 as described by FRANCOIS *et al.*[11]. Protein was quantitated by the method of LOWRY *et al.*[12]. That this method was equally applicable to all membrane fractions was confirmed by amino acid analysis.

Amino acid analyses were performed on acid hydrolyzates prepared in 6 M HCl in sealed tubes at 110° for 21 h. The hydrolyzates were filtered, concentrated to dryness on a rotary evaporator, and analyzed with a Spinco 120C amino acid analyzer according to SPACKMAN *et al.*[13]. N^{ε},N^{ε}-Adipamidino-bis-L-lysine was analyzed as described by HARTMAN AND WOLD[2].

A monosubstituted derivative of adipimidate with one lysine residue may also be formed[2]. This derivative would be expected to be N^{ε}-4-carboxybutyroamidino-L-lysine (I) or its methyl ester (II)

$$\mathrm{ROOC{-}(CH_2)_4{-}\underset{\underset{\mathrm{NH_2^+}}{\|}}{C}{-}NH{-}(CH_2)_4{-}\underset{\underset{\mathrm{NH_3^+}}{|}}{CH}{-}COOH} \quad (\mathrm{I},\ R = H;\ \mathrm{II},\ R = CH_3)$$

Compound I was synthesized by treating α-*N*-acetyl-L-lysine[14] with a 4-fold excess of dimethyl adipimidate in methanol–water–triethylamine (10:1:1, by vol.). After 2.5 h at room temperature, the solvent was evaporated, the residue was refluxed with 6 M HCl for 2 h, and the solvent was evaporated. The residue was dissolved in 0.35 M $NaHCO_3$ (pH 7.5) and chromatographed on a cation exchange resin as described by HARTMAN AND WOLD[2]. A major ninhydrin-positive peak, which was eluted before N^{ε}, N^{ε}-adipamidino-bis-L-lysine, was acidified, desalted on Sephadex G-10 and dissolved in water. Attempts to crystallize the product were unsuccessful and its identity and purity were therefore established by chromatography, degradation and titration. The sample was judged to be homogeneous by thin-layer chromatography (methanol–water, 1:1, by vol.) and by high-voltage paper electrophoresis (1 M formic acid–0.02 M ammonium acetate). Further evidence for the identity of the derivative was obtained by titration using a Radiometer automatic recording titrator. Four p*K*'s were observed at pH 3.2, 4.6, 9.4 and about 12. The titration at each p*K* consumed 1 equiv of base. The concentration of the derivative was determined by Kjeldahl nitrogen determination. Base hydrolysis of the derivative (1 M NaOH, 110°, 2 h) liberated lysine and ammonia as the only ninhydrin-positive compounds. The derivative was chromatographed on the Spinco 120C amino acid analyzer by applying it to the long column and eluting with pH 5.28 buffer. Tyrosine was eluted after 50 min, phenylalanine after 57 min, *p*-methylphenylalanine (internal standard) after 84 min, and the derivative after 67 min. Under these same conditions, glucosamine was eluted after 110 min and galactosamine after 125 min.

N^{ε}-Butyroamidino-L-lysine was synthesized from methyl butyroimidate and

the copper complex of L-lysine. On a normal short column analysis on the Spinco 120C analyzer, it was eluted 4 min after arginine.

Procedure for modification of erythrocyte membranes

To 2 ml of a membrane suspension in distilled water (4–5.5 mg protein/ml) was added buffer (0.1 M NaCl–0.05 M Tris·HCl buffer (pH 9.6)) *plus* reagent (0.05 M dimethyl adipimidate–0.1 M NaOH (pH 9.6) or 0.1 M methyl butyroimidate–0.1 M NaOH (pH 9.6)) to yield 3 ml at the desired reagent concentration. After 30 min at room temperature, 6 ml of ice water were added and the mixture was centrifuged at 25000 × *g* for 10 min. The supernatant fluid, which contained 17–24% of the total protein was discarded*. The membrane pellet was resuspended in cold water to a volume of 2.0 ml and 1.0 ml of cold pyridine was added[7]. The mixture was immediately chromatographed on a 50-ml column of Sephadex G-25-coarse which completely removed the pyridine. The pyridine concentration of the effluent was monitored by measuring the absorption at 250 mμ. Less than 5 min were required for the chromatography. The turbid effluent containing the membrane components was collected and a portion was removed for analyses (total fraction). The remaining sample was centrifuged for 60 min at 105000 × *g*. The clear supernatant fluid was removed by aspiration and assayed for the various constituents (soluble fraction). The pellet was resuspended in distilled water by homogenization or sonication (insoluble fraction). In all samples, the protein content of the soluble *plus* insoluble fractions equalled that of the total fraction. In some samples, the content of sialic acid, hexosamine, and hexose was determined for both the soluble and insoluble fractions. The sum of these values equalled the value for the total fraction.

RESULTS AND DISCUSSION

Bifunctional imidoesters have been shown to cause the formation of covalently linked protein dimers, either between identical protein molecules, *e.g.* ribonuclease[2], or between different proteins, *e.g.* bovine serum albumin–human γ-globulin, ferritin–human γ-globulin[3]. Since certain of the various protein components of a biological membrane are very probably within a few Å of one another, formation of covalent cross-links between dissimilar protein molecules should be a likely occurrence.

Our results (Table I) are consistent with the formation of such cross-links. Modification of the membrane proteins with dimethyl adipimidate results in a progressive decrease in the percentage of the soluble membrane protein as the concentration of dimethyl adipimidate is increased. This is presumably due to the formation of cross-links between insoluble membrane proteins and proteins which normally would appear in the soluble fraction. The resulting cross-linked protein would be expected to be insoluble. Modification of the membrane proteins with a monofunctional reagent (methyl butyroimidate) resulted in a slight increase in the percentage of the membrane protein subsequently rendered soluble (Table I). This effect might be due to the slight detergent action of methyl butyroimidate in solution.

* Preliminary experiments did not reveal any significant differences in the relative contents of sialic acid, hexosamine, hexose, cholesterol, or lipid phosphorus between the untreated membranes and the insoluble fraction resulting from the alkaline treatment. The solubilized protein was not characterized.

TABLE I

EFFECT OF METYL BUTYROIMIDATE AND DIMETHYL ADIPIMIDATE MODIFICATION ON THE SUBSEQUENT SOLUBILIZATION OF MEMBRANE PROTEIN

Figures represent percent ± range of values (number of samples).

Reagent	*Concn. (mM)*	*Lysine modified* (%)*	*Protein solubilized (soluble fraction per total fraction × 100)*	*Protein solubilized (% of control)*
Control	0	0	40 ± 7 (8)	100
Methyl butyroimidate	0.67	13 (1)	39 (1)	100
Dimethyl adipimidate	0.33	5 ± 2 (2)	32 ± 1 (2)	80
Methyl butyroimidate	1.33	22 ± 6 (3)	39 ± 3 (2)	100
Dimethyl adipimidate	0.67	18 ± 4 (3)	25 ± 4 (3)	60
Methyl butyroimidate	2.00	32 ± 8 (5)	45 ± 6 (5)	110
Dimethyl adipimidate	1.00	33 ± 4 (5)	24 ± 3 (5)	60
Methyl butyroimidate	2.67	48 (1)	48 (1)	120
Dimethyl adipimidate	1.33	39 ± 4 (2)	22 ± 3 (2)	55
Methyl butyroimidate	3.33	51 ± 3 (2)	48 ± 1 (2)	120
Dimethyl adipimidate	1.66	48 ± 5 (3)	21 ± 3 (3)	50

* This value represents the difference between lysine residues in the control and experimental samples, normalized with respect to the arginine contents. The percentage of lysine modified could be accounted for by the appearance of N^{ε}-butyroamidino-L-lysine in the methyl butyroimidate-treated samples, and by the appearance of $N^{\varepsilon}, N^{\varepsilon}$-adipamidino-bis-L-lysine and N^{ε}-4-carboxybutyroamidino-L-lysine in the dimethyl adipimidate-treated samples.

The condensation product with methyl butyroimidate was identified as N^{ε}-butyroamidino-L-lysine by amino acid analysis. This derivative accounted for the decrease in lysine content, within the error of the analysis.

Two condensation products are formed with dimethyl adipimidate. The cross-linked derivative, $N^{\varepsilon}, N^{\varepsilon}$-adipamidino-bis-L-lysine, was identified and quantitated as described by HARTMAN AND WOLD[2]. This product accounts for about 20% of the total number of lysine residues modified. The remainder of the modified lysine residues have presumably been modified monofunctionally, yielding either N^{ε}-4-carboxybutyroamidino-L-lysine (I) or, more probably its methyl ester (II). After acid hydrolysis the derivative cochromatographed with N^{ε}-4-carboxybutyroamidino-L-lysine on the amino acid analyzer. This derivative was not quantitated for all the samples, but in representative samples accounted for the remainder of the lysine deficiency of the sample.

The pyridine-solubilized fraction from untreated erythrocyte membranes has been reported to contain all of the membrane sialic acid, in addition to hexose and hexosamine[7]. Since these compounds are presumably bound to the proteins by covalent linkages, they would be expected to be transferred to the insoluble fraction, along with protein, as a result of intermolecular cross-linking. These substances were therefore measured in the total membrane fraction and in the soluble membrane fraction of untreated, monofunctionally modified, and cross-linked membranes (Table II). A smaller percentage of each component is found in the soluble fraction of the cross-linked membranes, corresponding to the smaller percentage of soluble protein in these samples. Although the quantitative recoveries of the insoluble fractions were more difficult, all these compounds were present in greater quantities

TABLE II

EFFECT OF CROSS-LINKING ON THE DISTRIBUTION OF MEMBRANE COMPONENTS AFTER SOLUBILIZATION

Figures represent percent ± range of values (number of samples).

Reagent	*Concn. (mM)*	*Soluble fraction per total fraction × 100*				
		Protein	*Sialic acid*	*Hexose*	*Glucos-amine*	*Galactos-amine*
Control	0	47 ± 2 (4)	70 ± 5 (4)	46 ± 1 (2)	24 ± 3 (2)	49 ± 1 (2)
Methyl butyroimidate	2.0	51 ± 2 (4)	78 ± 10 (4)	29 ± 1 (2)	35 ± 1 (2)	57 ± 1 (2)
Dimethyl adipimidate	1.0	22 ± 2 (4)	46 ± 1 (4)	16 ± 2 (2)	15 ± 0 (2)	31 ± 2 (2)

in the cross-linked samples relative to the control samples. In contrast to the results of BLUMENFELD[7], in all the untreated samples studied here, about 30% of the total sialic acid was found in the insoluble fraction. This difference may well be due to the use of Sephadex rather than prolonged dialysis to remove the pyridine. Also, using the assay procedure of WARREN[10], one must correct for the malonaldehyde produced by peroxidation of the unsaturated fatty acids present in the phospholipids of the insoluble fraction.

If specific protein components of the soluble fraction are cross-linked in a non-random fashion to insoluble proteins, one might expect an unequal redistribution of sialic acid, hexose, glucosamine, and galactosamine to the insoluble fraction. Although the distribution of hexose is different than that of the other compounds, much more precise data are required to investigate this possibility fully.

Intact red blood cells were also treated with dimethyl adipimidate (0–8 mM) at pH 9.0 for 15 min in isotonic solution. In agreement with other observations, erythrocytes treated with dimethyl adipimidate above 1.5 mM were not subject to hemolysis by distilled water, detergents, or sonication. It has been suggested that this resistance to hemolysis was due to an increase in the strength of the membrane due to cross-linking[4]. To investigate this possibility, red cells were treated with 1.0 mM dimethyl adipimidate, after which they would still undergo osmotic lysis. The cells were then lysed and the membrane fraction was washed repeatedly. Amino acid analysis was performed on the soluble protein (hemoglobin) and on the insoluble protein (membrane). There was no significant difference in the percentage of the lysines modified in these two fractions. The hemoglobin did not exhibit normal solubility properties, but tended to form a gel upon standing in dilute aqueous solution. The increased resistance of the erythrocytes to lysis is therefore likely due to two factors — cross-linking of the membrane, and the formation of an insoluble hemoglobin derivative by intermolecular cross-linking.

The effect of cross-linking on the physiological properties of the red cell was also investigated. Human red cells were treated with dimethyl adipimidate as above at concentrations ranging from 0 to 8 mM, washed and suspended in isotonic Tris–NaCl buffer (pH 7.4). To determine the glycolytic activity, the cells were incubated in a medium containing 2.5 mM glucose[15], and the glucose concentration was measured at 30-min intervals over a 2-h period. The rate and extent of glucose utilization by the modified cells was not significantly different from that of unmodified cells. A semiquantitative measure of O_2 binding by modified and unmodified cells was

performed. Deoxygenation was effected by stirring a suspension of cells under a stream of N_2 for 30 min. The extent of deoxygenation was estimated by measuring the difference spectrum of the N_2-treated cells *versus* O_2-treated cells, between 500 and 600 mμ. The difference spectra for cells treated with dimethyl adipimidate at concentrations below 0.8 mM were indistinguishable from the difference spectra for unmodified erythrocytes. Cells treated with dimethyl adipimidate at concentrations above 0.8 mM showed no peaks in the difference spectra. This indicated that O_2 was not released from the hemoglobin by treatment with N_2, even after several hours. The absorption spectra of these cells treated at the higher dimethyl adipimidate concentrations were identical with those of unmodified, oxygenated erythrocytes.

Dimethyl adipimidate would be expected to react with all the proteins within the erythrocyte to a similar extent. However, the overall rate of glycolysis is unaffected by dimethyl adipimidate treatment at concentrations which completely inactivate the hemoglobin. Further studies will be performed to characterize these inactive hemoglobin derivatives.

CONCLUSIONS

These results provide strong evidence for the formation of covalent cross-links between dissimilar protein components of human erythrocyte membranes. The fractionation method which was employed, yielding only two membrane fractions, did not enable us to characterize individual cross-linked dimers. More selective separation methods, which allow the isolation of homogeneous membrane proteins, are required. The use of cross-linking reagents, such as dimethyl adipimidate, should then provide a great deal of information regarding the spatial relationships of membrane proteins.

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