Journal of Supramolecular Structure 8:303–310 (1978)

Membrane Permeability of Bifunctional, Amino Site-Specific, Cross-Linking Reagents

Tokichi Miyakawa, Larry J. Takemoto, and C. Fred Fox

Department of Microbiology and the Molecular Biology Institute, University of California, Los Angeles, California 90024

The membrane permeability of a series of reversible cross-linking reagents which are diazide tartarate derivatives has been compared with that of dimethyl-3,3′-dithiobispropionimidate (DTBP). The diazide tartarate derivatives tested include tartryl-diazide (TDA), tartryl-di(glycylazide) (TDGA), tartryl-di(β-alanylazide) (TDAA), tartryl-di-(γ-aminobutyrylazide) (TDBA), tartryl-di (ε-aminocaproylazide) (TDCA). TDA, which has the shortest chain length of the diazide tartarate derivatives tested, proved to be readily permeable through the erythrocyte membrane. When added at equal concentration to unsealed ghosts, TDGA was at least as reactive as DTBP in its ability to cross link the internally displayed proteins 1, 2, 4.1, 4.2, and 6. Treatment of resealed ghosts by DTBP produced oligomeric complexes of these proteins plus apparent homooligomeric complexes of hemoglobin. TDGA at the same concentrations did not cross-link any of these components, indicating its membrane-impermeable nature. As the chain length of the homologous series increased from TDGA to TDCA, the cross-linkers became increasingly permeable through the erythrocyte membrane.

Key words: **membrane permeability, cross-linking reagents, erythrocyte membrane, tartryl-di(glycylazide), dimethyl-3,3′-dithiobispropionimidate**

Reversible, bifunctional cross-linking reagents can be powerful tools in assessments of neighbor protein interactions in membranes. Early experiments by Steck [1], who used glutaraldehyde to cross-link human erythrocyte membrane proteins, were the first to indicate the likely existence of oligomeric protein complexes in membranes. Cross-linking

The abbreviations for DTBP, TDA, TDGA, TDAA, TDBA and TDCA are given in Table I. Other abbreviations are PBS, 150 mM sodium chloride in 5 mM sodium phosphate (pH 8.0); NEM, N-ethylmaleimide; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane.

Received for publication May 9, 1978; accepted May 9, 1978.

Tokichi Miyakawa is now a member of the Faculty of Engineering, Hiroshima University, 3-8-12, Sendamachi, Hiroshima City, Japan, 730.

0091-7419/78/0803-0303$01.70 © 1978 Alan R. Liss, Inc

reagents which are not readily reversible, however, are of limited value, since it is often not possible to identify the component parts of the cross-linked oligomeric complexes. Wang and Richards [2, 3] were the first to employ a readily dissociable cross-linker in membrane studies. In an elegant series of experiments, they employed dimethyl-3,3′-dithiobispropionimidate (DTBP) in conjunction with two-dimensional SDS polyacrylamide gel electrophoresis to identify the nearest-neighbor associations of proteins in the human erythrocyte membrane. DTBP, which has also proved useful in the assessment of nearest-neighbor protein interactions in membrane-enveloped viruses [4, 6] has proved to be membrane-permeable in all membrane systems for which its use has been reported. Its use, for example, leads to the formation of homooligomers of polypeptides of hemoglobin and various hemoglobin-membrane protein complexes with intact red cells [5]. DTBP does, however, suffer from one distinct disadvantage: It cannot be used when reducing conditions are required in the first dimension of a two-dimensional gel electrophoresis system. This led us to investigate the applicability of the series of diazide tartarate derivate cross-linkers initially developed by Lutter, Ortanderl, and Fasold [7]. Acyl azide groups, like alkylimidate groups, are amino site-specific. Unlike the products formed during cross-linking with DTBP, however, these cross-linked oligomers once formed cannot be disrupted by reducing reagents. Instead, the vicinal hydroxyl center of these reagents is cleaved by oxidation with periodate [7]. Our first applications of one reagent of this type, TDGA (Table I), produced unanticipated results. TDGA should produce oligomers similar to those produced by DTBP; both are site-specific for amino groups and both have approximately the same distance between the reactive site-specific groups. The initial results suggested to us that TDGA might not be membrane-permeable, a hypothesis substantiated by the tests reported here. Having established the membrane-impermeable nature of TDGA, we also felt that a characterization of other tartryl diazides might be indicated, since not all members of the diazide tartarate derivative series might share this property. In this report, we provide a characterization of the permeability properties of diazide tartarate derivatives of maximal cross-linking separation capability varying from 6 to 23 Å.

METHODS

Preparation of Erythrocyte Ghosts

Sealed right-side-out ghosts and unsealed ghosts were prepared from freshly drawn, heparanized (50 units/ml) blood as described by Steck [8]. Whole blood (10 ml) was mixed with 20 ml of PBS, and the cells were sedimented at 1,000g for 10 min at 4°C. The supernatant solution and buffy coat were decanted, and the red cells were washed twice by suspension in 30 ml of PBS and sedimentation. Cells were then lysed by suspension in 150 ml of ice-cold 5 mM sodium phosphate buffer at pH 8.0, and the membrane ghosts were sedimented at 12,000g for 15 min at 4°C. The supernatant solution was removed by aspiration, leaving the loose ghost sediment. The tube was then rotated to separate the ghost sediment from the tightly packed "button." The firm pellet ("button") was removed by aspiration. The sediment was divided into two equal portions of 2 ml each to prepare sealed and unsealed ghosts. Unsealed ghosts were prepared by washing one portion of the sediment twice at 4°C by suspension in 60 ml of 5 mM sodium phosphate buffer at pH 8.0 and centrifugation as described above until a clean white pellet was obtained. Sealed ghosts were prepared by suspending the second portion of the crude ghost sediment in 60 ml of PBS prewarmed to 37°C. The suspension was kept at 37°C for 60 min and then

centrifuged at 12,000g for 15 min at 4°C. The sealed ghosts were washed twice more by suspension with 60 ml of cold PBS and centrifugation.

Cross-Linking Reagents

The cross-linking reagents used in the study are listed in Table I. The chemical nomenclatures, abbreviations, formulas, distances between the two functional groups, and the melting points, – both observed and literature values – are given. DTBP was prepared by the method of Ruoho et al. [9]. DTBP solutions were made in PBS and adjusted to pH 8.0 with 1 N NaOH immediately before use. The diazide tartarate derivative cross-linkers (TDA, TDGA, and TDCA) were prepared as described by Lutter, Ortanderl, and Fasold [7]. TDAA and TDBA were prepared by the procedures used for TDCA synthesis except that β-alanine ethyl ester and γ-amino-butyric acid ethyl ester were employed, respectively, in place of ϵ-aminocaproic acid ethyl ester. The diazide cross-linkers were prepared by activating the corresponding dihydrazide precursors immediately before use. The procedures for activation were basically the same as described by Lutter, Ortanderl, and Fasold [7], except for the following changes. The dihydrazide precursors were dissolved in 0.75 ml of 1 N HCl to make up 5 ml of reagent solutions for all the reagent concentrations used. Sodium phosphate was used at the final concentration of 5 mM instead of triethanolamine. The excess HCl was neutralized, and the pH was adjusted to 8.0 with 5 N NaOH instead of 5 N KOH.

TABLE I. List of Cross-Linking Reagents Used.

Abbreviation	Name of crosslinker	Formula	Length (Å)	Melting point[a] (°C)	
				Observed	Literature
DTBP	Dimethyl-3,3'-dithiobispropionimidate dihydrochloride	$\left(\begin{matrix}H_3O\\ \ominus\ \oplus\\ ClH_2N\end{matrix}\!\!\!\!\!>C-CH_2-CH_2-S-\right)_2$	11.0	174–176	175–176[b] 174–176[c]
TDA	Tartryl-diazide	$\left(N_3-\overset{O}{\overset{\Vert}{C}}-\overset{OH}{\overset{\vert}{C}}-\right)_2$	6.0	184–186	183[d]
TDGA	Tartryl-di-(glycylazide)	$\left(N_3-\overset{O}{\overset{\Vert}{C}}-CH_2-NH-\overset{O}{\overset{\Vert}{C}}-\overset{OH}{\overset{\vert}{C}}-\right)_2$	13.0	175–178	176–178[d]
TDAA	Tartryl-di-(β-alanylazide)	$\left(N_3-\overset{O}{\overset{\Vert}{C}}-(CH_2)_2-NH-\overset{O}{\overset{\Vert}{C}}-\overset{OH}{\overset{\vert}{C}}-\right)_2$	15.5	172–174	–
TDBA	Tartryl-di-(γ-aminobutyrylazide)	$\left(N_3-\overset{O}{\overset{\Vert}{C}}-(CH_2)_3-NH-\overset{O}{\overset{\Vert}{C}}-\overset{OH}{\overset{\vert}{C}}-\right)_2$	18.0	170–172	–
TDCA	Tartryl-di-(ϵ-aminocaproylazide)	$\left(N_3-\overset{O}{\overset{\Vert}{C}}-(CH_2)_5-NH-\overset{O}{\overset{\Vert}{C}}-\overset{OH}{\overset{\vert}{C}}-\right)_2$	23.0	163–165	164–166[d]

[a]Melting points given for diazide tartarate derivatives (TDA, TDGA, TDAA, TDBA, TDCA) are those of corresponding dihydrazide precursors.
[b]Wang and Richards [2].
[c]Ruoho et al [9].
[d]Lutter Ortanderl, and Fasold [7].

Chemicals

NEM, β-alanine, γ-aminobutyric acid, ε-aminocaproic acid, and Coomassie brilliant blue R were obtained from Sigma Chemical Co, St Louis. Acrylamide, N,N′-methylene-bisacrylamide, 2-mercaptoethanol, diethyltartarate, hydrazine hydrate, and glycine ethylester hydrochloride were obtained from Eastman Organic Chemicals, Rochester, New York. Tris was obtained from Fisher Scientific Co, Tustin, California. Sodium metaperiodate, thiourea, and glycine were obtained from J.T. Baker, Philipsberg, New Jersey. Hydrogen chloride was obtained from Matheson Gas Products, Lyndhurst, New York.

SDS Polyacrylamide Gel Electrophoresis

The discontinuous system described by Laemmli [10] was used. The 1.0-mm-thick slab gel consisted of a 10-cm-long resolving gel and a 1.2-cm-long stacking gel. Samples were prepared in buffer containing 62.5 mM Tris-HCl, 3% SDS, 30 mM NEM, and 10% sucrose at pH 6.8 and were solubilized by boiling for 1 min before electrophoresis.

Protein Determination

Proteins were estimated by the method of Lowry et al [11] using a bovine serum albumin standard.

RESULTS

Initially, intact erythrocytes were incubated for 30 min at 21°C with 10 mM DTBP or 10 mM TDGA. Analysis by SDS-PAGE of ghosts prepared from these cells indicated extensive cross-linking of hemoglobin and internally displayed membrane components after treatment with DTBP, but not after treatment with TDGA (data not shown). These results, although suggesting the possibility that TDGA was membrane-impermeable, were complicated by the presence of bulk quantities of cytoplasmic substances in the intact cell. Some of these, such as glutathione, could conceivably act as a "sink" by competing with hemoglobin for small amounts of TDGA that might cross the membrane.

To more rigorously test the permeability of TDGA, resealed ghosts devoid of at least 98% of the cytoplasmic proteins were prepared by the method described by Steck [8]. Figure 1 demonstrates that both sealed and unsealed ghosts showed similar changes in membrane protein gel profiles with increasing DTBP concentration – basically a large decrease in intensities of bands 1, 2, 4.1, and 6 and a smaller decrease in bands 3 and 7. Since bands 1, 2, 4.1, 6, and 7 are exclusively disposed on the inner membrane surface [12], these results confirm the membrane-permeable properties of DTBP. A similar decrease in the intensities of these bands both in unsealed and resealed ghosts, accompanied by an increase in high-molecular-weight oligomers in the stacking and resolving gels, strongly suggests that decreased intensities are a result of cross-linking and not of elution of protein from the cytoplasmic membrane surface. Among the oligomeric complexes formed in re-sealed ghosts at high (5 mM) DTBP concentrations were apparent dimers, trimers, and tetramers of hemoglobin polypeptides. The extent of cross-linking of membrane proteins was even greater in sealed ghosts than in unsealed ghosts when both were compared at the same reagent concentrations. This may be due to the cross-linking of hemoglobin to the membrane proteins, a phenomenon observed after DTBP treatment of intact erythrocytes [5].

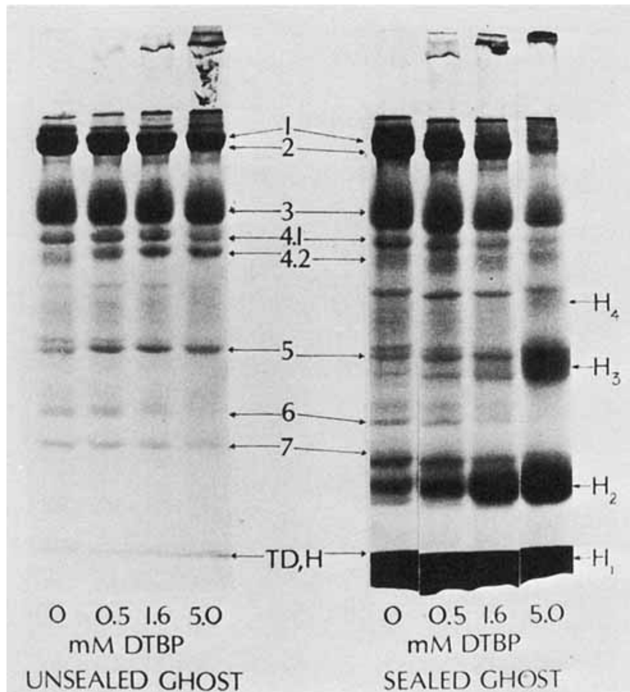

Fig 1. Cross-linking of proteins in sealed right-side-out and unsealed erythrocytes by DTBP. Resealed and unsealed ghosts were prepared as described in Materials and Methods. To 5 ml of DTBP solution made up in PBS, 150 μl (600 μg protein) of unsealed ghost or 100 μl of resealed ghost (1,200 μg protein) were added and mixed well. Incubations were for 30 min at 21°C with gentle rotary agitation. A 250-μl sample of 1 M ammonium acetate was added to terminate each reaction [3], and the membranes were sedimented at 12,000g for 15 min. Membrane pellets were dissolved in 0.3 ml of electrophoresis sample buffer containing 30 mM NEM, and aliquots of 35 μl were subjected to slab gel SDS-PAGE. Nomenclature of erthrocyte proteins was according to Fairbanks, Steck, and Wallach [20].

Figure 2 demonstrates that resealed ghosts treated with 0.5, 1.6, and 5.0 mM TDGA have gel profiles identical to those of the untreated controls, whereas treatment of unsealed ghosts at the same TDGA concentrations produced significant cross-linking of bands 1, 2, 3, 4.1, 6, and 7. It is possible that the disappearance of these bands upon TDGA treatment of unsealed, but not sealed ghosts, might arise from elution of the proteins rather than from cross-linking. Low concentrations (2 mg/ml) of the amino site-specific reagent 2,3-dimethylmaleic anhydride have been previously shown to selectively elute bands 1, 2, 4.1, 4.2, 5, and 6 [13]. However, the increase in protein not entering the stacking or resolving gels, which is noted when unsealed but not sealed ghosts are treated with TDGA, is a firm indication of the occurrence of protein cross-linking in our experiment. Hence, the ability of DTBP and TDGA to cross-link these proteins in unsealed ghosts indicates their similar reactivities toward protein amino groups and further supports the initial conclusion that the inability of TDGA to cross-link erythrocyte membrane proteins must be due to its membrane-impermeability.

TDGA extensively cross-linked a transmembrane protein, band 3 of erythrocytes, only when unsealed membrane ghosts were incubated with the reagent (Fig 2). No significant cross-linking of the protein was observed when sealed membrane ghosts (Fig 2) were

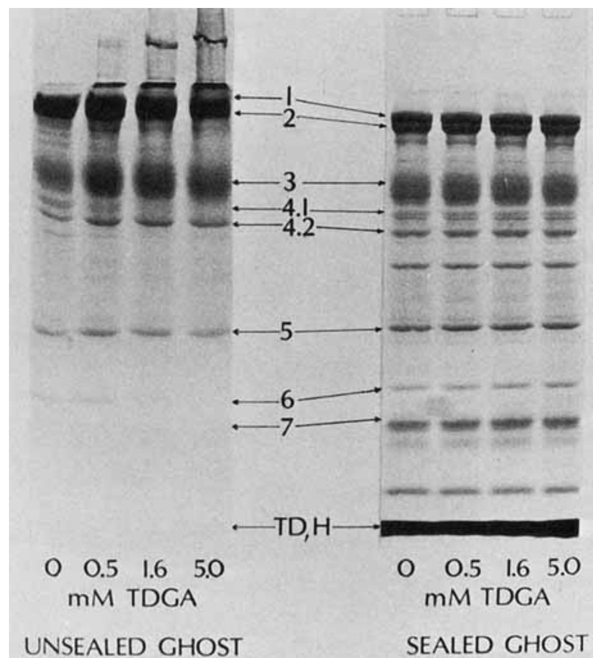

Fig 2. Cross-linking of proteins of right-side-out sealed and unsealed erythrocyte ghosts by TDGA. Experimental procedures were the same as those described in the legend to Figure 1 except that TDGA was used in place of DTBP.

treated. The cross-linked product of band 3 protein in the TDGA-treated unsealed membrane ghosts was found to be mainly the homodimer of this protein, through experiment using the technique of two-dimensional diagonal electrophoresis (data not shown; for the details of the technique, see Takemoto, Miyakawa, and Fox [14]). Since this protein is exposed on both sides of the membrane [12, 15, 21], this suggests that band 3 proteins are intermolecularly cross-linkable by this amino site-specific reagent exclusively at sites on band 3 protein accessible only at the cytoplasmic side of the membrane, an observation previously described after formation of intermolecular disulfide bonding by treatment of erythrocyte ghosts with o-phenanthroline-Cu^{2+} complex [16].

A series of diazide tartarate derivatives was tested to see whether the member-impermeable characteristic of TDGA is an exceptional or a general property of reagents of this group. The derivatives tested had bridging length distances between reactive acylazide groups ranging from 6.0 Å (TDA) to 23.0 Å (TDCA), and all members of this series except TDA differed only in the number of methylene carbons separating the reactive ends from the diol center (Table I). Each reagent was tested to determine the concentration required to obtain the same approximate amount of cross-linking products with unsealed erythrocyte ghosts as were formed with 5 mM TDGA (Fig 2), a concentration at which TDGA was totally ineffective in cross-linking proteins of intact erythrocytes or resealed ghosts. The reagents were then retested for their cross-linking capabilities with sealed erythrocyte ghosts (Fig 3). Under these conditions, TDA was the most effective cross-linker of proteins localized inside the membrane barrier. The effectiveness of TDA at 15 mM is unlikely to arise from an artifact such as destruction of the membrane barrier

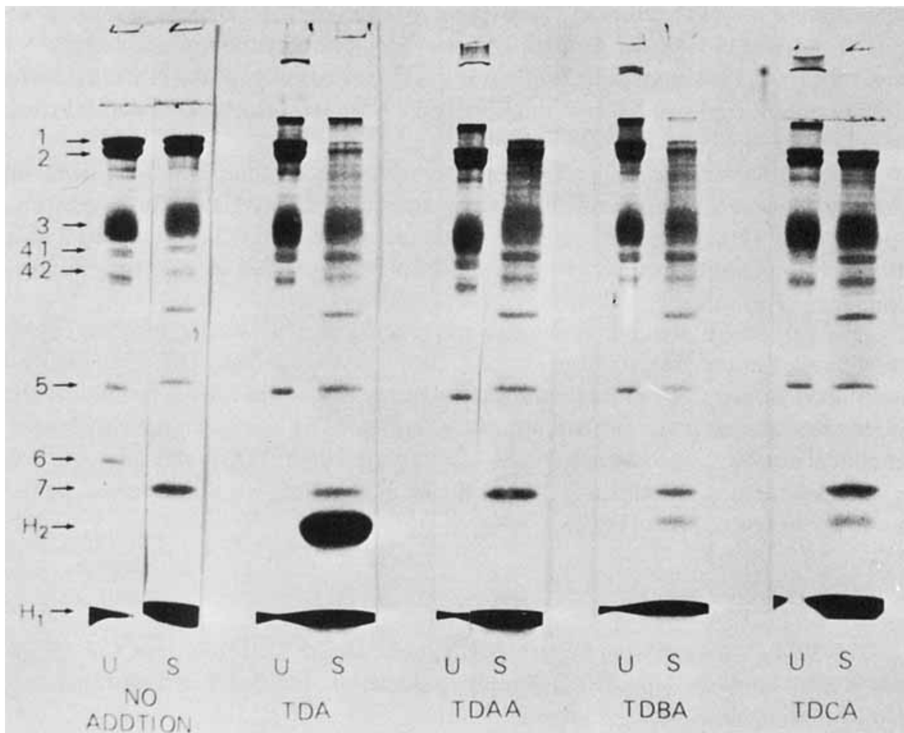

Fig 3. Cross-linking of proteins of sealed and unsealed ghosts by diazide tartarate derivatives with different cross-linking lengths. Experimental procedures were the same as those described in the legend to Figure 2 except that TDA, TDAA, TDBA, TDCA were employed at concentrations of 15 mM, 1.5 mM, 0.5 mM, and 0.05 mM, respectively. U) unsealed ghosts; S) sealed ghosts.

in sealed ghosts, since TDGA was not effective in cross-linking proteins of sealed ghosts even at this high concentration (data not shown). As indicated by the appearance of the hemoglobin dimer band, the members of the homologous series proceeding from TDGA to TDCA are increasingly effective in breaching the membrane barrier as the chain length, and thus the apolar nature of the cross-linkers, increases. It is noteworthy in this regard that only 0.05 mM TDGA was as effective as 1.5 mM TDAA in producing equivalent cross-linking of unsealed ghosts.

DISCUSSION

Of all the amino site-specific cross-linking reagents tested for membrane impermeability, only one, TDGA, appears to be essentially membrane-impermeable. This property does not appear to be associated solely with presence of the highly polar vicinal hydroxyl groups present on this reagent since tartryl diazide (TDA), the simplest member of the diazide tartarate derivative series, is readily membrane-permeable. The possibility exists, however, that TDA may have an affinity for a transport system sufficient to permit its facilitated passage through the red cell membrane barrier. Should this be the case, cross-linking of hemoglobin in intact red cells by TDA might have a temperature coefficient higher than that of other membrane-permeable crosslinkers, a possibility we have not tested.

Dubovi and Wagner [17], however, have recently reported that TDA can give rise to cross-linking of proteins in vesicular stomatitis virus (VSV), but they did not exclude the possibility that the initial cross-linking process occurred entirely on the extraviral surface, disrupting overall viral structure, or the VSV had a cell surface membrane-derived carrier protein capable of facilitating TDA transport.

Members of the diazide tartarate derivative series higher than TDGA are increasingly membrane permeable in direct relation to increasing molecular weight. This suggests that the property of TDGA which imparts its membrane-impermeable character is its hydrophilicity, since the molecular weight increase above TDGA in this series is entirely due to the presence of additional methylene residues (Table I).

A variety of membrane-impermeable site-specific protein-labeling reagents has been broadly applied in the assessment of sidedness of membrane protein [18, 19]. TDGA may prove to have similar value in establishing sidedness relationships for the functional groups involved in nearest-neighbor protein interactions revealed by amino site-specific homobifunctional cross-linking reagents. In one case reported here, TDGA was shown to cross-link band 3 protein of the erythrocyte membrane in unsealed, but not in sealed, right-side-out erythrocyte ghosts (Fig 2).

ACKNOWLEDGMENTS

This study was supported in part by US Public Health Research grant GM-18233 and by a grant from the Muscular Dystrophy Association, Inc. L.J.T. is a postdoctoral fellow of that organization.

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