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# INTRA- AND INTERMOLECULAR CROSS-LINKING OF MEMBRANE PROTEINS IN INTACT ERYTHROCYTES AND GHOSTS BY SH-OXIDIZING AGENTS

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## Summary

In intact human erythrocytes, SH-oxidizing agents exclusively cross-link spectrin via disulfide bonds. In ghosts, additional dimerization of the major intrinsic protein, band 3, is observed. After blockade of intracellular GSH the agents dimerize band 3 in the intact cell too, indicating that GSH may prevent band 3 dimerization under physiological conditions. The oxidizing agents reversibly oxidize 80% of the membrane SH-groups, suggesting that these groups are arranged close enough to each other to form disulfide bonds. This arrangement may protect other cell structures against free radicals or oxidative stress.

In the human erythrocyte membrane, cross-linking between the two components of spectrin (band 1 and 2 in SDS-gel electrophoresis [1]) and covalent dimerisation of band 3, the major intrinsic protein fraction (apparent molecular weight 100 000), has been observed [1—3]. The formation of such covalent dimers may indicate a noncovalent dimeric assembly of band 3 in the membrane, a view supported by the demonstration of noncovalent self-association of band 3 in vitro [4]. These cross-linking studies involved formation of intermolecular disulfide bonds by $Cu^{2+}$ · *o*-phenanthroline or cross-linking of amino groups by a bifunctional imidoester [1—3]. The latter reagent could be used with intact cells due to its high membrane permeability. Considerable bonding, however, between globin and membrane proteins greatly impeded the evaluation of the cross-linking pattern [3]. $Cu^{2+}$ · *o*-phenanthroline, on the other hand, produces intermolecular disulfide bonds only in leaky erythrocyte ghosts, due to the $Cu^{2+}$-impermeability of the intact erythrocyte.

In order to obtain further information on nearest neighbour relationships of membrane proteins in intact cells, cross-linking studies were performed

with two permeable [5,6] SH-oxidizing reagents, diamide and tetrathionate, in intact erythrocytes and erythrocyte ghosts.

Freshly obtained human erythrocytes, washed three times with 154 mM NaCl, and resealed ghosts prepared according to Lepke and Passow [7], were suspended in 10 vols. of a medium containing (mM) KCl 90, NaCl 45, $NaH_2PO_4/Na_2HPO_4$ 10 and sucrose 44, unsealed ghosts [8] in 10 vols. of a 10 mM sodium phosphate buffer. Erythrocytes and ghosts were treated at 37°C and pH 8.0 with diamide (diazine dicarboxylic acid bisdimethylamide (Calbiochem)), or sodium tetrathionate ($Na_2S_4O_6$, Fluka) or $Cu^{2+}$ · *o*-phenantroline. A 15 min incubation of leaky ghosts with each of these SH-oxidizing agents resulted in a decrease of the membrane protein SH-groups to 20—30% of the original value (data not shown). Simultaneously, considerable changes in the gel electrophoretic pattern of the membrane polypeptides became evident (Fig. 1A). The intensities of bands 1, 2 and 3 diminished and two new bands (marked by arrows) appeared, one on top of the gel, the other between bands 2 and 3. On the basis of its position in the gel, an apparent molecular weight of 180 000 can be assigned to the latter band, which has been shown [1,2] to represent dimers of band 3 polypeptides. The band on top of the gel may be assumed to consist at least to a large extent of oligomers of bands 1 and 2, since the intensities of other bands did not diminish to a notable extent (cf. also ref. 2). Similar results were obtained for resealed

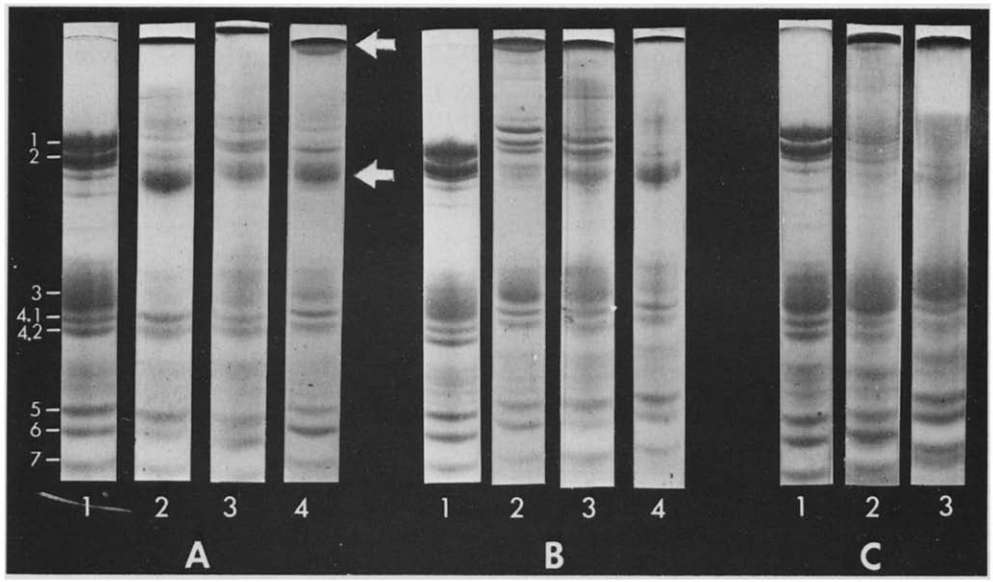



**Fig. 1. Intermolecular cross-linking of membrane proteins by treatment of ghosts and erythrocytes with SH-oxidizing agents. Leaky [8] or resealed [7] ghosts and intact erythrocytes, treated with SH-oxidizing agents were washed and the membranes isolated. Electrophoresis of the SDS-solubilized membranes was performed on 5.0% acrylamide/0.1% bis-acrylamide gels in 0.2% SDS according to ref. 9, but without reducing agents. 60—80 μg protein per gel. Gels were stained with Coomassie Blue. A and B: membrane polypeptides derived from leaky (A) and resealed (B) ghosts incubated for 15 min with (1), no addition; (2), $Cu^{2+}$ · *o*-phenanthroline, (0.01 mM and 0.05 mM, respectively); (3), tetrathionate (1 mM); (4), diamide (1 mM). C: membrane polypeptides from intact erythrocytes incubated with (1), no additives; (2), diamide (60 min, 5 mM); (3), diamide (120 min, 5 mM).**

ghosts treated with tetrathionate and diamide (Fig. 1B), while $Cu^{2+}$·*o*-phenanthroline had less pronounced effects, particularly on band 3. This difference can be explained by a low $Cu^{2+}$ permeability of the resealed ghost membrane. Treatment of intact erythrocytes with diamide or tetrathionate revealed a more complex situation: both reagents pass the membrane as indicated by a complete oxidation of intracellular GSH (Table I and refs. 5 and 6). Furthermore, they lower the SH-content of the membrane proteins to 25% of its initial value (Table I). Moreover, the spectrin bands disappear under appearance of a new band on top of the gel (Fig. 1C). These effects are reversible upon treatment of the membranes with sulfite or dithioerythritol (Table I and Fig. 2). In contrast to spectrin, band 3 is not crosslinked by diamide (and tetrathionate) in the intact erythrocyte.

The absence of dimerization of band 3 after exposure to the agents under native conditions might indicate a spatial separation of the monomeric units, but could also be due to the absence of favourable reaction conditions. Such unfavourable reaction conditions could result, e.g., from the presence of GSH in the intact erythrocyte. Iodoacetate pretreatment, which depletes the cells of GSH, had almost no effect on the membrane protein SH-groups (Table I). Subsequent exposure to diamide, produced dimerization of band 3 (Fig. 3), which was, however, less pronounced than that observed in ghost membranes (cf. Fig. 1A).

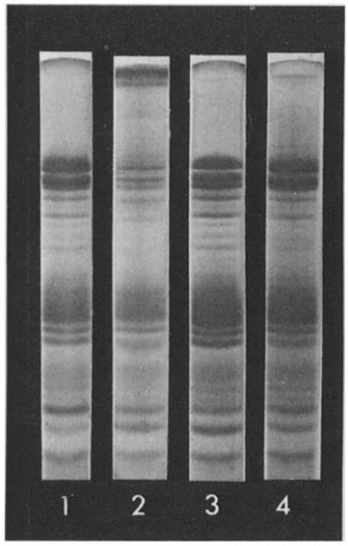
Iodoacetate-induced "sensitation" of band 3 to diamide-catalysed dimerization may be interpreted by two assumptions: on the one hand, diamide alone could lead to a predominant formation of disulfide bonds between GSH and SH-groups of band 3, precluding dimerization. Diamide-induced formation of asymmetric disulfides has previously been demonstrated for hemoglobin and GSH [13] as well as erythrocyte membrane proteins and GSH (Haest, unpublished results).

TABLE I

DECREASE OF INTRACELLULAR GSH AND MEMBRANE SH-GROUPS IN INTACT ERYTHROCYTES TREATED WITH SH-REAGENTS

Cells were incubated with: iodoacetate (10 mM), tetrathionate (20 mM) or diamide (5 mM) and washed 3 times. The GSH content was determined according to Beutler et al. [10], membrane SH-groups by the method of Habeeb [12] and membrane protein according to Lowry [11]. In order to substantiate the formation of disulfide bonds by diamide, erythrocytes treated with this agent were subsequently exposed to sulfite (30 mM) or dithioerythritol (10 mM). Note the reincrease, after these treatments, of the membrane SH-groups to the theoretically expected values.

| Cells treated with | Time of exposure (min) | Membrane SH-groups remaining (%) | GSH (%) |
|---|---|---|---|
| No additive | 0 | 100 | 100 |
| Iodoacetate | 15 | 90—95 | 0 |
| Tetrathionate | 60 | 35—40 | 10 |
| Tetrathionate | 120 | 25—30 | <5 |
| Diamide | 60 | 25—30 | 0 |
| Diamide | 120 | 20—25 | 0 |
| Iodoacetate | 15 | — | — |
| then diamide | 60 | 20 | 0 |
| Diamide | 60 | — | — |
| then sulfite | 60 | 60 | — |
| Diamide | 60 | — | — |
| then dithioerythritol | 30 | 100 | — |



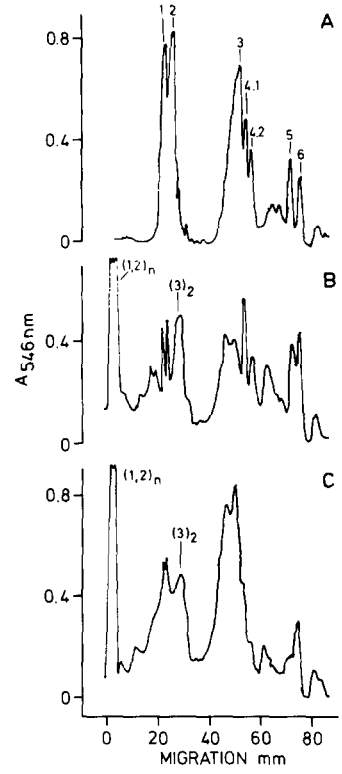




**Fig. 2. Reversibility of the diamide induced intermolecular cross-liriking of spectrin. Erythrocytes were incubated with 5 mM diamide (30 min, 37°C, pH 8.0) and washed 3 times. Membranes isolated [8] from the cells were treated (37°C, pH 8.0) with Na-sulfite (60 min, 30 mM) or dithioerythritol (30 min, 5 mM), solubilized in SDS and subjected to electrophoresis. See Fig. 1 for further details. (1), control; (2), diamide; (3), diamide, then dithioerythritol; (4), diamide, then sulfite.**

**Fig. 3. Dimerization of band 3 in intact erythrocytes. Erythrocytes were exposed (pH 8.0, 37°C) to iodoacetate (15 min, 10 mM) followed by diamide (15 min, 0.2 mM) or to *N,N'*-*p*-phenylenedimaleimide (30 min, 0.4 mM). The cells were washed 3 times, the membranes solubilized in SDS, subjected to electrophoresis, stained with Coomassie Blue and scanned at 546 nm. (A), control; (B), diamide after iodoacetate pretreatment; (C), *N,N'*-*p*-phenylenedimaleimide.**

Alternatively, iodoacetate could bring into closer proximity (<4 Å), by a minor structural alteration, SH-groups of two adjacent copies of band 3 and thereby render possible an intermolecular disulfide formation by diamide. This concept receives support from the observation that *N,N'*-*p*-phenylene-dimaleimide (Aldrich), a bifunctional SH-reagent, able to span a distance of about 10 Å [14], produces crosslinking of band 3 (and spectrin) in intact erythrocytes without iodoacetate pretreatment (Fig. 3).

These findings may be regarded as further support for a dimeric association of band 3 proteins in the membrane of the intact erythrocyte. The formation of disulfide-linked band 3 dimers in vivo, however, is unlikely in view of the ineffectivity of diamide. In contrast, spectrin oligomerization is not prevented by GSH and could therefore occur in vivo under conditions of oxidative stress. Of physiological interest is our finding that up to 80% of the protein SH-groups in the native membrane can be linked to each other via

disulfide bonds. It may be concluded that these SH-groups are close enough to each other in situ to build up disulfide bonds. The reversible formation of such disulfide bonds could have important implications for the proper function of the membrane in circulating erythrocytes, e.g., the protection of the membrane structure against radicals or oxidative stress.

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## References

1 Steck, T.L. (1972) J. Mol. Biol. 66, 295—305
2 Wang, K. and Richards, F.M. (1974) J. Biol. Chem. 249, 8005—8018
3 Wang, K. and Richards, F.M. (1975) J. Biol. Chem. 250, 6622—6626
4 Yu, J. and Steck, T.L. (1975) J. Biol. Chem. 250, 9176—9184
5 Kosower, N.S., Kosower, E.M. and Wertheim, B. (1969) Biochem. Biophys. Res. Commun. 37, 593—596
6 Haest, C.W.M. and Deuticke, B. (1976) Biochim. Biophys. Acta 436, 353—365
7 Lepke, S. and Passow, H. (1972) Biochim. Biophys. Acta 255, 696—702
8 Dodge, J.T., Mitchell, C. and Hanahan, D.J. (1963) Arch. Biochem. Biophys. 100, 119—130
9 Fairbanks, G., Steck, T.L. and Wallach, D.F.H. (1971) Biochemistry 10, 2606—2617
10 Beutler, E. Duron, D. and Mikus Kelly, B. (1963) J. Lab. Clin. Med. 61, 882—888
11 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
12 Habeeb, A.F.S.A. (1972) Methods Enzymol. XXV part B, p. 458
13 Srivastava, S.K., Awasthi, Y.C. and Beutler, E. (1974) Biochem. J. 149, 259—275
14 Burke, M., Reisler, E. and Harrington, W.F. (1976) Biochemistry 15, 1923—1927