Cross-Linking of Erythrocyte Membrane Proteins by Periodate and Intramembrane Particle Distribution

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(Received 3 November 1977)

Treatment of isolated human erythrocyte membranes at pH 7.4 with 0.1–0.5 mM-sodium periodate specifically cross-linked some of the spectrin polypeptides. Treatment with 2 mM-periodate resulted in complete cross-linking of spectrin and partial cross-linking of other polypeptides. The latter treatment also caused aggregation of the intramembrane particles made visible by freeze-fracturing. When membranes that had been treated with 2 mM-periodate were depleted of spectrin by treatment with 0.1 mM-EDTA, extensive aggregation of the intramembrane particles occurred.

In the erythrocyte membrane band 3, the sialoglycoproteins (for nomenclature see Fairbanks *et al.*, 1971) and many others (Gahmberg, 1976) are integral glycoproteins (Singer & Nicolson, 1971). At least band 3 and the sialoglycoproteins span the membrane (Bretscher, 1971a,b; Morrison *et al.*, 1974; Jenkins & Tanner, 1975). The peripheral proteins are more loosely bound to the cytoplasmic side of the membrane (Steck, 1974). Spectrin (Marchesi & Steers, 1968) belongs to this group of membrane proteins and can be released from the membrane by chelating agents (Marchesi & Steers, 1968; Tanner & Boxer, 1972). It consists of at least two polypeptides, with apparent mol.wts. of 240 000 and 220 000, and constitutes 20–25% of the total membrane protein (Marchesi & Steers, 1968). Spectrin resembles myosin (Sheetz *et al.*, 1976) and is implicated in the control of cellular shape and membrane integrity (Marchesi *et al.*, 1976; Kirkpatrick, 1976).

When membranes are studied by the freeze-fracturing method, characteristic intramembrane particles are seen on the fracture surfaces. There is strong evidence that these particles consist of integral membrane proteins (Zingsheim & Plattner, 1976). Several attempts have been made to elucidate the association between spectrin and the particles, but no conclusive results are available.

We here report that oxidation with 0.1–0.5 mM-periodate results in selective partial cross-linking of the spectrin polypeptides. This treatment does not lead to re-arrangement of the intramembrane particles. Only when all spectrin and part of other membrane proteins are cross-linked by 2 mM-periodate is an aggregation of the particles observed. When the membranes are subsequently treated with EDTA to deplete the membranes of spectrin, an extensive aggregation of the particles occurs.

Materials and Methods

Fresh erythrocytes were extensively washed in phosphate-buffered saline (0.15 M-NaCl/0.01 M-sodium phosphate, pH 7.4) before further treatment. Isolation of erythrocyte membranes was performed as described earlier (Gahmberg, 1976). A spectrin preparation was obtained by dialysis of membranes at +4°C against 0.1 mM-EDTA, pH 8.0 (Marchesi & Steers, 1968) and concentrated by ultrafiltration. The preparation contained a small amount of other proteins, mainly band 5 or erythrocyte actin (Tilney & Detmers, 1975).

Polyacrylamide-slab-gel electrophoresis was performed on 8% acrylamide gels as described by Laemmli (1970). Coomassie Blue-stained gels (Weber & Osborn, 1969) were scanned with a Joyce-Loebl Chromoscan apparatus.

For periodate oxidation, 1 ml of phosphate-buffered saline was added to 1 ml of packed cells, and periodate (Merck A.G., Darmstadt, Germany) was added to the final concentration indicated. After a 10 min incubation at 22°C in the dark, the cells were washed with 3 × 5 ml of phosphate-buffered saline and the membranes isolated. Membrane samples, corresponding to 1 ml of packed cells, were incubated as above with periodate in a total volume of 2 ml of phosphate-buffered saline, and the membranes washed twice in phosphate-buffered saline/water (1:9, v/v). Isolated spectrin (1 mg/ml) was treated as above with periodate, dialysed against water and freeze-dried.

Amino acid analysis was performed with a Beckman 120C amino acid analyser after hydrolysis in 6M-HCl under vacuum for 24 h at 110°C. Half-cystine and methionine were determined from parallel samples after performic acid oxidation (Hirs, 1967).

Protein was measured as described by Lowry *et al.* (1951), with bovine serum albumin as standard.

Six different samples of membranes were studied by the freeze-fracturing method: (a) isolated membranes which had not received periodate treatment, (b) membranes isolated from cells that had been treated with 2mM-periodate, (c)–(e) isolated membranes that had been treated with 0.1 mM-, 0.5 mM- or 2 mM-periodate, (f) isolated membranes that had been treated with 2 mM-periodate and were then depleted of spectrin by EDTA treatment. The membrane samples were then incubated at 37°C for 30 min, washed twice by centrifugation (10000g for 30 min) and fixed in 2.5% glutaraldehyde, buffered with 0.1 M-sodium cacodylate, pH 7.2, at 22°C for 30 min. The fixed membranes were washed with 2 × 10 ml of phosphate-buffered saline and impregnated with 30% (v/v) glycerol in phosphate-buffered saline at 4°C for 16 h. The samples of membrane suspensions were transferred to gold discs and rapidly frozen in liquid Freon 22 cooled to its freezing point by liquid N₂. The freeze-fracture replicas were prepared in a Balzers 360 M apparatus by electron-gun evaporation of Pt-C and C. Replicas were examined in a Jeol 100 B electron microscope.

Results

Effect of periodate on intact erythrocytes

When intact erythrocytes were treated with 2 mM-periodate and the membranes isolated and subjected to electrophoresis, no gross changes in the polypeptide patterns were observed (Figs. 1a and 1b).

Fig. 1. Polyacrylamide-gel-electrophoresis patterns of erythrocyte membrane proteins before and after treatment with periodate

(a) Pattern of membrane that did not receive any periodate treatment; (b) pattern of membrane isolated from cells that were treated while intact with 2 mM-periodate; (c) pattern of membrane without periodate treatment; (d) pattern of membrane treated with 0.1 mM-periodate; (e) pattern of membrane treated with 0.5 mM-periodate; (f) pattern of membrane treated with 2 mM-periodate.

Effect of periodate on erythrocyte membranes and isolated spectrin

Treatment of isolated erythrocyte membranes with periodate, even at 0.1 mM, resulted in partial disappearance of spectrin from the gels (Figs. 1d and 2b, Table 1). Treatment with 0.5 mM-periodate further decreased the spectrin bands, without much effect on other bands (Figs. 1e and 2c). Spectrin almost disappeared from the gels after treatment with 2 mM-periodate (Figs. 1f and 2d), but now there was also partial cross-linking of other proteins, notably band 4.1. It is noteworthy that band 1 was more easily cross-linked than band 2 at all concentrations

Fig. 2. Scanning patterns of Coomassie Blue-stained gels of erythrocyte membranes before and after treatment with periodate

(a) No periodate treatment; (b) after treatment with 0.1 mM-periodate; (c) after treatment with 0.5 mM-periodate; (d) after treatment with 2 mM-periodate.

Table 1. Quantitative distribution of erythrocyte-membrane proteins after treatment with periodate

Results are given as percentages of major components measured by scanning of Coomassie Blue-stained gels. Relative values were used because of difficulties in applying exactly the same amounts of protein to the gels.

Periodate concn. (mM)	Band	Distribution of proteins (% of total)			
		0	0.1	0.5	2.0
1	(spectrin)	26	18	10	0
2		18	19	13	2
3		20	22	28	36
4.1		10	10	9	5
4.2		6	8	9	11
4.4		4	6	7	12
5 (actin)		6	6	10	15
6		5	5	6	8
7		5	6	8	11

EXPLANATION OF PLATE I

Electron micrographs of freeze-fractured human erythrocyte membranes

(a) Isolated erythrocyte membranes that did not receive periodate treatment. The intramembrane particles have an even distribution. (b) Membranes isolated from cells that had been treated with 2 mM-periodate. A similar even distribution of the particles is seen. (c) Membranes that were treated with 0.1 mM-periodate. The particles are evenly distributed. (d) Membranes that were treated with 0.5 mM-periodate. The particles are evenly distributed. (e) Membranes that were treated with 2 mM-periodate. The particles now show a distinct aggregation. (f) Membranes that were treated with 2 mM-periodate before spectrin depletion. An extensive aggregation of the particles is obvious. Shadowing is indicated by the circled arrows. Magnification $\times 46000$.

of periodate. Polyacrylamide-gel electrophoresis of concentrated supernatants showed that no spectrin was released from the membrane. Though the sialoglycoproteins were also oxidized, no change in their electrophoretic mobilities occurred with these concentrations of periodate (results not shown). To exclude an indirect effect on spectrin by periodate, the isolated spectrin/actin preparation was treated with periodate. Spectrin was then more easily cross-linked than actin (Fig. 3).

The amino acid compositions of the spectrin preparation and of spectrin oxidized with 2mM-periodate are shown in Table 2. Two changes are evident: about half of the tyrosine residues are not recovered after periodate treatment and all cysteine/cystine residues are oxidized to cysteic acid.

Influence of periodate on the distribution of the intra-membrane particles

In freeze-fracture electron micrographs the intra-membrane particles had an even distribution in membranes that had not been treated with periodate (Plate 1a). When intact cells were treated with 2mM-periodate and the isolated membranes studied, no change in the particle distribution occurred (Plate 1b). Neither was there any change in membranes that were treated with 0.1 or 0.5mM-periodate (Plates 1c and 1d). However, in membranes treated with 2mM-periodate but not depleted of spectrin, the intra-membrane particles showed a distinct aggregation (Plate 1e). When membranes that had been subjected to 2mM-periodate were treated with EDTA to deplete them of spectrin, smaller membrane vesicles were formed, and the particles were extensively aggregated (Plate 1f).

Table 2. *Amino acid compositions of isolated spectrin and spectrin treated with periodate*

Results are means±s.d. of three analyses. Half-cystine and methionine were determined after performic acid oxidation.

	Content (nmol/1000nmol)	
	Spectrin	Periodate-treated spectrin (2mM)
Lysine	71.7±2.3	68.7±2.7
Histidine	29.5±0.5	28.1±0.6
Arginine	59.7±2.1	59.9±4.0
Aspartic acid	106.2±1.4	107.5±2.2
Threonine	45.7±0.2	45.6±0.5
Serine	57.4±5.1	58.2±4.3
Glutamic acid	187.2±2.9	189.2±2.6
Proline	22.6±2.2	21.7±0.6
Glycine	53.4±1.3	57.0±4.0
Alanine	87.9±3.2	89.7±1.8
Valine	52.1±4.2	54.4±2.0
Isoleucine	43.7±1.3	43.9±1.0
Leucine	120.2±2.2	120.4±6.6
Tyrosine	23.1±0.3	10.6±1.8
Phenylalanine	34.1±0.9	32.9±0.5
Cysteic acid	0	8.8±0.4
Methionine sulphone	0	0
Half-cystine	9.2±0.6	—
Methionine	19.8±0.9	20.1±1.0

Discussion

Spectrin is closely associated with and shows specific interaction with the cytoplasmic aspect of the erythrocyte membrane (Nicolson *et al.*, 1971; Bennett & Branton, 1977). The spectrin polypeptides may form a network at the membrane and can easily be cross-linked by a variety of protein-reactive reagents, which indicates a close interaction between the spectrin molecules (Steck, 1972; Wang & Richards, 1974). The polypeptides are also cross-linked partially by exposure to O₂ at pH4.5–5.5, which is near the isoelectric point of spectrin (Liu *et al.*, 1977). This cross-linking was due to formation of inter-chain disulphide bonds.

Periodate is known to oxidize vicinal hydroxy groups in carbohydrates, but its effects on membrane proteins have not been much studied. When rabbit thymocyte membranes were treated with periodate, certain glycoproteins were cross-linked (Schmidt-Ullrich & Wallach, 1977). This effect was interpreted as being due to the formation of reactive aldehyde groups in the carbohydrate portions of the glycoproteins.

In the present report we demonstrate that spectrin, which does not contain carbohydrate, is partially cross-linked by periodate at concentrations hardly affecting other membrane proteins. A concentration

Fig. 3. *Scanning patterns of Coomassie Blue-stained gels of the spectrin/actin preparation before and after treatment with periodate*

(a) No periodate treatment; (b) after treatment with 0.1mM-periodate; (c) after treatment with 0.5mM-periodate; (d) after treatment with 2mM-periodate.

of 2mM-periodate results in cross-linking of most membrane proteins, though spectrin remains the most sensitive. The mechanism of cross-linking is unclear but it could be due to formation of tyrosine dimers, as oxidizing agents are known to dimerize tyrosine (Waykole & Heidemann, 1976). This question needs extensive further experimentation to be solved.

It is well established that in the freeze-fracture method, membranes are cleaved along their hydrophobic interiors (Branton, 1966). The cleaved membrane planes contain particles that in the human erythrocyte are probably mainly formed by the major membrane-penetrating polypeptide, band 3 (Yu & Branton, 1976). Also the sialoglycoproteins may contribute to the particles (Segrest *et al.*, 1974).

It has been shown that there is a transmembrane connection between spectrin and the surface portions of the membrane-penetrating glycoproteins. Anionic sites on the surface are clustered after treatment of membranes with anti-spectrin antibodies (Nicolson & Painter, 1973), and when surface glycoproteins are cross-linked with lectins, spectrin more easily forms high-molecular-weight aggregates by treatment with cross-linking agents (Ji & Nicolson, 1974). The association between intramembrane particles and spectrin is not clear. Elgsaeter & Branton (1974) claimed that depletion of spectrin is necessary to induce particle aggregation. In their experiments the aggregation of the particles occurred most extensively by lowering the pH below 6. On the other hand it is probable that some aggregation of remaining spectrin had taken place (Liu *et al.*, 1977).

We did not observe any particle aggregation when spectrin was only partially cross-linked. At higher periodate concentrations, other polypeptides were also affected. Therefore we cannot draw absolute conclusions about the role of spectrin in the aggregation phenomenon by this experiment. However, treatment of the more extensively oxidized membranes with dilute EDTA resulted in strong particle aggregation. Therefore it appears reasonable to suggest that spectrin is involved, but that the effect is observed only after most of the spectrin is either cross-linked or detached from the membrane. A direct role for other proteins, such as band 4.1, remains to be elucidated.

The skilful technical assistance of Marja Wilkman, Anneli Asikainen and Elina Waris is acknowledged. This work was supported by the Academy of Finland, the Finnish Cancer Society and the Sigrid Jusélius Foundation.

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