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Cross bonding and stiffening of the red cell membrane

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Cross bonding and stiffening of the human red cell membrane was studied using treatments with SH, amino, and carboxyl reagents, oxidizing and denaturing treatments and acidification. Membrane cross bonding was initiated when, after red cell treatment, opposite areas of the cytoplasmic face of the red cell membrane were brought into contact by cell shrinking. Membrane cross bonding was detected by light microscopy when this contact persisted upon swelling the cells in a hypotonic medium. Membrane stiffening was recorded as a decrease in elongation of red cells in the shear field of a viscous dextran solution. No correlation was found between membrane cross bonding and membrane stiffening. The results are explained by the existence of two modifications of spectrin, type I causing solely membrane stiffening, type II causing membrane cross bonding as well as membrane stiffening. The amino and carboxyl reagents caused only type I modification. The other treatments caused both types of modification although with varying proportions. The results support the previously suggested mechanism of membrane cross bonding which involves a rearrangement of spectrin similar to denaturation by heat or urea, a decrease in associations within the membrane skeletal network, and a lateral aggregation of membrane proteins. These changes are proposed to occur by the type II modification. The data further substantiate the membrane stiffening effect of inter- and intra-molecular cross linking of spectrin which is identified with the type I modification. Finally, hypotheses are presented concerning the mechanism of membrane stiffening due to type II modifications of spectrin.

Introduction

In two preceding papers, the phenomenon of membrane cross bonding in human red cells has been described [1,2]. Membrane cross bonding requires a modification of the cell membrane as well as contact of opposing areas of the cytoplasmic membrane face. This contact was achieved by cell shrinking. Concerning membrane modifications, treatment with diamide, *N*-ethylmaleimide (NEM), heat, urea and ATP-depletion have so far been used. Membrane cross bonding becomes apparent when the membrane areas cross bonded in the shrunken cell withstand the peeling force exerted by isotropic membrane tensions due to cell swelling. The structure formed by the cross-bonded membrane areas has been called an (internal) membrane bridge. The distance between the cross-bonded membranes has been found to be in the order of 100 nm. It has been shown by exclusion that spectrin provides the bonding between the membranes in a membrane bridge [2].

At the same time spectrin is the major protein constituting the membrane skeleton that imparts shear stiffness to the red cell membrane. It is therefore not surprising that the same treatments which induce membrane cross bonding also influence membrane stiffness. Indeed, treatment with diamide or NEM has been shown to decrease the elongation of red cells measured in the rheoscope [3]. For heat treatment, a decrease in the shear modulus of the red cell membrane was demonstrated by micropipette methods [4].

In the present work the correlation between cross bonding and stiffening of the red cell membrane was studied using a variety of membrane modifications: treatments with SH, amino and carboxyl reagents, further oxidizing and denaturing treatments and finally an acidification. No correlation was found between membrane cross bonding and membrane stiffening which is explained by two different kinds of spectrin modification. Hypotheses as to the molecular nature of these modifications as well as to the molecular mechanism of

Abbreviations: APH, 1-acetyl-2-phenylhydrazine; BHP, *t*-butylhydroperoxide; CDE, L-cystine dimethyl ester; EDC, 1-ethyl-3-[dimethylaminopropyl]carbodiimide; diamide, diazene dicarboxylic acid bis[*N*,*N*'-dimethylamide]; DMS, dimethylsuberimidate; DTDP, 4,4'-dithiodipyridine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; $IANH_2$, iodoacetamide; MDA, malondialdehyde; Mes, 4-morpholineethanesulfonic acid; NEM, *N*-ethylmaleimide.

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membrane cross bonding and membrane stiffening are presented.

Materials and Methods

Reagents

NEM, iodoacetamide ($IANH_2$), 1,1,3,3-tetramethoxypropane, and *t*-butylhydroperoxide (BHP) were purchased from Fluka, Neu-Ulm, F.R.G. 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), 4-morpholineethanesulfonic acid (Mes), bovine serum albumin fraction V, 4,4′-dithiodipyridine (DTDP), L-cystine dimethyl ester (CDE), dithioerythritol, 1-ethyl-3-[dimethylaminopropyl]carbodiimide (EDC), 1-acetyl-2-phenylhydrazine (APH) and butylated hydroxytoluene were purchased from Sigma, Deisenhofen, F.R.G. Diazene dicarboxylic acid bis[*N*,*N*′-dimethylamide] (diamide) was from Calbiochem, Frankfurt, F.R.G. Dimethylsuberimidate (DMS) was from Serva, Heidelberg, F.R.G. Dextran (M_r 60 000) was from Knoll, Ludwigshafen, F.R.G. All other chemicals were from Merck, Darmstadt, F.R.G.

Suspending media

Suspending media are designated by M carrying as a subscript their osmolarity in mosM. M_{300} contains (mM) KCl (90), NaCl (45), Na_2HPO_4/NaH_2PO_4 (10). For the treatment with EDC and for acidification Hepes and Mes were used instead of phosphate. For the treatment with DMS the phosphate concentration was 20 mM with compensatory decreases in chloride. M_{200} was prepared by diluting M_{300} with H_2O. M_{250} and M_{600} were produced by adding appropriate amounts of sucrose to M_{200}. The osmolarity of the solutions was checked by freezing point depression (Osmomat 30, Gonotec, Berlin, Germany). The pH of all solutions was adjusted to 7.4 except for the acidification experiments. Bovine serum albumin was added to prevent crenation of cells (0.3 mg/ml for M_{100} and M_{250}; 1.3 mg/ml for M_{600}.

Incubation procedures

Erythrocytes from freshly collected heparinized human blood were washed three times with M_{300}. To avoid internal membrane contact and thus premature cross bonding, modifications were performed in either of the two hypotonic media M_{200} or M_{250}. To allow for cross bonding, the cells were incubated in M_{600} for 15 min after the respective treatment. Some treatments were also performed directly in M_{600} to determine whether membrane contact during the treatment, instead of afterwards, had an additional effect. Incubations were performed at 37 °C except for heat treatment and the incubation with BHP. The hematocrit was always 10%. When a treatment with modifying agents was followed by a subsequent incubation, the cells were washed three times. When a subsequent incubation required a change in osmolarity the cells were washed once in the new medium before resuspension.

Treatment with SH reagents

Except for the treatment with $IANH_2$, the cells were pretreated with 10 mM iodoacetate in M_{200} for 15 min to block intracellular GSH and the reducing capacity of the cell metabolism. Subsequently the cells were treated in M_{250} with NEM, diamide, DTDP or CDE for 15 or 30 min. The concentrations used and the incubation times are given in Tables I and II or in the legends to the figures. DTDP was dissolved in *N*,*N*-dimethylformamide at a concentration of 100 mM. The iodoacetate treated red cells served as controls. They gave the same results as untreated red cells. Treatment with 20 mM $IANH_2$ was performed in M_{250} for 30 min without iodoacetate pretreatment.

To study reversibility after a disulfide-forming treatment, the cells were washed at 4 °C and then incubated for 60 min with and without 10 mM dithioerythritol to reduce disulfide bonds.

Treatment with amino reagents

Malondialdehyde (MDA) was prepared by dissolving 1,1,3,3-tetramethoxypropane in 0.1 M HCl, followed by a 10 min incubation at room temperature and subsequent neutralization. Acid hydrolysis yields 4 molecules methanol for one molecule MDA. The final concentration of MDA was adjusted to 5 or 10 mM by addition of M_{250}. The incubation time was 30 min. Controls were treated with 40 mM methanol. They did not differ from controls without methanol.

Red cells were treated in M_{250} with 5 mM DMS for 30 min. Since DMS rapidly hydrolyses in water, the reagent was dissolved in the incubation medium just prior to the addition of red cells. To facilitate pH adjustment after addition of DMS, the concentration of phosphate was twice as large as usual.

Treatment with carboxyl reagents

Red cells were treated in M_{250} at pH 7.4 with 5 or 10 mM EDC for 30 min in Hepes-buffered solution, since EDC reacts with phosphate [5].

Oxidizing treatments

Red cells were treated in M_{250} with 9 mM APH for 2 h. Treatment with 1 mM H_2O_2 was performed in M_{250} for 30 min. The incubation medium contained 1 mM azide to inhibit the cytoplasmic catalase [6]. Treatment with 2 mM BHP was carried out in M_{250} for 30 min. 1 mM azide, which complexes with ferric ion of hemoglobin [7], was present to delay the termination of radical chain reactions. The time course of membrane stiffening was strongly non-linear. Therefore, the incubation was carried out at 25 °C because it was easier

to reproduce a certain extent of membrane stiffening. At the end of the incubation with BHP, the peroxidative chain reaction was stopped by adding butylated hydroxytoluene, dissolved in a small amount of ethanol, to a final concentration of 100 μM. The suspension was then immediately cooled to 4°C and washed at this temperature. Controls were incubated in the same way except that no H_2O_2 or BHP was added. Azide or butylated hydroxytoluene alone showed no effect.

Spectrin-denaturing treatment

For thermal denaturation of spectrin, cells were heated by incubation in M_{250} for 15 min at 45 or 47°C. To probe reversibility, cells heated for 30 min at 46°C were incubated for 60 min at 37°C to allow for spontaneous reversion. For chemical denaturation of spectrin, cells were treated with 1.1 to 1.54 M urea in M_{250} for 30 min. To study reversibility, the cells were exposed to 1.35 M urea for 30 min. The subsequent washing was carried out at 4°C. To avoid osmotic hemolysis, the urea concentration was lowered in steps of 0.3 M before washing with urea-free M_{250}. To allow for spontaneous reversion, the cells were then incubated for 60 min at 37°C.

Acidification

Red cells were suspended in M_{300} (without phosphate) at a hematocrit of 15%. No hypotonic medium was used because the cells swell upon decrease in pH. At 37°C the extracellular pH (pH_e) was decreased from 7.4 to 5.7 within 15 min by addition of 100 mM Mes (final concentration 10 mM) and appropriate amounts of 0.3 M HCl. M_{300} (without phosphate) was then added to adjust the hematocrit to 10%. The suspension was then incubated for 15 min and the pH_e was kept at 5.7. An intracellular pH (pH_i) of 5.78 was determined after packing the red cells in an Eppendorf centrifuge and lysis by addition of saponin. For cross bonding, 2 ml of the suspension at pH_e 5.7 were added to 300 mg sucrose and incubated for 15 min at 37°C. For reversion of the acidification, the red cells were resuspended in Hepes-buffered M_{300} at a hematocrit of 15% and titrated back with KOH/NaOH to pH_e 7.4 following the same schedule as above.

Assessment of membrane cross bonding

After the treatments described above (except acidification), the cells were washed and then incubated in M_{600} for 15 min at 37°C. This provided internal membrane contact and allowed for membrane cross bonding. For counting of bridged cells, a drop of the cell suspension was mixed with 1 ml M_{200}. Swelling in this medium stopped bridge formation and opened up some weak membrane bridges [1]. After standing for 5 min at room temperature a drop of this suspension was placed between two plastic coverslips and inspected under a microscope. Due to the sharp edge of the membrane at the perimeter of the membrane bridges, these were clearly visible by differential interference contrast microscopy. H_2O_2-treated cells are shown as an example in Fig. 1. Between 100 and 300 cells were evaluated directly under the microscope to determine the percentage of bridged red cells. In order to select a random sample, the field to be counted was positioned according to a fixed scheme without looking through the microscope.

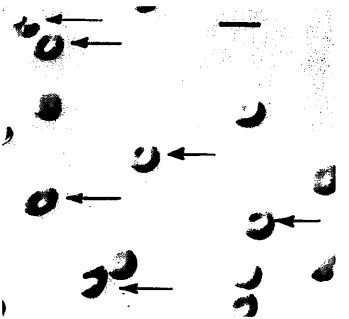

Fig. 1. Morphology of cross-bonded red cells (arrows) swollen in a hypotonic medium. Red cells were treated with H_2O_2 under the conditions shown in Table I. Membrane cross bonding was induced by incubation in M_{600}. Membrane bridges (arrows) become apparent upon swelling in M_{200} (differential interference contrast, objective 40×, Leitz). Scale bar 10 μm.

Assessment of membrane stiffening

After the treatments described above, the cells were washed and resuspended (hematocrit was 4%) in solutions of dextran in M_{300} (16–46 cP, at 24°C). The concentration of NaCl and KCl was reduced appropriately to compensate for the osmotic activity of dextran. 20 μl of the suspension were transferred into the cone-plate-chamber of a rheoscope. The red cells elongate when the suspension is sheared [8]. If the aperture of the microscopic illumination is small enough, an elliptical diffraction pattern, as in ectacytometry [9], can be observed in the back focal plane of the microscope lens (25 × 0.50). This diffraction pattern represents the average elongation of all red cells in the field of view. To photograph the diffraction pattern a Bertrand lens was added to the microsope [10]. The elongation, E', as defined in the legend of Fig. 2, was evaluated by comparing the shape of the diffraction patterns with ellipses graded in steps of 0.025 in E'.

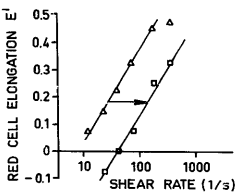

Fig. 2. Red cell elongation, E', of red cells as a function of the logarithm of the mean shear rate in the rheoscope. Red cells were incubated for 30 min in M_{250} at a concentration of 1.54 M urea. Then the cells were washed, suspended in a dextran solution of 37 cP (at 24 °C), and sheared in the rheoscope (△, controls; □, urea). E' is equal to $(L - B)/(L + B)$, where L and B are the long and the short axis of the diffraction pattern. The arrow indicates the stiffening index (for definition see text).

Elongations, E', were plotted versus the log of the mean shear rate in the cone-plate chamber. As an example the elongation of urea-treated red cells is shown in Fig. 2. The shift (parallel to the abscissa) of a hand-drawn straight line for the treated sample with respect to the line for the control corresponds to the factor by which the shear rate has to be increased to compensate for the increased stiffness of the treated sample. In the following this factor is called 'stiffening index'.

Results and Interpretation

Correlation between membrane cross bonding and membrane stiffening

Modifications of spectrin have been suggested to cause membrane cross bonding [2] and membrane stiffening [3]. It is therefore interesting to ask whether, on a

TABLE I

Correlation between membrane cross bonding and membrane stiffening

Incubations were carried out in M_{250} except for the acidification experiment which was in M_{300} buffered with Mes, the treatment with DMS where the phosphate concentration was doubled, and the treatment with EDC where Hepes was used instead of phosphate. Before treatment with diamide, DTDP, CDE and NEM, the cells were pretreated with 10 mM iodoacetate for 15 min in M_{200}. For cross bonding, the cells were incubated for 15 min in M_{600} and then counted in M_{200}. One general control value is given because percentages of bridged red cells after treatment with 1 mM azide, 40 mM methanol, or 10 mM iodoacetate were not significantly different from those after a incubation in M_{250} alone. For membrane stiffening, the cells were resuspended in a dextran salt solution and sheared in a rheoscope. The stiffening index for the control is 1 per definition. pH_i, intracellular pH.

Treatment		Incubation time (min)	Percentage of bridged cells		Stiffening index	
			mean value (n)	range	mean value (n)	range
Control		15–120	2 (40)	0– 6	1.0	
Diamide	(0.1 mM)	15	8 (4)	4–10	1.5 (3)	1.3– 1.7
	(0.2 mM)	15	17 (4)	15–18	2.4 (6)	1.8– 3.4
	(0.5 mM)	15	34 (4)	29–44	4.5 (3)	4.0– 4.9
	(1 mM)	15	56 (8)	46–69	9.7 (6)	6.5–11.5
DTDP	(0.05 mM)	15	10 (4)	9–13	2.6 (3)	1.5– 3.8
	(0.1 mM)	15	29 (4)	20–42	4.1 (6)	2.3– 6.0
	(0.2 mM)	15	63 (4)	57–67	4.9 (6)	4.5– 5.5
	(1 mM)	15	83 (4)	75–93	4.9 (10)	4.2– 5.8
NEM	(1 mM)	15	69 (4)	59–80	2.6 (3)	2.5– 2.8
CDE	(9 mM)	30	57 (8)	45–73	2.6 (4)	2.1– 3.8
$IANH_2$	(20 mM)	30	17 (4)	14–24	1.7 (5)	1.3– 2.1
DMS	(5 mM)	30	2 (6)	0– 4	2.3 (4)	1.8– 2.9
MDA	(5 mM)	30	2 (2)	1– 2	2.6 (2)	2.5– 2.8
	(10 mM)	30	2 (5)	1– 3	4.5 (5)	4.0– 6.0
EDC	(5 mM)	30	3 (5)	0– 6	7.2 (3)	6.8– 7.4
	(10 mM)	30	18 (3)	9–29	13.8 (3)	13.0–14.7
APH	(9 mM)	120	22 (3)	17–25	1.8 (3)	1.4– 2.1
H_2O_2	(1 mM) + azide (1 mM)	30	39 (5)	32–56	2.2 (3)	2.0– 2.6
BHP	(2 mM) + azide (1 mM)	30	63 (5)	55–72	12.1 (5)	2.5–30.7
Heat	(45 °C)	15	33 (4)	14–45	1.3 (4)	1.0– 1.6
	(47 °C)	15	78 (16)	55–90	2.4 (4)	1.8– 3.0
Urea	(1.1 M)	30	19 (4)	14–23	1.1 (3)	1.0– 1.2
	(1.35 M)	30	31 (7)	23–40	2.2 (4)	1.1– 3.5
	(1.54 M)	30	49 (4)	44–56	4.5 (3)	3.7– 5.1
Acidification (pH_i 5.78)		30	32 (3)	26–42	5.3 (3)	2.8–10.2

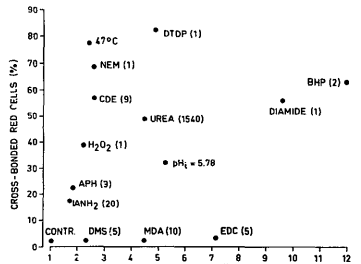

Fig. 3. Correlation between the percentage of cross-bonded red cells and the stiffening index (for definition see text and Fig. 2). Mean values are given. Concentration of reagents (in brackets) are given in mM. The conditions for treatment as well as the scatter of the experimental data are given in Table I.

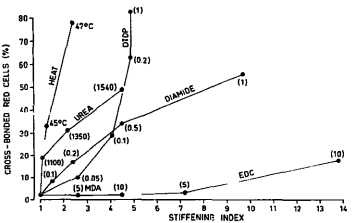

Fig. 4. Dose dependence of the correlation between the percentage of cross-bonded red cells and the stiffening index. Mean values are given. Concentration of reagents (in brackets) are given in mM. The conditions for treatment as well as the scatter of the experimental data are given in Table I.

molecular level, the same modification is responsible for both effects. To answer this question, identically modified red cells were used to assess membrane cross bonding and membrane stiffening. To induce membrane cross bonding, the modified cells were incubated in M_{600} for 15 min. For quantification, the percentage of bridged red cells was determined after swelling the cells in M_{200}. For assessment of membrane stiffening it was important that cell swelling in M_{250} during the various treatments prevented membrane cross bonding because cross-bonded red cells do not show the tank-tread motion of the membrane which is a prerequisite for the steady-state elongation in a shear field [11]. The factor by which the shear rate had to be increased to obtain the same elongation in modified cells as in controls served as an index of membrane stiffening.

Six different sorts of treatment were employed: chemical modifications specific for SH, amino or carboxyl groups, oxidizing treatments involving formation of O_2-derived reactive species, denaturing treatments that primarily derange the tertiary structure of proteins, and acidification that changes the cytoplasmic pH towards the isoelectric point of spectrin, thus decreasing the net charge of the molecule. Percentages of bridged red cells and stiffening indices are listed in Table I for the various treatments. Fig. 3 presents the mean values, plotted against each other.

The conditions for the treatments shown in Fig. 3 were chosen so that either the percentage of cross-bonded cells or the stiffening index were strongly increased. However, concentrations of the respective reagents above 20 mM were not used. An increase of the concentration of DMS was limited by its low solubility. For urea treatment and acidification, the conditions were just subcritical with respect to vesiculation.

All treatments shown in Fig. 3 increased membrane stiffness. This has been reported for most treatments, although the conditions were quite different in many cases [3–5,12–17]. Membrane stiffening after treatment with CDE or urea is reported for the first time. There is no correlation between the extent of membrane cross bonding and the extent of membrane stiffening. In contrast to membrane stiffening, not all treatments induced membrane cross bonding. This shows that the spectrin modifications that lead to membrane cross bonding are of a different kind, compared to those that induce solely membrane stiffening. On the other hand, all treatments that induced bridges caused membrane stiffening as well. It is therefore likely that spectrin modifications causing membrane cross bonding inevitably increase membrane stiffness. Taken together these results suggest that we can distinguish two kinds of modification of spectrin:

(a) type I that induces solely membrane stiffening
(b) type II that induces membrane cross bonding as well as membrane stiffening.

The great variation in the slope of hypothetical lines connecting each data point in Fig. 3 with that for the respective control treatment can then be explained by varying contributions of the two types of modification. A slope of zero found after treatment with the carboxyl reagent EDC (5 mM) and the amino reagents MDA and DMS represents a pure type I modification. Steep slopes as observed after treatment with $IANH_2$, APH, H_2O_2, CDE, NEM or heat can be interpreted as a consequence of a pure type II modification, its membrane stiffening effect being relatively small. Intermediate slopes indicate contributions of both types of modification.

A variation of the contributions of the two types of modification is not only found when effects of the

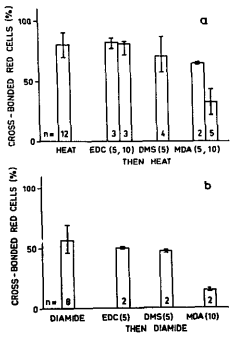

Fig. 5. Membrane cross bonding with heat or diamide after pretreatment with EDC, DMS or MDA. Red cells were first treated in M_{250} with EDC, DMS or MDA for 30 min. Concentration of reagents (in brackets) are given in mM. Red cells were then treated for 15 min in M_{250} either by heat (47°C) (a) or with iodoacetate (10 mM) and subsequently with diamide (1 mM) (b). Numbers of experiments are shown on each column. Bars indicate ranges.

various treatments are compared, but even when the dose dependence of a single treatment is considered. Fig. 4 shows for a few treatments that upward as well as downward curved deviations from a linear correlation between membrane cross bonding and membrane stiffening can occur. In pure type II modifications, a variation in slope can be ascribed to a varying portion of its effect on membrane cross bonding and membrane stiffening. Fig. 4 also shows that EDC at 10 mM has a small cross bonding effect.

Binding of hemoglobin to the membrane

The absence of membrane cross bonding after treatment with EDC, DMS and MDA might be alternatively explained by a massive deposition of hemoglobin at the cytoplasmic face of the membrane. A layer of hemoglobin might prevent the spectrin–spectrin interaction required for bridge formation. To investigate this possibility, membrane-bound hemoglobin was determined using essentially the turbidimetric method introduced by Winterbourn [18]. The only change was that after treatment with H_2O_2 or BHP the lysing solution contained in addition 5 mM azide.

Controls had absorbances between 0.006 and 0.016. The values for the treated samples ranged from 0.014 to 0.026. The only exception was H_2O_2 with a mean value of 0.054. These results indicate that the alternative explanation considered above can be dismissed, since treatment with H_2O_2 which bound most hemoglobin still induced bridges.

Suppression of membrane cross bonding by pretreatment with amino and carboxyl reagents

The idea behind the following experiments was that the treatment with EDC, DMS and MDA that did not induce membrane cross bonding might even stabilize the red cell against the cross bonding capacity of a subsequent treatment. Fig. 5a shows that pretreatment with EDC and DMS did not suppress the cross-bonding effect of a subsequent heat treatment. Pretreatment with MDA, on the other hand, lowered the heat effect in a dose-dependent fashion. Corresponding results were obtained when membrane cross bonding was induced with diamide instead of heat treatment (Fig. 5b). These observations indicate that the type I modification of spectrin induced by treatment with EDC and DMS does not prevent a subsequent type II modification. The type I modification of spectrin after treatment with MDA, on the other hand, appears to interfere with a subsequent type II modification.

Reversibility of membrane cross bonding and membrane stiffening

Among the six sorts of treatment (chemical modification of SH, amino or carboxyl groups, oxidizing treatments, denaturing treatments, and acidification) used in this work, the effects of only three are possibly reversible. Among the covalent modifications only S–S bonds (produced by the reagents DTDP, diamide and CDE) can be cleaved by reduction with dithioerythritol. A partial reversibility of heat, urea and pH effects reported in the literature (see below) lead us to try to determine whether a reestablishment of control conditions would spontaneously reverse membrane cross bonding and membrane stiffening after heat or urea treatment or acidification.

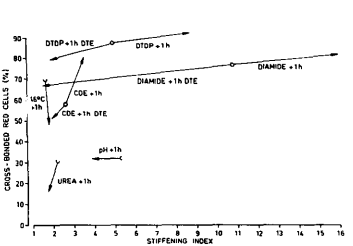

Fig. 6. Reversibility of membrane cross bonding and membrane stiffening. The conditions for treatment as well as the scatter of the experimental data are given in Table II. The arrows indicate the change observed on the 1 h reversal incubation.

TABLE II

Reversibility of membrane cross bonding and membrane stiffening

Incubations were carried out in M_{200} for membrane cross bonding and M_{250} for membrane stiffening except for the acidification experiment which was in M_{300} buffered with Mes or Hepes. Before treatment with diamide, DTDP and CDE, the cells were pretreated with 10 mM iodoacetate for 15 min in M_{200}. For membrane stiffening, the cells were resuspended in dextran salt solutions and sheared in a rheoscope. For cross bonding, the cells were incubated for 15 min in M_{600} and then counted in M_{200}. DTE, dithioerythritol; pH_i, intracellular pH; pH_e, extracellular pH.

Treatment	Reversion	Percentage of bridged red cells			Stiffening index		
		incubation time (min)	mean value (*n*)	range	incubation time (min)	mean value (*n*)	range
Diamide (1 mM)		30	77 (5)	75–79	15	10.7 (3)	9.0–11.5
	DTE (10 mM)	60	67 (5)	58–72	60	1.5 (3)	1.4– 1.7
	no DTE	60	82 (5)	77–87	60	15.9 (3)	8.0–25.0
DTDP (1 mM)		30	88 (4)	81–98	15	4.9 (7)	4.5– 5.8
	DTE (10 mM)	60	79 (4)	62–90	60	1.8 (7)	1.5– 2.3
	no DTE	60	93 (4)	86–99	60	8.7 (7)	6.5–12.5
CDE (9 mM)		30	58 (4)	48–73	30	2.6 (4)	2.1– 3.8
	DTE (10 mM)	60	51 (4)	40–69	60	1.9 (4)	1.3– 2.6
	no DTE	60	81 (4)	72–93	60	3.5 (4)	2.5– 5.5
Heat (46 °C)		30	70 (4)	52–83	30	1.6 (4)	1.2– 1.7
	37 °C	60	48 (4)	38–56	60	1.8 (4)	1.5– 2.2
Urea (1.35 M)		30	31 (7)	23–40	30	2.2 (4)	1.1– 3.5
	no urea	60	16 (7)	9–25	60	1.7 (4)	1.0– 2.3
Acidification (pH_i 5.78)		30	32 (3)	26–42	30	5.3 (3)	2.8–10.2
	pH_e 7.4	60	32 (3)	28–37	60	3.9 (3)	2.6– 6.5

It turned out that the effects of SH oxidation, heat or urea treatment, or acidification were all partially reversible. The conditions for incubation and the numerical results are listed in Table II. The changes in membrane cross bonding and membrane stiffening upon reversion are displayed graphically as arrows in Fig. 6. The foot of the arrows represents the values measured after the initial treatment. The arrowhead points to the values observed after the reversal incubation. For the treatments with SH reagents there is a second arrow corresponding to an incubation without dithioerythritol. These arrows point in the opposite direction, indicating that a mere incubation after removal of the SH reagents did not revert but actually enhanced their effect.

The different sorts of treatment behaved differently upon 'reversion'. Arrows pointing downwards indicate exclusive reversion in membrane cross bonding. Arrows pointing to the left indicate exclusive reversion in membrane stiffening. It is tempting to associate the latter with exclusive reversion of a type I modification of spectrin.

Dose dependence of membrane stiffening and membrane cross bonding after treatment with bifunctional SH reagents

The membrane stiffening effect of DTDP was much smaller at a concentration of 1 mM than that of diamide (Fig. 3). This is at variance with earlier results where smaller concentrations were employed [3]. To examine this discrepancy dose-response curves in membrane stiffening were established for DTDP and diamide (Fig. 7a). At low concentrations, DTDP had a greater membrane stiffening effect than diamide which is in keeping with earlier findings [3]. With increasing concentrations the effect of DTDP levelled off, whereas that of diamide increased and eventually became greater than that of DTDP. This is interpreted as follows.

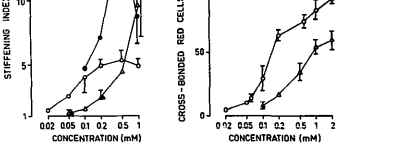

Fig. 7. Dose-response curves for treatments with diamide and DTDP, stiffening index (a), and percentage of cross-bonded red cells (b). Red cells were treated in M_{200} with iodoacetate (10 mM) for 15 min. Subsequently, they were treated in M_{250} for 15 min with DTDP (○) or diamide (△) at the indicated concentrations (open symbols). In some cases the cells were then washed and incubated for 1 h in M_{250} (closed symbols). In a, $n = 6$ where a standard deviation is plotted, otherwise $n = 3$. In b, $n = 4$.

With an average of 100 nmol SH groups per mg membrane protein, and considering the mass of membrane proteins per red cell, the cell volume and the hematocrit during the treatment it can be calculated that a concentration of 0.05 mM corresponds to one molecule of SH reagent per membrane SH group. Note that the intracellular glutathione was blocked by pretreatment with iodoacetate. For concentrations below 0.05 mM, membrane SH groups are in abundance. This favours the formation of S–S bonds instead of mixed disulfides. The stronger effect of DTDP at these concentrations indicates that it is more reactive than diamide. The levelling off at the higher concentrations of DTDP is interpreted by the formation of an increasing percentage of mixed disulfides between membrane SH groups and thiopyridine. This interpretation is based on the finding that bifunctional modification is more effective in membrane stiffening than monofunctional modification [3]. The increasing slope of the curve for diamide, on the other hand, can be explained by assuming that only disulfide bonds are formed by this reagent. The reason for this difference in behavior between DTDP and diamide is unclear at present.

The low effectivity of CDE in membrane stiffening, which is comparable to that of the purely monofunctional NEM, can be explained along the same lines.

Membrane stiffening was, in addition, measured after a mere incubation in buffer for 1 h subsequent to the 15 min treatment with DTDP or diamide. Fig. 7a shows that the additional incubation did not significantly change the values for the diamide-treated cells. For the DTDP-treated cells, on the other hand, a considerable increase was observed so that the resulting curve was now parallel to that of diamide up to a concentration of 0.5 mM. This increase can be explained as follows. After the 15 min treatment not all SH groups were modified by DTDP. During the 1 h incubation, monovalent adducts reacted to disulfide bonds with previously unmodified SH groups. The absence of a significant increase after diamide treatment is in keeping with the assumption of exclusive formation of disulfide bonds during the 15 min treatment.

In contrast to membrane stiffening, treatment of cells with DTDP was at all concentrations more effective in membrane cross bonding than diamide (Fig. 7b). This indicates that bifunctional modification by diamide is not more effective in membrane cross bonding than monofunctional modification by DTDP.

Effect of blockade of SH groups by a pretreatment with NEM

The effects of a diamide treatment on leak formation [19], and on transbilayer reorientation of phospholipids [20,21] have been shown to be suppressed after a moderate pretreatment with NEM. This pretreatment obviously blocked a small fraction of spectrin SH groups

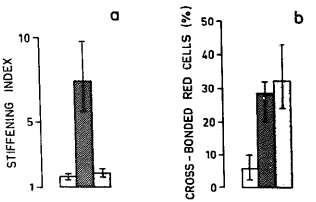

Fig. 8. Blockade of SH groups by pretreatment with NEM: stiffening index (a), and percentage of cross-bonded red cells (b). Red cells were treated in M_{200} with iodoacetate (10 mM) for 15 min. Subsequently, they were treated in M_{250} with NEM (0.5 mM) for 2 min and finally in M_{250} with diamide (0.5 mM) for 15 min (dotted columns). Controls were either treated with iodoacetate and NEM (blank columns) or with iodoacetate and diamide (cross-hatched columns). Bars indicate ranges. $n = 4$.

which appear to be of crucial importance for the effects of diamide. It was therefore interesting to ask whether this would also apply to membrane cross bonding and membrane stiffening.

To block membrane SH-groups red cells pretreated with iodoacetate were treated in M_{250} with 0.5 mM NEM for 2 min. After this time the reaction was stopped by addition of GSH stock solution to a final concentration of 0.5 mM. The time of treatment with NEM was chosen so that its effect on membrane stiffening and membrane cross bonding was small (Fig. 8a and b, left column).

The pretreatment with NEM blocked membrane stiffening, but not membrane cross bonding due to a subsequent treatment with 0.5 mM diamide for 15 min (Fig. 8). The strong increase in membrane cross bonding when NEM-pretreated red cells were treated with diamide (Fig. 8b, left and right columns) indicates that only a few SH groups were alkylated by the NEM pretreatment. This is in accordance with earlier measurements under similar conditions [20]. The suppression of membrane stiffening after this NEM pretreatment (Fig. 8a, middle and right columns), indicates that the membrane stiffening effect of a treatment with diamide depends on cross linking of a few crucial SH groups. The absence of a blocking effect on membrane cross bonding (Fig. 8b, middle and right columns) indicates that the spectrin modification responsible for bridge formation is much less specific.

The membrane stiffening effect of a 15 min treatment with diamide after blocking the crucial SH groups by a 2 min treatment with NEM is similar to that of the mere NEM treatment (Fig. 8a, left and right columns). This suggests that the type I modification of spectrin by diamide was completely blocked and that the type II

modification which is responsible for the increase in membrane cross bonding (Fig. 8b, left and right columns) is to a very small extent effective in membrane stiffening.

Cross bonding during membrane contact

The idea behind the following experiments was to find out whether type I modifications can induce membrane cross bonding by covalent cross linking of spectrin. To this end, the treatment was performed in M_{600} to bring opposing parts of the membrane into contact. All treatments that produced solely type I modifications (EDC, MDA and DMS) were used, because it is likely that all of them induce cross links in the plane of the membrane (see below).

The conditions for treatment were the same as in Fig. 3 except that the incubation medium was M_{600} instead of M_{250}. Before counting membrane bridges, the cells were washed three times with M_{250} because they were used in elongation measurements. After the washing, the percentage of bridged cells was in all cases no greater than 1%. Although these data may not be directly comparable to those in Fig. 3, since the washing procedure could have opened up some weak bridges, they do not support the possibility of membrane cross bonding by covalent cross linking by type I modifications of spectrin.

Discussion

Mode of action of the various treatments

The SH reagents can be grouped in the purely monofunctional and the potentially bifunctional reagents. The latter (diamide, DTDP and CDE) react by forming an adduct with a protein SH group which can then react with a further protein SH group to form a protein disulfide. The monofunctional reagents (NEM, $IANH_2$ and iodoacetate) add a small chemical group to a single protein SH group. The action of diamide, DTDP or CDE can be fully reversed by dithioerythritol, irrespective of whether the reaction was mono- or bifunctional. The effects of the purely monofunctional reagents are irreversible. As detailed above, DTDP at low concentrations acts bifunctionally, whereas with increasing concentrations, the portion of monofunctional modification appears to increase. CDE at the concentration used appears to induce mainly monofunctional modification. Diamide appears to produce exclusively protein disulfides. Similar conclusions have been reached by measuring the proportion of mixed and of protein disulfides after treatment with DTDP, CDE and diamide (Haest, RWTH Aachen, personal communication).

Two (potentially bifunctional) amino reagents (MDA and DMS) were used. MDA can form Schiff's bases with amino groups of proteins. This can lead to cross linking of membrane proteins, preferentially spectrin [22]. The imidoester DMS can react with primary amines of proteins which results in either cross linking or monofunctional modification of proteins [23]. Cross linking of spectrin was observed for dimethyl adipimidate [13], a closely related compound.

EDC reacts with carboxyl groups to form an unstable intermediate. This intermediate can be stabilized by isomerization or can react with an amino group [24]. The first case results in a monofunctional modification, the second results in cross linking of proteins. The proportion of the occurrence of these two reactions in EDC-treated red cells is unknown. Extensive cross linking between membrane proteins by EDC at acidic pH has been reported [25]. The strong effect in membrane stiffening indicates a fair amount of bifunctional modification even at pH 7.4.

Treatment with the oxidizing agents APH, H_2O_2 and BHP has been shown to deposit oxidized hemoglobin at the membrane [26,15,7]. Under the present conditions, the amount of membrane-bound hemoglobin was on average about the same after treatment with APH and BHP as after the non-oxidizing treatments. This indicates that membrane cross bonding and membrane stiffening by APH and BHP are due to membrane protein modifications, presumably by oxidative cross linking. In contrast, after treatment with H_2O_2 membrane-bound hemoglobin was considerably higher than in all other treatments applied. An additional effect of spectrin-hemoglobin cross linking [15] can therefore not be excluded.

Two treatments (by heat and with urea) were employed that denature proteins. Heat denaturation of spectrin in solution has been shown to start at very low temperature [27], to be partially reversible [28] and to concern only a small part of the molecule [29], the rest of the molecule being stable up to relatively high temperatures. Denaturation of spectrin in solution by urea has been shown to start at small concentrations and to be largely reversible [30].

A decrease of cytoplasmic pH towards the isoelectric point of spectrin reduces the net charge on this molecule. This has been shown to lead to aggregation of membrane skeletal proteins in red cell ghosts which was partially reversible upon restoration of normal pH [31].

Interpretation of spectrin modifications on a molecular level

Based on earlier data (for treatment with diamide, NEM, heat and urea and ATP depletion) the following working hypothesis for the mechanism of membrane cross bonding has been put forward [1,2]. Unspecific modifications of spectrin, which by analogy to heat and urea treatment were called 'denaturation', expose amino acid side chains hidden within the native molecule that mediate unspecific spectrin–spectrin interactions. If the interacting spectrin molecules belong to opposing mem-

brane areas in the discoid red cell, a membrane bridge can be formed. It was further hypothesized [2] that a partial breakup of the native associations of spectrin within the membrane skeleton and lateral aggregation of skeletal proteins which appear to be a consequence of spectrin 'denaturation' are involved in the process of membrane cross bonding. These working hypotheses are further substantiated by the results of the present work as detailed in the following.

The low specificity of spectrin modifications leading to membrane cross bonding is supported by the following observations. (i) Two additional sorts of membrane modification, oxidizing treatments (with APH, BHP and H_2O_2) and acidification produced membrane cross bonding as well (Fig. 3). (ii) Pretreatment with NEM which blocked specifically the membrane stiffening effect of a subsequent treatment with diamide (Fig. 8a) did not prevent membrane cross bonding due to diamide (Fig. 8b).

That breakup of network associations and lateral protein aggregation might not only be involved in membrane cross bonding, but might be a prerequisite for that event is suggested by the following findings. (i) Pretreatment with MDA partially blocked membrane cross bonding by a subsequent heat or diamide treatment (Fig. 5). This can be interpreted by a suppression of lateral aggregation as a consequence of stabilization of the membrane skeleton by protein cross linking with MDA. However, EDC (5 mM) did not block membrane cross bonding although its membrane stiffening effect was stronger. This may be due to an additional type II modification by EDC which becomes apparent at 10 mM. (ii) No membrane cross bonding was observed when the treatments with EDC, DMS and MDA were performed in M_{600}, where opposing parts of the membrane are in contact. If mere spectrin–spectrin interactions were sufficient for membrane cross bonding, one would have expected membrane bridges due to covalent cross linking in these cases. The absence of membrane cross bonding does at least not falsify the above hypothesis that additional factors are necessary for cross bonding.

The evidence presented above suggests that type II modification comprises spectrin 'denaturation' as well as a breakup of its network associations and subsequent lateral aggregation. Type I modification is suggested to be related to a covalent intra- and intermolecular cross linking within or between spectrin molecules. This is supported by the cross linking ability of MDA, DMS and EDC and by the suppression of the membrane stiffening effect of diamide by blocking a few SH groups with NEM.

Mechanisms of membrane stiffening

Membrane stiffening as a consequence of type I modification of spectrin can be explained in straightforward way on a molecular level [3]. Cross links within a single spectrin tetramer can be envisaged to decrease its flexibility. Cross linking between different tetramers introduces additional linkages within the membrane skeletal network. Both effects increase the stiffness of the network. The relatively large membrane stiffening effect of the acidification indicates that electrostatic interactions might have the same effect.

Membrane stiffening as a consequence of type II modification of spectrin is not self evident on a molecular level. Alkylation of SH-groups or other monofunctional modifications have been suggested to induce membrane stiffening by decreasing the flexibility of spectrin molecules [3]. However, if the modified spectrin tetramers desintegrate into dimers, as observed after treatment with NEM [32], a decrease in membrane stiffness is expected, because only spectrin tetramers that are linked at both ends with the network should contribute to its stiffness. Similarly, Chasis and Mohandas [33], after treatment with NEM, observed a decrease in membrane stability, which they attributed to a decrease in membrane skeletal associations, and at the same time an increase in membrane stiffness. To explain this contradiction, they postulated that membrane stability and membrane stiffness were independently regulated. However, they suggested no molecular mechanism for the increased membrane stiffness after treatment with NEM.

In the following, two molecular mechanisms for membrane stiffening after treatments that decrease membrane skeletal associations are presented which are purely hypothetical at present and which need substantiation in the future. (i) Denatured spectrin which is partly disconnected from the membrane skeleton becomes attached to intact spectrin tetramers that are still connected at both ends to the skeleton. This could impair the flexibility of these intact tetramers to such an extent that the loss of the contribution of the denatured spectrin is overcompensated and the net membrane stiffness increases. (ii) Lateral aggregation of the denatured spectrin molecules leads to a partial stretch of the remaining native spectrin tetramers between the aggregated patches. A complete stretch which can be visualized in the electron microscopic pictures obtained by Byers and Branton [34] would not allow any elastic shear deformation. It is therefore conceivable that in the intermediate stages the shear modulus is increased.

The two mechanisms suggested above take into account the molecular changes comprised in type II modification: denaturation of spectrin, lateral aggregation of skeletal proteins and splitting of network associations. The latter could also occur between spectrin and actin. The two mechanisms form a common molecular basis for membrane cross bonding, membrane stiffening and a decrease in membrane stability.

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