Sulfhydryl Reagents Induce Altered Spectrin Self-Association, Skeletal Instability, and Increased Thermal Sensitivity of Red Cells

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Incubation of erythrocytes with the sulfhydryl reagent N-ethyl-maleimide (NEM) results in altered spectrin self-association and formation of dimers on the membrane. Skeletons isolated from these cells exhibit marked skeletal instability. In addition, NEM treatment induces increased thermal sensitivity of both cells and purified spectrin. These effects were not produced in aerobically incubated cells. Treatment with NEM results in microspherocytosis, poikilocytosis, and increased red cell mechanical instability. These effects were not due to depletion of intracellular reduced glutathione. These effects were produced by another permeant sulfhydryl reagent, monobromobimane, but not by its membrane-impermeant derivative. We conclude that spectrin sulfhydryl groups play an important role in spectrin self-association and thermal stability.

MATERIALS AND METHODS

Incubation With Sulfhydryl Reagents

Erythrocytes were obtained from healthy volunteers by venipuncture, using heparin as anticoagulant. Cells were washed with saline and Buffy coats were removed. Cells were resuspended to 10% hematocrit in 5 mM sodium phosphate buffer, pH 7.4, containing 150 mM NaCl, for all treatments with sulfhydryl reagents. Cells were incubated with either N-ethylmaleimide (NEM), monobromobimane (MB; Thiolyte, Calbiochem, La Jolla, CA), monobromotritiated bimane (MB; Thiolyte, Calbiochem), or buffer for 15 min at 37°C, then washed and incubated with 5 mM dithiothreitol (DTT) for an additional 15 min at 37°C to remove unreacted reagent. Cells were then washed 3 times with buffer.

Membrane Protein Analyses

Red cell membranes were prepared by hypotonic lysis by the method of Dodge et al. Spectrin was extracted from these ghosts by overnight incubation at 0°C with 0.1 mM NaPO4, pH 8.0, containing 0.1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM N-α-p-tosyl-L-lysine chloromethyl ketone, and 0.1 mM β-mercaptoethanol. The supernatant extract and ghost residues were separated by centrifugation at 250,000 g for 35 min. Extracts were analyzed for spectrin species by nondenaturing gel electrophoresis, as previously described. Proteins of ghosts, ghost residues, and extracts were analyzed by electrophoresis on 5.6% polyacrylamide gels containing sodium dodecyl sulfate (SDS). Crosslinked complexes were analyzed on 1% agarose–2.5% acrylamide gels containing SDS in reducing conditions.

Skeletal Instability

Membrane skeletons were prepared from erythrocyte ghosts by incubation with 2% Triton X-100 for 15 min at room temperature. Mechanical stability was examined as previously described by shaking of samples at 1,500 oscillations/min at 4°C for varying times. Aliquots of skeletons were stained with 0.5% uranyl acetate and examined by phase-contrast microscopy.

Thermal Sensitivity

Thermal stability of erythrocytes was determined by heating 200-μl aliquots of intact cells (10% hematocrit in 5 mM NaPO4 buffer, pH 7.4, containing 150 mM NaCl) to 42°–52°C for 10 min. Heated cells were fixed with 1% glutaraldehyde in isotonic buffer and examined for vesiculation and fragmentation by phase-contrast microscopy.
Membrane Protein Crosslinking

Cells were preincubated with 2 mM NEM, washed, and then heated to 46°C for 15 min. Ghosts isolated from these fragmented cells were incubated at 50% suspension (v/v) with 0.4% glutaraldehyde in 5 mM NaPO₄ buffer, pH 7.4, for 10 min at 20°C. Ghosts were washed in the same buffer containing 2 mM EDTA, and membrane proteins were analyzed on SDS-agarose-acrylamide gels in reducing conditions.

Circular Dichroism (CD) Measurements

Crude spectrin was extracted from ghosts of NEM-treated cells by overnight incubation of ghosts in 0.1 mM NaPO₄ buffer, pH 8.0, containing 0.1 mM EDTA. Ghost residues were removed by centrifugation at 250,000 g for 35 min, and the spectrin-containing supernatant was made isotonic by addition of 1/3 volume of 390 mM NaPO₄, pH 7.4. CD measurements were made on a Cary 61 spectropolarimeter using a water-jacketed 1.0-mm cell. Cell temperature was determined by a thermistor attached to the sample holder during all measurements. Ellipticities of heated samples were measured at 222 nm, and molar ellipticity was calculated using 114 as average residue weight.

Quantitation of Free Sulphydryl Groups

Free sulphydryl groups of spectrin were quantitated colorimetrically by reaction with 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB; Eastman Kodak, Rochester, NY) by modifications of the method described by Wadsworth and Bloboda. Protein in 5 mM sodium phosphate, pH 7.4, was added either to an equal volume of denaturing solution, containing 8 M urea, 2.5 M EDTA, and 0.1 M sodium phosphate, pH 7.4, or to an equal volume of 5 mM sodium phosphate, pH 7.4. Tubes were shaken and an equal volume of 1 mM DTNB in 0.1 M imidazole, pH 7.4, was added. Samples were incubated in a 25°C water bath for 15 min, then absorbance was measured in a spectrophotometer at 412 nm versus a reagent blank. Sulphhydryl content was calculated using ε = 13,600 M⁻¹cm⁻¹ for the resultant DTNB product. Accuracy of the assay was monitored by quantitation of sulphydryl groups of reduced glutathione solutions or fresh preparations of 6s tubulin purified from calf brain (a gift of Dr. Brian Herman).

RESULTS

Spectrin Species in the Membrane

Figure 1 shows the spectrin species in 0°C extracts of ghosts prepared from red cells that were exposed to varying amounts of NEM at 37°C. As previously shown, spectrin extracts from untreated cells contained primarily spectrin tetramers (SpT), spectrin oligomers (SpO), and a high molecular weight complex (HMW) of spectrin, actin, and polypeptide 4.1. As measured by the intensity of Coomassie blue staining, SpT of untreated red cells comprised 51% ± 10% of the total extracted material, with HMW and SpO representing 44% ± 10%. Spectrin dimers (SpD) were 4% ± 2% of the total. In contrast, extracts from cells treated with ≥0.5 mM NEM contained significant amounts of SpD, with a concomitant decrease in SpT. The 0°C extract from 1 mM NEM-treated cells consisted of ~35% SpD, with SpT reduced to ~25% and HMW plus SpO remaining at 40%. The amount of SpD increased and SpT decreased proportionately to the NEM concentration, while the amount of HMW plus SpO remained relatively constant.

Although normal spectrin species are known to be kinetically trapped in solution at 0°C, we questioned whether increased dimers in 0°C extracts from NEM-treated cells might result from abnormal dissociation of tetramers at 0°C after release of spectrin from the membrane. To test this possibility, hypotonic spectrin extracts from untreated cells were incubated with NEM at 0°C for up to 3 hr. Under these conditions, no dimers were formed, suggesting that NEM treatment does not alter the behavior of spectrin in solution at 0°C. Therefore, the NEM-induced increase in dimers in low ionic strength 0°C extracts is presumably reflective of dimer formation on the membrane.

The protein composition of ghosts from treated cells was identical to that of controls. Although adherence of cytoplasmic proteins to the membrane was previously observed after prolonged (24-hr) incubation of cells with NEM, no adherence was apparent after the brief incubation used in our studies. Extractability of spectrin from NEM-treated cells was similar to that of controls (70%-80% of spectrin extracted) at NEM levels ≤2 mM. At higher concentrations, spectrin extractability was decreased, presumably due to interference of NEM treatment with low salt-induced release of spectrin from its binding site(s) on the membrane. NEM did not cause release of spectrin.
from the membrane prior to low ionic strength extraction in our studies, as evidenced by normal amounts of spectrin relative to band 3 in SDS-polyacrylamide gels of membranes from NEM-treated cells. This is consistent with previous reports.26,27

Mechanical Stability of Membrane Skeletons

The transformation of spectrin tetramers to dimers in the membrane is associated with mechanical instability of isolated skeletons.22 Therefore, we investigated whether NEM-induced perturbation of SpD self-association is also associated with a similar skeletal defect.

Isolated skeletons were subjected to mechanical shaking at 0°C for periods of up to 1 hr. After 15 min, skeletons from untreated cells were intact and retained their normal morphology (Fig. 2). However, skeletons from cells incubated with ≥0.5 mM NEM showed fragmentation, which was more pronounced with increased NEM concentrations, and the extent of fragmentation was proportional to the amount of spectrin dimers on the membrane in these cells (Fig. 2). All skeletons disintegrated completely with longer periods of shaking, with the lower doses of NEM resulting in 100% fragmentation only after 1 hr, similar to the fragmentation pattern of controls. The protein composition of isolated skeletons was similar to controls, consisting primarily of spectrin, actin, and polypeptide 4.1.

Thermal Stability of Intact Cells

In HPP, the defective spectrin self-association is accompanied by increased susceptibility of red cells to thermal damage, due to the altered thermal denaturation and aggregation of spectrin in these cells.11,12 We therefore investigated whether the changes in spectrin self-association in NEM-treated cells are accompanied by thermal sensitivity of these cells. Aliquots of cells were heated to temperatures between 42°C and 52°C (Fig. 3). As shown previously, untreated cells began to vesiculate at 49°C–50°C. However, cells treated with 2 mM NEM began to fragment at 46°C. Below this temperature, NEM-modified cells exhibited a normal discoid shape. Cells treated with concentrations of NEM that did not result in dimer formation in the membrane behaved as controls, and cells treated with an intermediate concentration of NEM (1 mM) vesiculated at 47°C–48°C. Therefore, the thermal instability of intact cells, the changes in SpD/SpT ratio, and membrane skeletal instability all exhibited a similar NEM concentration dependence.

Nearest-Neighbor Crosslinking of Heated Cells

We further investigated whether the increased sensitivity of NEM-modified cells to thermal fragmentation was associated with increased propensity of spectrin to aggregate in the membrane. Ghosts isolated from heated cells were subjected to mild crosslinking with 0.4% glutaraldehyde. Figure 4 shows gels of

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**Fig. 2.** Mechanical stability of membrane skeletons from NEM-treated cells. Skeletons prepared by Triton solubilization of ghosts from NEM-treated cells were subjected to mechanical shaking at 0°C for 15 min. Skeletons were stained with uranyl acetate and observed by phase-contrast microscopy. Dimers are expressed as percent of total protein in spectrin 0°C extracts from cells incubated with or without NEM.
membrane proteins from these cells. Membranes from heated NEM-treated cells showed significant amounts of crosslinked protein, whereas very little crosslinking occurred in either buffer-treated or unheated cells. Appearance of the crosslinked complexes corresponded with a slight reduction in intensity of spectrin bands 1 and 2 (mol wt = 240,000 daltons and 220,000 daltons, respectively), suggesting that spectrin is contained in the complex. Similar complexes could be formed in normal red cells by glutaraldehyde treatment of cells heated at 50°C, but not at lower temperatures (not shown). Disulfide crosslinkers, which could be cleaved to allow second dimension gel resolution of components in the complex, were not used, since they are themselves sulfhydryl reagents and could complicate interpretation of such results. However, in HPP cells, it was shown previously that similar complexes formed by oxidative crosslinking of cells heated to 46°C consisted primarily of spectrin. Our data suggest that NEM treatment lowers the thermal denaturation temperature of spectrin, which results in aggregation of spectrin in the membrane upon heating and thermal instability of the cells similar to that found in HPP.

Circular Dichroism of Spectrin From NEM-Treated Cells

To investigate directly whether thermal sensitivity of intact cells reflected altered temperature sensitivity of the spectrin molecule, the thermal denaturation of spectrin was examined by the technique of circular dichroism. Spectrin extracts from untreated cells and cells pretreated with 1 mM NEM were heated to temperatures between 40°C and 70°C. Molar ellipticity ([θ]) at 222 nm decreased with increasing temperature, reflecting unfolding of the spectrin molecule (Fig. 5). Normal spectrin showed a major structural transition centering at 49°C, as previously demonstrated. However, this structural transition was centered at 47°C for spectrin from cells treated with 1 mM NEM, which is similar to the transitional temperature...
Fig. 5. Circular dichroism of spectrin from NEM-treated cells. Spectrin isolated from NEM-treated cells was heated to temperatures between 40°C and 70°C, and ellipticity (θ) was measured at 222 nm (solid line). (A) Spectrin from cells treated with 1 mM NEM; (B) spectrin from cells incubated with buffer. First derivative (Δθ/ΔT) graphs generated by computer reveal a transitional midpoint for controls of ~49°C, with the midpoint for spectrin from NEM-treated cells ~47°C.

reported for HPP spectrin. Therefore, both sulphydryl-modified spectrin and HPP spectrin show altered temperature sensitivity.

Effect of GSH Depletion

Since NEM is known to alkylate intracellular reduced glutathione (GSH), we investigated the possibility that NEM-induced effects on red cells were due to indirect effects on spectrin through perturbation of intracellular GSH levels. To test this, we used red cells from patients with glucose-6-phosphate dehydrogenase (G6PD) deficiency, which are unable to regenerate GSH due to their inability to reduce NADP. G6PD-deficient red cells from 5 patients (G6PD Dothan28 and G6PD Birmingham,29 kindly supplied by Dr. J. Prchal; and 2 Black A variants) were subjected to GSH depletion by aerobic incubation at 37°C in ATP-maintaining conditions for up to 24 hr.30 Spectrin species extracted at 0°C from G6PD-deficient cells were similar to species extracted from normal cells, and there was no increase in dimer due to GSH depletion (not shown). Therefore, effects of sulphydryl reagents on spectrin equilibrium in the membrane were presumably not due to indirect effects on GSH depletion.

Effect of Reagent Permeability

Cells were also treated with the sulphydryl reagent monobromobimane (MB), which was previously shown to permeate intact red cells.31 As with NEM treatment, incubation of cells with MB resulted in appearance of SpD in 0°C extracts (Fig. 6). Extracts from cells treated with 1 mM MB contained 37% HMW + SpO, 38% SpT, and 25% SpD. These cells also exhibited skeletal instability and thermal sensitivity (not shown). Such effects were therefore not restricted to induction only by NEM.

Red cells were also incubated with a derivative of monobromobimane (MQ, see Materials and Methods), which is impermeant to intact erythrocytes.31 This reagent did not result in the presence of SpD in low ionic strength extracts from membranes of these cells (Fig. 6), suggesting that NEM and MB must react with a component inside the red cell (presumably

Fig. 6. Incubation of cells with permeant or impermeant sulphydryl reagents. Cells were incubated with 1 mM monobromobimane (MB), a permeant SH reagent, or with monobromotrimethylammonobimane (MQ), an impermeant derivative. The permeant MB resulted in dimer formation whereas the impermeant derivative was ineffective.
specrin itself) to induce effects on specrin self-association.

Spectrin Sulfhydryl Group Quantitation

To investigate directly whether spectrin sulfhydryl groups were involved in NEM-induced alteration of spectrin self-association, we examined the number of free sulfhydryls in spectrin isolated from NEM-treated cells. Unmodified spectrin, when assayed in denaturing conditions, contained approximately 28 sulfhydryl groups, of which 21 were accessible in the native molecule (Table 1). Spectrin extracted from cells pretreated with 0.1 mM NEM contained approximately the same number of free SH-groups as unmodified spectrin, within the resolution of the assay. However, incubation of cells with 0.5 mM NEM reduced the number of free SH-groups by approximately 13 in both denatured and native molecules. This concentration of NEM (0.5 mM) is near the minimum dose resulting in dimer formation on the membrane (see Fig. 1); therefore, SH oxidant-induced alterations of spectrin self-association may involve all or a fraction of these modified moieties.

DISCUSSION

Incubation of red cells with the sulfhydryl reagent NEM produced altered spectrin self-association, resulting in the presence of dimers on the red cell membrane. This was associated with mechanical instability of skeletons similar to that described for dimer-enriched ghosts and with thermal instability of intact cells. These effects were also produced by another membrane-permeant sulfhydryl reagent, monobromobimane, but not by the membrane-impermeant derivative, monobromotrimethylammoniobimane, suggesting that intracellular sulfhydryl groups are responsible for these changes. Altered spectrin self-association and skeletal properties were presumably not due to diminished intracellular GSH levels resulting from oxidation by NEM, since GSH depletion produced in G6PD-deficient cells by aerobic incubation did not induce such alterations. The altered thermal denaturation profile of spectrin extracted from cells treated with sufficient NEM to produce dimer formation suggests that the spectrin molecule itself is altered. In fact, approximately half the free sulfhydryl groups of spectrin were shown to be blocked after incubation of cells with 0.5 mM NEM.

Since dimer–dimer interaction involves noncovalent associations at the end of the dimer molecule, sulfhydryl group modification may have an effect on the structure of this region, resulting in altered affinity of dimers. Whether the effect of alkylation is steric or conformational is presently under investigation in our laboratory. Since the amount of α-helix in spectrin from untreated and dimer-enriched cells is similar at 37°C (from CD in Fig. 5), such conformational changes may be limited. Preliminary data from our laboratory suggest that binding of spectrin from NEM-treated cells to other red cell membrane proteins is unaffected under our conditions.

The similarities of NEM-induced effects and the characteristics of HPP suggest that changes in dimer–dimer association induced by NEM or through a separate mechanism by the HPP molecular defect result in similar instabilities. Since the defect in HPP spectrin has been localized to the 80,000-dalton tryptic peptide containing the dimer–dimer binding site, it will be of interest to examine the structure and properties of purified NEM-alkylated spectrin. Such studies are in progress in our laboratory.

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REFERENCES

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