Metabolic Dependence of Protein Arrangement in Human Erythrocyte Membranes. II. Crosslinking of Major Proteins in Ghosts From Fresh and ATP-Depleted Red Cells

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We have investigated changes in membrane protein organization of ATP-depleted red cells by employing intermolecular crosslinking of the neighboring membrane proteins with glutaraldehyde or catalytic oxidation. After oxidative crosslinking, the complexes of crosslinked proteins were separated by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS PAGE) and treated with a reducing agent, dithiothreitol (DTT), which subsequently cleaved the intermolecular disulfide bonds. The individual protein components of the crosslinked complexes were subsequently identified by two-dimensional SDS PAGE. Catalytic oxidation of ghosts from both fresh and anaerobically ATP-depleted red cells produced crosslinked oligomers of spectrin (heterodimers, trimers, and tetramers), oligomers of band 3 (dimers, trimers, and tetramers), complexes of 2 + 4.9, and dimers of 4.1, 4.5, and 5 (actin). In addition, crosslinked ghosts from ATP-depleted cells, but not from fresh red cells, contained an additional large molecular weight (>10^6 daltons) complex that was enriched in spectrin, suggesting a rearrangement of spectrin in the membrane to closer contacts. Maintenance of intracellular ATP stores during anaerobic incubation prevented the formation of this complex after subsequent crosslinking. In contrast, the propensity of ghosts to form this complex after crosslinking was not reversed by restoration of ATP level or by EDTA extraction of membrane-bound calcium. In addition to reducible membrane protein complexes, introduction of calcium (>0.5 mM) into fresh red cells by ionophore A23187 or into their ghosts produced a nonreducible large molecular weight polymer, which was shown by others to result from membrane protein crosslinking catalyzed by Ca^{2+}-stimulated transglutaminase. This crosslinking is markedly decreased in ATP-depleted cells due to inactivation of the cytoplasmic transglutaminase during ATP depletion. Our data indicate that the organization of spectrin and other polypeptides in red cell membranes depends on intracellular ATP and calcium levels.

Spectrin and actin, which form a two-dimensional network at the inner surface of the red cell membrane, are thought to participate in several ATP-dependent membrane phenomena, which include discocyte-echinocyte equilibrium, changes in viscoelasticity, and surface topology of membranes. In a previous communication, we reported that ATP-depleted red cells contained several heteropolymers of membrane proteins that were absent in fresh erythrocytes. These complexes resulted from spontaneous intermolecular disulfide couplings between the nearest membrane protein neighbors during aerobic ATP depletion. One of these complexes, the size of which was greater than 10^6 daltons,
contained high amounts of spectrin relative to band 3 and appeared to reflect directly an altered organization of spectrin and other proteins in ATP-depleted red cell membranes. In this article we analyze directly membrane protein organization of ATP-depleted red cells employing crosslinking of the nearest protein neighbor amino groups and sulfhydryl groups with glutaraldehyde and catalytic oxidation (CuSO₄, o-phenanthroline), respectively. We avoided the interference of concomitant spontaneous oxidation of membrane proteins (which occurs during aerobic ATP depletion) by incubating the cells under anaerobic conditions.

MATERIALS AND METHODS

Incubations
To deplete intracellular ATP, washed red cells (10% hematocrit) were incubated at 37°C under N₂ with shaking in buffer containing 50 mM glycylglycine (pH 7.4), 5 mM KCl, 2 mM CaCl₂, and NaCl to a final osmolity of 290-300 mosmol. To maintain (or rejuvenate) intracellular ATP, fresh (or ATP-depleted) red cells were incubated at 37°C under N₂ in the buffer containing adenine, inosine, and glucose. The details were described previously.³

Glutaraldehyde Crosslinking
Packed ghosts were prepared as previously described³ and suspended in 30 vol of 10 mM N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid (HEPES), pH 7.4, containing 2 mM glutaraldehyde and incubated at 25°C for 10 min. After terminating the reaction with 2 mM EDTA in 10 mM TRIS-HCl, pH 7.4, ghosts were recovered by centrifugation and subjected to sodium dodecylsulfate (SDS) polyacrylamide electrophoresis (PAGE), as described.³

Crosslinking by Catalytic Oxidation
Ghosts were normally reacted with 30 vol of a mixture containing CuSO₄ (10 μM) and o-phenanthroline (50 μM) at 25°C for 10 min. Reaction was terminated and membrane proteins dissolved in SDS without DTT and electrophoresed as described above. To examine the effect of Ca²⁺ on catalytic oxidation of isolated membranes, different concentrations of Ca²⁺ were added directly into the crosslinking mixture.

Transamidative Crosslinking
A23187 (final concentration of 10 μM) and Ca²⁺ (0-5 mM) were added to the fresh, ATP-depleted and ATP-repleted cell suspensions and incubated at 37°C for 30 min to induce the transamidative crosslinkings. To study the effect of hemolysate from fresh red cells on transamidative crosslinkings, ghosts from ATP-depleted cells were resuspended in hemolysate of fresh cells and incubated with 5 mM CaCl₂ at 37°C for 30 min. Ghosts were then isolated, dissolved in SDS with DTT (25 mM), and subjected to PAGE as described before.³

Two-Dimensional SDS-PAGE
The method has been described in detail previously.⁵ The first dimensional gel contained 2.5% polyacrylamide and 0.3% agarose. The second dimensional slab gel was composed of 8% polyacrylamide in a discontinuous buffer system of Laemmli.⁸ Densitometric scans were carried out with Scan Jr. of Helena.

Measurements of ATP and GSH
Red cell ATP and GSH were determined as previously described.¹⁰

RESULTS

Membrane Protein Crosslinking With Glutaraldehyde or Catalytic Oxidation
Membrane proteins from fresh and anaerobically ATP-depleted erythrocytes were subjected to SDS PAGE before and after crosslinking with glutaraldehyde.
ATP-DEPLETED RED CELLS

Fig. 1. Protein crosslinkings in ghosts from fresh and ATP-depleted erythrocytes. Ghosts (25 μg of protein) were reacted with 2 mM glutaraldehyde (gels III, IV) or CuP (10 μM CuSO₄ + 50 μM o-phenanthroline; gels V, VI) and fractionated in SDS-agarose (0.3%)-polyacrylamide (2.5%) composite gels. Gels I, III, and V were derived from fresh erythrocytes; gels II, IV, and VI were derived from ATP-depleted erythrocytes (37°C, 24-hr incubation under N₂ without glucose). Ghosts before crosslinking (gels I, II) or after CuP catalytic oxidation were electrophoresed without reduction. Glutaraldehyde-treated ghosts were reduced with DTT before electrophoresis. The major gel bands were numbered after Stock.¹ Molecular weight estimates on large protein complexes were obtained from the linear extrapolation of plots of unreacted membrane proteins.

(2 mM, 25°C, 30 min) or catalytic oxidation with CuSO₄ (10 μM) plus o-phenanthroline (50 μM, 25°C, 10 min) (Fig. 1). Under anaerobic conditions, intracellular GSH level was maintained at about 1.5 mM during ATP depletion. No oxidized protein complexes were detected in ghosts from anaerobically incubated cells (Fig. 1, gel II). This differs from aerobic incubation, which has been previously shown to decrease the intracellular GSH level (to <15% of preincubation value after 16 hr at 37°C) and produce spontaneously several oxidized protein complexes in the membrane.³

Crosslinking of fresh and anaerobically ATP-depleted cells with glutaraldehyde resulted in a formation of a set of new bands in the region of 450,000 daltons in both cell types. In ATP-depleted red cells, an additional large molecular weight complex (>10⁶) appeared at the origin of the gel (Fig. 1, gel IV); this complex was not present in fresh erythrocytes (Fig. 1, gel III). During the course of ATP depletion, the intensity of this component progressively increased (not shown). In addition, the intensity of several bands (such as 1, 2, 3, and 4.2) decreased in all the crosslinked samples.

Catalytic oxidation of isolated membranes with CuSO₄ plus o-phenanthroline, which induces membrane protein crosslinking by disulfide couplings of adjacent protein SH groups,¹¹–¹³ resulted in the formation of several bands in the 450,000, 670,000, and 900,000 dalton regions and a diffuse band of 175,000 daltons both in fresh and ATP-depleted cells (Fig. 1, gels V and VI). As in the case of glutaraldehyde crosslinking, catalytic oxidation of ghosts from ATP-depleted red cells produced an additional large molecular weight complex (>10⁶) that was absent in fresh erythrocytes. In ATP-depleted cells, this complex was first noted at approximately 16 hr of incubation, when ATP decreased to about 15% of the preincubation value and 68% ± 4% of cells lost their biconcave shape.

The reproducibility of the above differences between fresh and ATP-depleted cells was critically dependent on time and temperature during crosslinking and the concentration of catalysts or crosslinkers. Low concentrations of glutaraldehyde or CuSO₄ plus o-phenanthroline failed to produce the >10⁶ dalton aggregate in
A TP-depleted erythrocytes, in particular when the temperature during crosslinking was decreased to near 0°C (Fig. 2, gel II). In contrast, increased catalyst concentrations, temperature, or time of the reaction produced a similar large molecular weight complex in fresh erythrocytes (Fig. 2, gel V), but the amount of this complex was less than that in ATP-depleted red cell ghosts subjected to the same treatment. Thus, the major difference between fresh and ATP-depleted red cells appears to be in the kinetics of the crosslinking reaction.

**Identification of Membrane Proteins Crosslinked by Catalytic Oxidation**

To study the specific proteins involved in the CuSO₄ plus o-phenanthroline catalyzed membrane protein oxidation, the protein complexes in the first dimension were dissociated by DTT reduction, and the released constituents were analyzed electrophoretically in the second dimension. The results of the two-dimensional analysis of oxidized ghosts (10 μM CuSO₄, 50 μM o-phenanthroline, 25°C, 10 min) from fresh erythrocytes is illustrated in Fig. 3A. The stained replica of the first dimensional cylindrical gel is positioned horizontally on top of the slab gel. A reference sample of membrane proteins in the second dimension is shown on the left. The released constituents migrated as “off-diagonal” spots or accents, positioned in the same vertical line as the original complex. The complex of 450,000 daltons in the first dimension can be identified as spectrin I₂ heterodimer, as indicated by the presence of these polypeptides as “off-diagonal” spots positioned under the original complex. In addition, the agreement between the apparent molecular weight of the complex (450,000 daltons) and the sum of released constituents (240,000 and 215,000 daltons) further supports this conclusion. Likewise, the 260,000 dalton complex represented the crosslinking of spectrin component 2 and band 4.9 (molecular weight 46,000). The specific involvement of spectrin component 2 can be clearly identified when small amounts of ghost protein are applied for electrophoresis. Several additional complexes were also detected, which included (A) hetero-trimers and tetramers of spectrin; (B) dimers, trimers, and tetramers of band 3; and (C) dimers of bands 4.1, 4.2, and 5. A schematic diagram of the results is illustrated in Fig. 3B. Similar crosslinking results have
Fig. 3. Two-dimensional gel electrophorogram of CuP crosslinkings of ghosts from fresh erythrocytes (A) Ghosts (100 μg of protein) were oxidized catalytically with CuP and fractionated in the first dimension without DTT, as described in Fig. 1. Electrophoresis in the second dimension was performed in a slab, incorporating a DTT zone to cleave disulfide bonds. The pattern of a membrane sample applied directly is shown at the left for reference. The pattern obtained by electrophoresis in the first dimension is shown in a stained replicate gel placed at the top of the slab. (B) A schematic diagram of the crosslinking pattern. The spot under (5) is a glycoprotein.

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Fig. 4. Two-dimensional gel electrophorogram of CuP crosslinkings of ghosts from ATP-depleted erythrocytes. (A) A crosslinked sample (100 μg of protein), corresponding to gel VI in Fig. 1, was run as in Fig. 3A. (B) A schematic diagram of crosslinking pattern. (C) Densitometric scans of Coomassie blue stained proteins of a reference membrane sample (............), and protein constituents released from the >10^6 dalton aggregate after DTT reduction [______] as indicated by a star in gel A.
been reported with different oxidation conditions or different analytical gel systems.\textsuperscript{11,12}

Results of two-dimensional PAGE of membrane proteins from ATP-depleted red cells exposed to crosslinking by catalytic oxidation are shown in Fig. 4 A and B. ATP depletion was achieved by 24-hr incubation at 37°C under anaerobic conditions (N\textsubscript{2}). In addition to slow migrating complexes 1 + 2 (450,000 daltons) and 2 + 4.9 (260,000 daltons), which were also seen in crosslinked fresh red cell membranes, ATP-depleted erythrocytes exhibited a large molecular weight complex of >10\textsuperscript{6}. This complex was fully dissociated by DTT and contained spectrin, bands 3, 4.1, 4.5, 4.9, 5, 6, 8, and globin. The relative distribution of the individual membrane polypeptides in this complex was compared to the reference sample of normal membrane proteins. It can be seen from densitometer scans (Fig. 4C) that this complex was selectively enriched in spectrin; the ratio of spectrin to band 3 in this complex was about 2.2, which is considerably more than the value of 1.0 found in normal red cell membranes.

\textbf{The Role of ATP and Ca\textsuperscript{2+} in Protein Crosslinking}

The formation of the >10\textsuperscript{6} dalton complex in ATP-depleted cells suggested the possibility that spectrin rearranges into closer contacts, thereby increasing its propensity to crosslinking. We investigated to determine whether or not this process is reversible with ATP repletion, as are some of the cellular properties such as shape and deformability.\textsuperscript{14}

The maintenance of intracellular ATP content during incubation effectively prevented the subsequent formation of spectrin-enriched polymer in the membrane (Fig. 5, gel I). However, when red cells were first depleted in ATP and their ATP content was subsequently restored by incubation with adenine, inosine, and glucose, the crosslinking of ghosts from such repleted cells by catalytic oxidation still produced a significant amount of the same large molecular weight aggregate (>10\textsuperscript{6} daltons) as that of ATP-depleted cells (Fig. 5, gel II–V). Unlike the crosslinking results, most of echinocytes were reversed to discocytes after ATP rejuvenation (Fig. 5).
Red cells undergoing ATP depletion in calcium-containing media exhibit a net calcium accumulation.\textsuperscript{14} We examined the effect of external calcium on crosslinking pattern of ghosts from ATP-depleted erythrocytes. The absence of external Ca\textsuperscript{2+} in the incubation medium did not prevent the formation of the spectrin-enriched polymer in CuSO\textsubscript{4}, o-phenanthroline crosslinked ghosts from ATP-depleted cells (data not shown), indicating that a net Ca\textsuperscript{2+} accumulation was not necessary for the formation of the spectrin-enriched polymer. Since normal red cells contain approximately 3 \textmu{mole}/liter of exchangeable calcium, presumably in the form of a Ca\textsuperscript{2+}–ATP complex,\textsuperscript{15} we have conducted additional experiments to test the possibility that the changes in membrane protein organization of ATP-depleted erythrocytes were related to interaction of these small amounts of calcium with the membrane. We have therefore examined oxidative membrane protein crosslinking of fresh hemoglobin-free ghosts in the presence of Ca\textsuperscript{2+} (0–2 mM). Figure 6 shows that Ca\textsuperscript{2+} did not promote formation of large molecular weight complexes of membrane proteins unless very high concentrations of Ca\textsuperscript{2+} (>2 mM) were added.

We have further tested to discover whether or not the propensity of the catalyst crosslinked ATP-depleted red cell ghosts to form the spectrin-rich polymer can be abolished by extracting calcium which accumulates in membranes of such cells. EDTA extraction of membrane-bound calcium from ATP-depleted red cell ghosts...
Fig. 7. The effect of EDTA on protein crosslinking of ghosts from ATP-depleted erythrocytes. Fresh and ATP-depleted red cell ghosts (25 μg of protein) were subjected to CuP crosslinking. Ghosts from ATP-depleted red cells were treated with 2 mM EDTA at 0°C for 5 min, washed 3 times with ice-cold 10 mM HEPES buffer to remove EDTA (pH 7.4), and then reacted with CuP as in Fig. 1 gel IV.

failed to decrease their original propensity to form a spectrin-enriched polymer after oxidative crosslinking (Fig. 7).

Captured Transamidative Membrane Protein Crosslinkings

The introduction of Ca²⁺ into fresh red cells in concentrations higher than 1 mM with ionophore A23187 or into ghosts during hypotonic hemolysis has been shown to induce formation of large molecular weight (>10⁶ daltons) membrane protein polymers, which were resistant to dissociation by DTT reduction. These have been recently shown to result from a crosslinking of γ-glutamyl and ε-lysine residues of the nearest neighbor proteins by a calcium-activated cytoplasmic transglutaminase. We explored transglutaminase-mediated protein crosslinkings in fresh and ATP-depleted cells exposed to increased Ca²⁺ concentrations. Fresh, ATP-depleted, and ATP-repleted red cells were treated with ionophore A23187 and Ca²⁺ at concentrations up to 5 mM, and their ghosts were dissolved in SDS in the presence of DTT and fractionated by SDS-PAGE. We observed that the Ca²⁺-induced nonreducible protein crosslinkings diminished dramatically in metabolically depleted cells and was not restored by ATP repletion (Fig. 8). The propensity of ATP-depleted cells to form this nonreducible polymer was in part corrected when a ghost-free hemolysate from fresh red cells was added to ghosts from ATP-depleted cells (Fig. 9). This suggested that the decreased transamidative crosslinking of ghosts from ATP-depleted cells was related to decreased transglu-
Fig. 8. Ca\(^{2+}\)-induced transamidative protein crosslinkings in fresh, ATP-depleted, and ATP-repleted erythrocytes. Washed erythrocytes were treated with A23187 (10 \(\mu\)M) and Ca\(^{2+}\) (0-5 mM) for 30 min at 37°C. Ghosts (25 mg of protein) from these cells were dissolved in SDS with DTT (25 mM) and electrophoresed as in Fig. 1 gel III.

Fig. 9. Effect of fresh hemolysate on transamidative crosslinkings in ghosts of ATP-depleted erythrocytes. (A) Control ghosts from fresh erythrocytes lysed and incubated in 10 mM Tris, 5 mM Ca\(^{2+}\), pH 7.4, at 37°C for 30 min. (B) Control ghosts from ATP-depleted erythrocytes after incubation, as in (A). (C) Ghosts from ATP-depleted erythrocytes incubated with ghost-free hemolysate from fresh erythrocytes in the presence of Ca\(^{2+}\) (5 mM) as in (A).

taminase reactivity rather than changes in accessibility of the membrane protein substrates in ATP-depleted cells. The failure of ATP-repleted cells to restore their propensity to form the transamidative crosslinkings (Fig. 8) suggests an irreversible inactivation of the enzyme.

DISCUSSION

In a previous communication, we reported that human erythrocytes depleted in ATP under aerobic conditions exhibited a large molecular weight (>10⁶) complex of membrane proteins that was selectively enriched in spectrin relative to band 3.³
The formation of this complex required a depletion of red cell ATP, GSH, and aerobic incubation. We proposed that this aggregate formed as a result of a rearrangement of spectrin and other membrane proteins to closer contacts followed by a spontaneous oxidative coupling of the neighboring protein sulfhydryl groups. To support this hypothesis we now directly examine membrane protein organization of ATP-depleted erythrocytes by the technique of exogenous protein crosslinking of ghosts from fresh and ATP-depleted erythrocytes. We employed anaerobic incubation, under which intracellular GSH remained at the preincubation level and membrane proteins remained reduced. The use of anaerobic conditions somewhat delayed the onset of discocyte–echinocyte transformation and increase in viscosity during ATP depletion as previously reported; however, after 16–18-hr incubation, 90% of ATP-depleted erythrocytes transformed to stage 2–3 echinocytes.

Ghosts from such ATP-depleted cells, as compared to fresh red cells, exhibited an increased propensity to form a large molecular weight polymer (>10^6 daltons) of membrane proteins both upon glutaraldehyde or oxidative crosslinkings. The composition of the polymer produced by catalytic oxidation with CuSO₄, o-phenanthroline was similar to that produced spontaneously by aerobic ATP depletion; namely, it exhibited a spectrin to band 3 ratio of about 2.2, which is significantly higher than the value of 1.0 found in the membrane.

This increased propensity of spectrin and other proteins to intramolecular crosslinking can be attributed to the following possibilities: (1) an increased lateral mobility of these proteins in the membrane increasing their random thermal collisions, (2) an exposure of previously masked –SH and –NH₂ groups resulting from protein conformational changes, and (3) a rearrangement of spectrin into closer contacts. The first possibility appears unlikely because the increased propensity of ATP-depleted red cell ghosts to crosslinking was not normalized by an increase of catalyst concentration (i.e., 50 μM CuSO₄, 250 μM o-phenanthroline) at the lower temperature (0°C) (Liu and Palek, unpublished observation) at which the lateral mobility of membrane proteins was dramatically reduced. Furthermore, lateral mobility of band 3, which is indirectly associated to spectrin via a polypeptide 2.1, was found to be reduced rather than increased after metabolic depletion.

The second and third possibilities cannot be easily distinguished at the present time. However, we favor the explanation of rearrangement of spectrin into closer contacts in ATP-depleted cells, because the increased propensity of protein crosslinkings was observed with two different classes of crosslinking agents, e.g., those crosslinking –NH₂ and –SH groups, respectively.

The formation of the >10^6 dalton polymer is apparently not controlled by intracellular Ca²⁺. This is supported by the observations that (1) the formation of the spectrin-rich polymer in ghosts from ATP-depleted cells was not influenced by exclusion of calcium from the incubation medium; (2) treatment of washed, white ghosts from fresh red cells with Ca²⁺ concentrations lower than 2 mM failed to produce similar polymer; and (3) extraction of calcium accumulating in membranes of ATP-depleted red cells with EDTA failed to prevent a subsequent formation of this polymer.

The incubation of red cells in ATP-maintaining buffer effectively prevented the formation of the spectrin-rich polymer, indicating that the abnormal protein crosslinkings in ATP-depleted cells was not an artifact of incubation. However, the
changes in crosslinking propensity of ATP-depleted cells were not reversed by regeneration of ATP (Fig. 5). This suggests that spectrin and other membrane proteins undergo an irreversible aggregation during ATP depletion. The reason for this irreversibility is not known and can be speculatively attributed to a denaturation of a small fraction of spectrin in ATP-depleted red cell membranes followed by its aggregation. However, such spectrin aggregation does not inhibit the restoration of biconcave shape of ATP-depleted erythrocytes after a subsequent regeneration of ATP stores, presumably because only a small fraction of spectrin is aggregated.

In addition to membrane protein crosslinking by exogenous agents, an endogenous crosslinking has been recently identified that is catalyzed by a Ca\textsuperscript{2+}-activated cytoplasmic transglutaminase. Others speculated that this membrane protein crosslinking may contribute to increased deformability of red cells containing increased amounts of calcium, such as ATP-depleted red cells. We have therefore compared the susceptibility of fresh and ATP-depleted red cells to Ca\textsuperscript{2+}-mediated transamidative crosslinking. ATP-depleted red cells exhibited a decreased propensity to transamidative membrane protein crosslinking, which was not reversible after ATP repletion. This decreased crosslinking appears to result from an inactivation of a cytoplasmic transglutaminase in ATP-depleted red cells rather than changes in accessibility of the protein substrates in the membrane (Figs. 8 and 9).

Although the organization of spectrin in the membrane depends on red cell ATP content, our data suggest that it does not directly contribute to changes in red cell shape. Likewise, a recent reevaluation failed to demonstrate any changes of intrinsic membrane deformability of red cells undergoing ATP depletion. This suggests that the above reorganization of spectrin in membranes of ATP-depleted cells does not have major effects on intrinsic deformability properties of normal erythrocytes. Discocyte-echinocyte transformation is characteristically produced by multiple factors, which include the changes in pH, charge, osmolarity, incorporation of various amphipathic agents into the membrane bilayer, and changes in metabolic state of the cells. Likewise, metabolically depleted red cells exhibit multiple alterations which, in addition to changes in spectrin organization, include a decrease in spectrin phosphorylation and in spectrin extractability, attachment of cytoplasmic proteins to the membrane, changes in phospholipid composition, and asymmetric loss of membrane proteins and, in part, lipids from the membrane. Although most of these alterations have been well characterized, none of these can be directly implicated in the shape and deformability alterations of ATP-depleted erythrocytes and their individual contribution remains to be defined.

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