Protein 4.1 in Sickle Erythrocytes

EVIDENCE FOR OXIDATIVE DAMAGE*

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Sickle erythrocytes are known to undergo excessive auto-oxidation, resulting in the generation of increased intracellular levels of several species of free radical oxidants. This environment is likely to enhance the accumulation of oxidative lesions by membrane components, although, as yet, this has been shown directly only for the sickle membrane phospholipids.

We examined the oxidative status of protein 4.1, a major component of the human erythrocyte protein skeleton. We found that protein 4.1 isolated from sickle erythrocytes bound ~4-fold less to protein 4.1-stripped membranes than did the normal protein. The binding defect was inherent in the sickle protein and not in its membrane-binding site(s) since normal protein 4.1 bound to sickle protein 4.1-stripped inside-out vesicles similar to normal protein 4.1-stripped inside-out vesicles. Sickle membranes, in particular spectrin-depleted inside-out vesicles, contained less protein 4.1 than normal membranes. Purified sickle protein 4.1 contained 20–40% high molecular weight aggregated protein (M, > 200,000), whereas the purified normal protein contained ~10% high molecular weight protein. The high molecular weight sickle protein was immunoreactive with antibodies to protein 4.1 but not with antibodies to spectrin, ankyrin, band 3, glycoporphin, or hemoglobin, suggesting that the high molecular weight protein was cross-linked protein 4.1 and not a complex of protein 4.1 and some other membrane protein(s).

Purified sickle protein 4.1 was eluted from an anion-exchange resin at a higher salt concentration than normal protein 4.1. Oxidizing normal protein 4.1 with diamide resulted in an anion-exchange elution pattern similar to the sickle protein, suggesting that oxidation can affect protein surface charge. Activated thiol beads bound one-half as much sickle protein 4.1 as normal protein 4.1 when both were solubilized directly from membranes, demonstrating that thiol oxidation had occurred in vivo. Direct quantification of protein thiols revealed that the sickle protein contained ~1–2 mol% fewer cysteines/protein 4.1 monomer than did the normal protein. By amino acid analysis, sickle protein 4.1 was found to contain less methionine and tyrosine than did the normal protein and contained ~1 mol% cysteic acid, whereas the normal protein did not contain any cysteic acid.

Taken together, our results strongly suggest that sickle protein 4.1 has sustained oxidative damage in vivo. This damage can alter the functional properties of the sickle protein and may be an underlying factor in the myriad of membrane abnormalities reported in sickle erythrocytes.

The erythrocyte membrane skeleton, a filamentous network of proteins underlying the cytoplasmic membrane surface, is essential for the maintenance of erythrocyte membrane integrity (1). The major components of the human erythrocyte membrane cytoskeleton are the proteins spectrin, actin, and protein 4.1 (1). Abnormal interactions between these proteins may result in a compromised cytoskeleton, one manifestation of which could be a reduced erythrocyte life span (1). Considerable evidence suggests that chemical agents which alter protein redox status can adversely affect cytoskeletal protein-protein interactions. For example, thiol-reactive reagents, such as diamide (azodicarboxylic acid bis[dimethylamide]) or N-ethylmaleimide (NEM), can adversely affect self-association interactions between spectrin dimers (2), as well as interactions between spectrin dimers and protein 4.1 (3). In addition, diamide-induced oxidation of erythrocyte cytoskeletal proteins results in a partial interbilayer rearrangement of membrane phospholipids (4), and exposure of erythrocytes to hydrogen peroxide results in increased cross-linking between spectrin and hemoglobin, morphologic alterations, decreased cell deformability, and increased membrane rigidity (5).

Since sickle erythrocytes are known to generate excessive amounts of activated oxygen species (6), we suspected that sickle cytoskeletal proteins might be particularly vulnerable to oxidative damage. We focused our studies on examining the redox status of protein 4.1 from sickle cells. We reasoned that the close physical proximity of protein 4.1 to cytoplasmic membrane phospholipids, which by virtue of their increased unsaturation (7) are particularly sensitive to peroxidative damage (8), could make protein 4.1 particularly vulnerable to oxidative damage. We found that protein 4.1 isolated from sickle erythrocytes was oxidatively damaged. This damage manifested itself both structurally (increased amount of high molecular weight protein aggregates > 200,000, abnormal thiol redox status, and generation of oxidized amino acids) and functionally (decreased content of the protein in sickle ghosts and IOVs, decreased binding to protein 4.1-stripped membranes).

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†The abbreviations used are: NEM, N-ethylmaleimide; DTT, dithiothreitol; IOVs, inside-out vesicles; NaDODSO4−PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; DFP, diisopropyl fluorophosphatase; HSVs, high salt vesicles; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; NaNs, sodium azide.
branes, and abnormal binding to anion-exchange and thiol-binding resins).

**EXPERIMENTAL PROCEDURES**

**Materials**—[3H]Tricine, 125I-labeled Bolton-Hunter reagent, and N-ethyl-N-(3-cysteinyl)maleimide were from Du Pont-New England Nuclear. Dialysis tubing was Spectrapor-2 from Spectrum Medical (Los Angeles, CA). DTT, diamide (azodicarboxylic acid bis(dimethylamide)), and Ficoll 400 were from Sigma. DE52 anion-exchange resin was from Whatman, Ltd. (Maidstone, Great Britain). Thiol-activated Sepharose 4B was from Pharmacia Biotechnology, Inc. All other chemicals were ultrapure from Dionex Corp. (Sunnyvale, CA). All gel electrophoresis and immunoblotting reagents were electrophoresis-grade from Bio-Rad. All other chemicals were reagent-grade from standard sources.

**Blood Collection and Ghost Preparation**—Fresh blood samples (400 ml) from patients with sickle cell disease (Hbs) were obtained during exchange transfusions after obtaining informed consent. The units were collected into acid/citrate/dextrose, and a sample of each was analyzed by either celluose acetate strip electrophoresis in Tris/ Veronal buffer (9) or isoelectric focusing on agarose gels (10) to verify Hbs homogeneity. All samples were washed with PBS containing 0.2% DTT followed by PBS containing a new methylen blue staining (11). Only sickle cell patients with normal glucose-6-phosphate dehydrogenase levels were used in these studies. For some experiments, blood was also obtained from a high reticulocyte patient (autoimmune hemolytic anemia, ~25% reticulocytes).

**Preparation of Erythrocyte Membranes**—Erythrocyte ghost membranes were prepared from PBS (5 mM sodium phosphate, 150 mM sodium chloride, pH 7.4) washed by hypotonic hemolysis (12). Protease inhibitors (0.4 mM DFP and 0.5 mM EDTA) were present throughout the preparation. Spectrin-depleted IOVs were prepared by low ion strength extraction of ghosts (13). The siddead of IOVs was determined by glyceraldehyde-3-phosphate dehydrogenase activity measured in the presence or absence of 0.2% Triton X-100 (14). Protein 4.1-depleted high salt vesicles (HSVs) were obtained by extraction of IOVs with 1 M potassium iodide (15). These HSVs were >90% depleted of protein 4.1.

**Assay of Protein 4.1 Content of Erythrocyte Membranes**—The amount of protein 4.1 in erythrocyte ghost and IOV membranes was determined by subtracting 20-μg samples to NaDodSO4-PAGE (9% acrylamide) under reducing conditions using the discontinuous buffer system of Laemmli (16). The separated proteins were stained with Coomassie Brilliant Blue, and the stained gels were analyzed by densitometry using an LKB 2222 GSXL laser densitometer and GSXL/ID software (LKB, Uppsala, Sweden).

**Purification of Protein 4.1**—Protein 4.1 was purified from erythrocytes by the method of Tyler et al. (17), with modifications by Cohen and Foley (18). For most experiments, protein 4.1 was purified from PBS-washed ghosts that had been pretreated with 5 mM DTT and 350 mM sodium chloride. After washing, the washing was removed by dialysis in quick-dialysis membranes (Spectrapor-2) against Buffer B for 2 h immediately prior to its addition to the [35S]NEM-containing incubation mixture. This procedure resulted in a concentration of DTT in the dialyzed protein of <0.1 mM. Removal of DTT prior to incubation with [35S]NEM was necessary since DTT competes with the protein for NEM-binding sites. Binding of [35S]NEM to the reduced (+DTT) protein, both in the presence and absence of 2% NaDodSO4, was determined as for the −DTT protein. Following the incubation, aliquots of the reaction mixture containing similar amounts of protein were spotted onto cellulose filters, and the bound labeled protein eluted using steps gradients of potassium chloride, and analyzed by NaDodSO4-PAGE under reducing conditions as described above.

**Chemical Labeling of Protein 4.1 Thiols**—Purified protein 4.1 was dialyzed at 4 °C for 18 h against 5P8 containing 1 mM DTT, 0.1 mM EGTA, and 2 μg/ml phenylmethylsulfonyl fluoride (Buffer A), followed by extensive dialysis for 48 h against a similar buffer without DTT (Buffer B). Portions (5 μg) of this −DTT protein were then reacted with [35S]NEM (13 μM) in 5P8 with or without 2% NaDodSO4 for 1 h at 25 °C (23). The molar ratio of NEM to protein 4.1 in the reaction mixture was 100:1. The remainder of the −DTT protein 4.1 was dialyzed at 4 °C overnight with Buffer B (+DTT), and then removed by dialysis in quick-dialysis membranes (Spectrapor-2) against Buffer B for 2 h prior to its addition to the [35S]NEM-containing incubation mixture. This procedure resulted in a concentration of DTT in the dialyzed protein of <0.1 mM. Removal of DTT prior to incubation with [35S]NEM was necessary since DTT competes with the protein for NEM-binding sites. Binding of [35S]NEM to the reduced (+DTT) protein, both in the presence and absence of 2% NaDodSO4, was determined as for the −DTT protein. Following the incubation, aliquots of the reaction mixture containing similar amounts of protein were spotted onto cellulose filters, and the bound labeled protein eluted using steps gradients of potassium chloride, and analyzed by NaDodSO4-PAGE under reducing conditions as described above.

**Thiol-Dissulfide Exchange Chromatography**—NaDodSO4-solubilized (2% final concentration) ghost membranes (20 μg) were boiled for 1 min and mixed with Sepharose 4B thiol-activated gel (1 ml of semidry gel/2 ml of protein solution in 0.2 M NaCl, 0.2 M sodium phosphate, pH 6.4, containing 2% NaDodSO4, 0.4 mM DFP, and 0.1 mM EDTA) in tightly capped 10 ml-stripped test tubes. The tubes were flushed with argon essentially as described by Rank et al. (24). For some experiments, the solubilized proteins were reduced with 10 mM DTT for 48 h at 4 °C in sealed argon-flushed tubes prior to mixing with the gel. In this case, the reduced proteins were dialyzed against a large excess of gel buffer to remove the DTT immediately prior to mixing with the gel since DTT interferes with protein-gel binding. The gel/protein mixture was then gently agitated for 18 h at 25 °C, following which time the columns were drained and the filtrate was collected (unbound protein), dialyzed against 5P8 for 2 h using Spectrapor-2 dialysis membranes, buphosphilized, resolved in 0.1 ml of 5P8, and analyzed by NaDodSO4-PAGE (20 μg) on 9% acrylamide gels. The column was then washed with 30 ml of gel wash buffer (5P8 containing 2% NaDodSO4, 0.4 mM DFP, and 0.1 mM EDTA) which was discarded. The gel-bound protein was eluted from the column by incubation with 1 ml of gel wash buffer containing 2.5 M ammonium thiocyanate under argon for 1 h to 25 °C, followed by dialysis and washing the column twice with 3 ml of the same buffer. The
Molecular Weight

Aggregates—We characterized the purified protein 4.1 as the ratio of protein 4.1 to total membrane protein or to band 3 and protein 4.1 stripped from normal or sickle IOVs was evaluated by analyzing binding sites was further demonstrated by the similar binding density of Coomassie Blue-stained gels. Results are presented with quantitative densitometry and autoradiography. As shown in Table I, protein 4.1 represented 4.5 ± 0.8% of normal ghost and 7.3 ± 1.5% of normal IOV protein. The amount of protein 4.1 in sickle membranes was found to be decreased compared to normal membranes; sickle ghosts contained 3.9 ± 1.1% protein 4.1, whereas sickle IOVs contained 5.1 ± 0.6% protein 4.1 (differs from normal IOVs at p < 0.001). The decreased amount of protein 4.1 in sickle IOVs was not simply a function of reticulocytosis since high reticulocyte control (25% reticulocytes) had normal amounts of protein 4.1 (data not shown).

Decreased Binding of Sickle Protein 4.1 to Protein 4.1-stripped Membranes—The binding of radiiodinated purified protein 4.1 to protein 4.1-stripped membranes (HSVs) prepared from normal or sickle IOVs was evaluated by analyzing the reassociated membranes on NaDodSO4-PAGE combined with quantitative densitometry and autoradiography. As shown in Figs. 1 and 2, purified sickle protein 4.1 bound 4-fold less to both normal and sickle HSVs than did purified normal protein 4.1 (p < 0.001). That the binding defect was associated with the sickle protein 4.1 and not its membrane-binding sites was further demonstrated by the similar bindings of purified normal protein 4.1 to either normal or sickle HSVs. These results agree with the sickle membrane analysis which showed that the protein 4.1 content of sickle IOVs was significantly reduced. The decreased membrane binding of sickle protein 4.1 was not due to increased proteolysis during the binding assay since autoradiograms of the protein-bound membranes did not demonstrate any increased amount of sickle compared to normal protein 4.1 degradation products (Fig. 2B).

Sickle Protein 4.1 Contains Increased Amounts of High Molecular Weight Aggregates—We characterized the purified sickle protein 4.1 to determine whether its reduced membrane binding was related to abnormalities in its physical state. As shown in Table II, both the normal and sickle protein contained some high M, aggregates (>200,000). For the normal protein, high M, protein represented ~10% of the total protein; whereas for the sickle protein, high M, protein represented between 20 and 40% of the total protein depending on whether or not the protein was isolated under reducing or nonreducing conditions. The high M, aggregate was not simply a function of reticulocytosis since protein 4.1 isolated from high reticulocyte controls (autoimmune hemolytic anemia, ~25% reticulocytes) did not contain above-normal levels of high M, protein (data not shown). To determine whether sickle high M, protein was artifically generated during its purification, we compared the amount of high M, protein present in protein 4.1 isolated from erythrocytes that had been pretreated with NEM prior to protein purification. Our rationale was that by covalently binding to protein thioles in the native protein, NEM pretreatment should prevent the artifactual oxidation of thiols during protein purification. We found that whereas NEM pretreatment had no effect on the amount of high M, protein in the normal protein, it reduced to ~20% the amount of high M, protein in the sickle protein. The high M, protein 4.1 that could not be reduced with DTT presumably represents cross-linking between amino acids other than cysteine.

Immunologic Characterization of Sickle Protein 4.1 High M, Aggregates—The presence of sickle protein 4.1 high M, aggregates was also demonstrated on immunoblots of the purified sickle protein (Fig. 3). That the high M, protein did not represent some other membrane protein, such as spectrin, ankyrin, band 3, glycophorin, or hemoglobin, was shown by the absence of immunoreactivity with antibodies against these proteins (data not shown).

Sickle Protein 4.1 Has an Altered Surface Charge—Purification of normal or sickle protein 4.1 under reducing condiments showed that the purified protein 4.1 from normal or sickle erythrocytes was radiiodinated with Bolton-Hunter reagent. Various concentrations of 125I-protein 4.1 (0-60 µg/ml) were incubated with 20 µg of normal or sickle protein 4.1-stripped IOVs (HSV, >90% depleted of protein 4.1) in 0.2 ml of 5 mM sodium phosphate, pH 8.0, 130 mM KCl, 10 mM NaCl, 1 mM EDTA, 0.2 mM DTT, 1 mg/ml bovine serum albumin, and 2 mM NaN3 (incubation buffer) for 2 h at 4°C. Following the incubation, the HSVs were collected by centrifugation, washed once with 20 volumes of incubation buffer, and analyzed by NaDodSO4-PAGE and autoradiography. Combinations tested include normal protein 4.1 and normal HSVs (O), normal protein 4.1 and sickle HSVs (●), sickle protein 4.1 and normal HSVs (□), and sickle protein 4.1 and sickle HSVs (X). Results are the average of duplicate experiments which varied by <5%.

![Fig. 1: Binding of purified protein 4.1 to protein 4.1-stripped IOVs.](image)
FIG. 2. A, NaDodSO₄-PAGE (9% acrylamide) of protein 4.1-HSV binding shown in Fig. 1. Gels were run under reducing conditions. Lanes 1–9 contained normal HSVs (20 µg) and increasing amounts of added normal ¹²⁵I-protein 4.1 (0–60 µg/ml). Lanes 10–19 contained normal HSVs (20 µg) and increasing amounts of added sickle ¹²⁵I-protein 4.1 (0–60 µg/ml). Lane 20 contained 2.5 µg of bovine serum albumin that was used to quantitate protein 4.1 in the gels. B, autoradiogram of A. The vacuum-dried gel was exposed to Kodak XAR-15 film for 90 min at 25 °C. HMW, high Mr.

TABLE II
High molecular weight aggregated protein in normal and sickle protein 4.1 preparations

<table>
<thead>
<tr>
<th>Protein 4.1 source</th>
<th>Purification conditions</th>
<th>n</th>
<th>Protein 4.1 high Mr. M₄ (%) of total protein 4.1</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA erythrocytes</td>
<td>−NEM, −DTT</td>
<td>7</td>
<td>10 ± 4</td>
</tr>
<tr>
<td></td>
<td>−NEM, +DTT</td>
<td>19</td>
<td>13 ± 2</td>
</tr>
<tr>
<td></td>
<td>+NEM, −DTT</td>
<td>4</td>
<td>9 ± 5</td>
</tr>
<tr>
<td>SS erythrocytes</td>
<td>−NEM, −DTT</td>
<td>7</td>
<td>40 ± 10⁺</td>
</tr>
<tr>
<td></td>
<td>−NEM, +DTT</td>
<td>19</td>
<td>23 ± 4⁺</td>
</tr>
<tr>
<td></td>
<td>+NEM, −DTT</td>
<td>4</td>
<td>22 ± 6⁺</td>
</tr>
</tbody>
</table>

⁺Statistically different from the AA value at p < 0.001.

FIG. 3. Immunoblot of normal erythrocyte ghost membranes (lane 1) and protein 4.1 purified from normal (lane 2) and sickle (lane 3) erythrocytes under reducing conditions (5 mM DTT). The protein 4.1 (20 µg) was analyzed by NaDodSO₄-PAGE (9% acrylamide), transferred to nitrocellulose (45 V for 18 h at 4 °C), and reacted first with rabbit anti-human protein 4.1 and then with goat anti-rabbit IgG coupled to horseradish peroxidase. The complex was visualized with 4-chloro-1-naphthol. HMW indicates protein 4.1 aggregates with Mr > 200,000.

FIG. 4. Anion-exchange chromatography elution profiles of normal or sickle protein 4.1. High salt (1 M KCl) extracts of spectrin-depleted normal or sickle IOVs were dialyzed against 5P8 containing 20 mM KCl, 1 mM EDTA, 0.2 mM DTT, 0.4 mM DFP, 0.3 mM NaN₃ and loaded onto a DE52 anion-exchange column (6 X 2.5 cm) equilibrated with the same buffer. The protein was eluted using a continuous linear gradient of 60–300 mM KCl (120 ml each). The composite chromatograph shows four individual samples: two normal (NL) protein 4.1 (O—O, ——–) and two sickle protein 4.1 (O—O, ——–).
unoxidized protein eluted from the DE52 resin at ≥125 mM KC1 (Fig. 5A), the diamide-oxidized protein eluted at ≥175 mM KC1 (Fig. 5B).

**Thiol-Diuldisulfide Exchange Chromatography**—Protein 4.1 in NaDodSO4-solubilized ghost membranes from normal or sickle erythrocytes bound differently to thiol-activated Sepharose 4B gel (Table III). Under nonreducing conditions, 58 ± 5% of the solubilized normal ghost protein 4.1 bound to the gel, and reducing the proteins had no effect on gel binding (65 ± 6%). In contrast, only 29 ± 2% of the nonreduced solubilized sickle ghost protein 4.1 bound to the gel, and reducing the proteins significantly increased the amount of gel-bound protein 4.1 (41 ± 4%, p < 0.001). However, even under reducing conditions, the percentage of gel-bound sickle protein 4.1 was only approximately two-thirds the percentage of gel-bound normal protein 4.1.

**Chemical Labeling of Protein 4.1 Cysteines**—The number of cysteine residues in purified protein 4.1 was quantitated using radiolabeled NEM. Although at high NEM/protein ratios NEM will covalently label both thiol and nonthiol residues, its use at low NEM/substrate ratios (100:1, as was the case in our experiments) results in cysteine-specific labeling (23). As shown in Table IV, purified normal protein 4.1 bound ~4 mol of NEM/mol of protein 4.1 monomer under denaturing conditions and ~2 mol of NEM/mol of protein 4.1 monomer under nondenaturing conditions. Reducing the normal protein had no effect on NEM labeling.

A similar examination of sickle protein 4.1 revealed that the denatured protein bound ~3 mol of NEM/mol of protein 4.1.

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**Table IV**

Quantitation of protein 4.1 cysteine residues

<table>
<thead>
<tr>
<th>Protein</th>
<th>DTT</th>
<th>NaDodSO4</th>
<th>[14CNEM bound to protein 4.1]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal 4.1</td>
<td>+</td>
<td>+</td>
<td>4.0 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>-</td>
<td>2.1 ± 0.1</td>
</tr>
<tr>
<td>Sickle 4.1</td>
<td>+</td>
<td>+</td>
<td>2.9 ± 0.3*</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>+</td>
<td>2.1 ± 0.1</td>
</tr>
</tbody>
</table>

*Statistically different from the normal values at p < 0.001.

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**Table V**

Total amino acid composition of protein 4.1

<table>
<thead>
<tr>
<th>Amino acid residue</th>
<th>Normal protein</th>
<th>Sickle protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid</td>
<td>12.2 ± 0.9</td>
<td>12.5 ± 0.2</td>
</tr>
<tr>
<td>Threonine</td>
<td>8.2 ± 0.3</td>
<td>7.9 ± 0.2</td>
</tr>
<tr>
<td>Serine</td>
<td>6.9 ± 0.2</td>
<td>7.0 ± 0.2</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>15.1 ± 0.2</td>
<td>16.2 ± 0.1*</td>
</tr>
<tr>
<td>Glycine</td>
<td>4.8 ± 0.8</td>
<td>5.8 ± 0.6</td>
</tr>
<tr>
<td>Alanine</td>
<td>8.3 ± 0.8</td>
<td>8.5 ± 0.6</td>
</tr>
<tr>
<td>Valine</td>
<td>6.1 ± 0.4</td>
<td>5.7 ± 0.2</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.86 ± 0.02</td>
<td>0.70 ± 0.02*</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>6.0 ± 0.2</td>
<td>6.0 ± 0.1</td>
</tr>
<tr>
<td>Leucine</td>
<td>10.8 ± 0.3</td>
<td>11.0 ± 0.2</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>3.5 ± 0.0</td>
<td>2.4 ± 0*</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>3.4 ± 0.1</td>
<td>3.4 ± 0.1</td>
</tr>
<tr>
<td>Histidine</td>
<td>2.3 ± 0.0</td>
<td>2.2 ± 0.0</td>
</tr>
<tr>
<td>Lysine</td>
<td>7.2 ± 0.1</td>
<td>5.9 ± 0.1*</td>
</tr>
<tr>
<td>Arginine</td>
<td>4.3 ± 0.1</td>
<td>3.8 ± 0.1*</td>
</tr>
<tr>
<td>Cysteic acid</td>
<td>None</td>
<td>0.74 ± 0.02*</td>
</tr>
</tbody>
</table>

*Statistically different from the normal protein at p < 0.001.

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![Diagram](image-url)
4.1 monomer, whereas the nondenatured protein bound ~2 mol of NEM/mol of protein 4.1 monomer. In contrast to the situation for the normal protein 4.1, where removal of reducing agents had no effect on NEM binding, removal of reducing agents from the sickle protein decreased NEM binding by ~1 mol/mol of protein 4.1 monomer under both denaturing and nondenaturing conditions; the denatured protein bound ~2 mol of NEM/mol of protein 4.1 monomer, whereas the native protein bound ~1 mol of NEM/mol of protein 4.1 monomer.

Amino Acid Composition of Protein 4.1—To obtain direct evidence of oxidative damage to sickle protein 4.1, we analyzed the total amino acid composition of protein 4.1 purified from normal or sickle erythrocytes. We were careful to include a reducing agent (0.1% β-mercaptoethanol) and an antioxidant (0.09% phenol) during protein hydrolysis to avoid artifactual loss of easily oxidizable amino acids. The results of this analysis are presented in Table V. Differences between the normal and sickle protein were most apparent when comparing amounts of tyrosine and methionine; the sickle protein contained ~50 mol% less (p < 0.0001) and ~20 mol% less (p < 0.0001), respectively, of these residues. In addition, the sickle protein contained ~1 mol% cysteic acid (the ultimate oxidation product of cysteine), whereas none was detected in the normal protein. Sickle protein 4.1 also contained ~7 mol% more glutamic acid (p < 0.001), ~13 mol% less arginine (p < 0.001), and ~8 mol% less lysine (p < 0.001) than did the normal protein.

**DISCUSSION**

It is becoming increasingly evident that the plasma membrane of sickle erythrocytes is markedly abnormal (25–29). That the membrane cytoskeleton may also be abnormal was first suggested by Lux et al. (20) who found that cytoskeletons from irreversibly sickled cells retained their sickled morphology. Recent evidence by Platt et al. (31) further suggests that acquired damage to specific components of the sickle cell cytoskeleton adversely affects some aspects of protein function. The mechanisms by which these abnormalities are acquired is still largely speculative; however, a role for oxidative damage in the mechanism of acquired membrane lesions is becoming increasingly apparent. This is a particularly attractive hypothesis since sickle erythrocytes have been shown to generate spontaneously twice-normal levels of free radical oxidants (8), and sickle membranes contain increased levels of lipid peroxidation by-products (32). Rank et al. (24) have obtained direct evidence for protein oxidation in sickle membranes by showing that most of the cytoskeletal proteins contained oxidized or blocked thiols. Our evidence essentially confirms the observations of these authors and further suggests that oxidation can adversely affect protein function.

We focused our attention on protein 4.1, one of the major components of the membrane cytoskeleton. In addition to stabilizing the spectrin-actin lattice (18), this protein has also been shown to bind preferentially to cytoplasmic leaflet phospholipids (33, 34), suggesting that protein 4.1 may be intimately involved in maintaining membrane integrity.

Evaluation of the protein composition of erythrocyte membranes revealed that sickle membranes, in particular spectrin-depleted IOVs, contained significantly less protein 4.1 than did normal membranes (p < 0.001). When the rebinding of purified protein 4.1 to protein 4.1-stripped membranes was evaluated, we found a 4-fold decrease in the rebinding of sickle protein 4.1 compared to the normal protein. This abnormality was observed using protein 4.1-stripped normal or sickle membranes, strongly suggesting that the binding defect resided in the sickle protein 4.1 and not in its membrane-binding site(s).

Biochemical characterization of purified protein 4.1 demonstrated that the sickle protein contained a significant amount of high molecular weight aggregates > 200,000. The high M<sub>p</sub> protein was detected by immunoblotting and represented between 20 and 40% of total sickle protein 4.1 depending on whether the protein was purified under reducing or nonreducing conditions. Normal protein 4.1 also contained some high M<sub>p</sub> protein; however, it never amounted to more than 10% of total protein. The high M<sub>p</sub> aggregate in purified protein 4.1 was probably not a complex of protein 4.1 and some other membrane proteins, such as spectrin, ankyrin, hemoglobin, glycophorin, or band 3, since it did not react immunologically with antibodies to any of these proteins. It is unlikely that the decrease in protein 4.1 membrane binding or the increase in high M<sub>p</sub> protein was secondary to reticulocytosis since protein 4.1 isolated from high reticulocyte controls did not exhibit either abnormality. Our attempts to detect high M<sub>p</sub> protein 4.1 aggregates in erythrocyte ghosts and IOVs were complicated by the requirement to analyze large amounts of membrane protein. Since, under reducing conditions, the high M<sub>p</sub> aggregate in purified sickle protein 4.1 constituted ~20% of total protein and protein 4.1 in ghost and IOV membranes represents ~5 and 12% of total protein, respectively, we would anticipate that 50 µg of membrane protein would contain between 0.5 and 1 µg of high M<sub>p</sub> protein 4.1. When we analyzed >50 µg of membrane protein by NaDodSO₄-PAGE and immunoblotting, we always observed protein 4.1 aggregates in both the normal and sickle membranes. These aggregates had a M<sub>p</sub> < 200,000 and appeared to arise as a function of the amount of protein analyzed, i.e. incomplete protein solubilization, and did not appear to be related to alterations in protein redox status.

The 2-fold increase in the amount of high M<sub>p</sub> sickle protein 4.1 compared to normal protein 4.1 cannot, by itself, explain the 4-fold decrease in membrane binding using the purified sickle protein and protein 4.1-stripped membranes. We therefore examined other parameters of protein 4.1 character including protein charge. We found that the sickle protein reproducibly eluted from anion-exchange resin (DE52) at a higher salt concentration than the normal protein. This result indicates that the sickle protein has a net increase in surface negative charge, one consequence of which could be abnormal interactions with other membrane components including both proteins and phospholipids. In support of the latter, we have preliminary evidence that sickle protein 4.1 binds less well to phosphatidylserine model membranes than does normal protein 4.1 (35).

Several lines of evidence suggest that protein thiol oxidation may be a contributing factor in the functional and structural abnormalities observed in the sickle protein. For example, oxidation of normal protein 4.1 thiols with diamide results in both the appearance of high M<sub>p</sub> aggregates and the expression of an abnormal DE52 elution profile similar to the pattern observed with sickle protein 4.1. In addition, purified sickle protein 4.1 has a reduced interaction with thiol-activating Sepharose beads, only ~30–40% of the sickle protein binds. Although these results do not rule out the possibility that nonoxidative processes may also be contributing factors, since protein aggregates induced by nonoxidative events would similarly affect protein-resin/gel binding, they do strongly suggest that oxidation can affect protein 4.1 functional properties.

To evaluate directly protein oxidative status, we measured
protein 4.1 thiols by chemical labeling with NEM. We found that the sickle protein was labeled by NEM to a lesser extent than the normal protein; the sickle protein contained between 1 and 2 fewer cysteines/protein 4.1 monomer than did the normal protein depending on whether or not NEM labeling was evaluated under reducing or nonreducing conditions. This result is similar to the results of the binding of sickle protein 4.1 to thioll-activated Sepharose beads, where only a portion of the decrease was reversible by pretreating the protein with reducing agents.

Examination of protein 4.1 by amino acid analysis revealed that the sickle protein contained fewer tyrosine and methionine residues than did the normal protein ($p < 0.001$). Furthermore, the sickle protein contained 0.74 mol% cysteic acid, the cystine oxidation product; whereas the normal protein did not contain any cysteic acid. Since both tyrosine (36) and methionine (37) are known to be susceptible to oxidation, it is not unreasonable to speculate that lower amounts of these amino acids in the sickle protein, as well as the increased amount of cysteic acid, came about as a consequence of protein oxidation in vivo. The reasons for the decreased amount of lysine and arginine and the increased amount of glutamic acid in the sickle protein are not clear at this time. Different amounts of amino acids in the normal and sickle protein are not due to different proportions of 4.1a and 4.1b protein since amino acid analysis of electrophoretically purified 4.1a and 4.1b protein failed to reveal any significant differences in their total amino acid content.

If the acquired oxidative lesions in sickle cytoskeletal proteins are due to the instability of sickle hemoglobin, one might expect that similar oxidative lesions would be found in membranes of erythrocytes containing other unstable hemoglobins. In support of this hypothesis, evidence for membrane protein oxidation has been reported in hemoglobin Köln (38) and hemoglobin H (39) erythrocytes as well as in β-thalassemic erythrocytes (40). In addition, membrane protein lesions have been reported in glucose-6-phosphate dehydrogenase-deficient erythrocytes (41), where a compromised antioxidant system renders the cells more susceptible to oxidant damage. Thus, cytoskeletal protein oxidative damage may be a common pathway by which certain unstable hemoglobinopathies or antioxidant compromised erythrocytes acquire membrane abnormalities.

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References