Increased Membrane Binding of Erythrocyte Catalase in Hereditary Spherocytosis and in Metabolically Stressed Normal Cells

By David W. Allen, Shirley Cadman, Shaun R. McCann, and Barbara Finkel

Normal red blood cell (RBC) membranes were compared with (1) RBC membranes from six patients with hereditary spherocytosis (HS), (2) normal membranes after hemolysis of the RBC in the presence of calcium, or (3) normal membranes after incubation of RBC for 24 hr in phosphate-buffered saline containing calcium without added glucose. When compared with normal controls, polyacrylamide gel electrophoresis with sodium dodecyl sulfate (PAGE SDS) of all three preparations showed an increase in membrane binding of globin and protein band 4.5 (60,000 molecular weight). In an attempt to identify band 4.5, 14 enzymes were assayed in the RBC membranes. Of these, catalase and lactate dehydrogenase were increased in membranes from HS RBC and from normal cells exposed to calcium. Only catalase, however, was present in sufficient quantity and had the correct subunit molecular weight on PAGE SDS and calcium-dependent membrane binding to account for an appreciable portion of 4.5. Catalase was further identified with a component of band 4.5 by double immunodiffusion using a specific anti-catalase antibody.

It has been shown by Weed et al. that incubation of RBC in vitro without added glucose produces a decrease in red blood cell (RBC) filterability and deformability associated with an increase in membrane calcium, hemoglobin, and nonhemoglobin protein. Hereditary spherocytosis (HS) cells have an increased tendency to accumulate calcium and thus become rigid. Changes in membrane polypeptide composition determined by polyacrylamide gel electrophoresis with sodium dodecyl sulfate (PAGE SDS) that occur on sterile incubation in vitro include an increase in membrane-bound globin and can be partly reproduced by hemolysis of fresh RBC with calcium-containing buffers. The addition of calcium to the hemolyzing solution has also been shown to increase the recovery of at least one enzyme, lactate dehydrogenase (LDH), in RBC membranes. Patients with HS, especially when combined with glyceraldehyde-3-phosphate dehydrogenase (G3PD) deficiency, have been observed to have changes in PAGE SDS of their RBC membranes similar to those of metabolically stressed RBC and, in particular, an increase in band 4.5 [molecular weight (MW) 60,000]. Thus it is of interest to compare in detail calcium-dependent metabolically induced changes in the polypeptide and enzymatic composition of membranes from RBC in model systems with observations of RBC membranes from HS patients. As a result of this study, a significant portion of band 4.5 has been found to be catalase.

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MATERIALS AND METHODS

Clinical data. Cell counts, hemoglobins, hematocrits, and red cell indices were determined on a Coulter Model S cell counter. Blood smears, reticulocyte counts, incubated and unincubated osmotic fragilities, and autohemolysis tests were performed by standard methods. Two patients with HS were described previously. Four more unrelated patients were studied who likewise showed spherocytes on peripheral smear, had elevated reticulocyte counts and indirect bilirubin prior to splenectomy, and had positive unincubated and incubated osmotic fragility and autohemolysis tests. Three HS patients had enlarged spleens; one patient had had a splenectomy. One patient with an enlarged spleen was studied before and after splenectomy.

Preparation and incubation of RBC. Using aseptic techniques, heparinized whole blood was freshly obtained from normal healthy donors and centrifuged at 1200 g for 10 min; the plasma, buffy coat, and top one-fifth of the RBC was removed. Similar efforts to remove white cells were made after three subsequent washes of the RBC with Dulbecco and Vogt’s phosphate buffered saline (PBS) with or without 10 mg/dl (0.9 mM) calcium chloride. Calcium-free PBS, made from distilled deionized water, was used unless otherwise specified. Washed RBC were suspended in two volumes of calcium-free or calcium-containing PBS with or without glucose (500 mg/dl) and incubated at 37°C for 24 hr. Under these conditions the pH of the media was maintained at 7.4 ± 0.1.

Preparation of membrane proteins. Following incubation, the RBC were again washed with PBS; the membranes were prepared for PAGE SDS by hemolysis in most experiments in 20 vol-

Fig. 1. Comparison of PAGE SDS of membranes hemolyzed without calcium (control, ——) with membranes prepared in the presence of 1 mM calcium (hemolyzed with calcium, ——) and with membranes from RBC incubated in the absence of added glucose but in Dulbecco’s PBS containing calcium chloride (incubated in PBS + calcium, · · · · ·). 25-µg samples of protein applied.
Volumes of 5 mM phosphate buffer, pH 8, by the method of Dodge et al.,12 as modified by Fairbanks et al.;13 the aliquots were stored at -70°C. In certain experiments, the 5 mM phosphate buffer was also made 1 mM in calcium chloride. In other experiments membranes were prepared in 10 mM Tris, pH 7.4, with varying calcium concentrations.6 Total protein was measured by the procedure of Lowry et al.14 hemoglobin by the O-tolidine method,15 and nonhemoglobin membrane protein reported as the difference.

Polyacrylamide gel electrophoresis. Prior to electrophoresis the protein was dissolved in 1% SDS, containing 1% mercaptoethanol, and heated to 100°C for 2 min to destroy proteases.13 PAGE SDS of 10-30 μg of protein were performed on 0.5 x 10-cm gels. The gels, containing 5% acrylamide in 0.1%, SDS and 0.1 M phosphate buffer, pH 7, were run at 25°C (10 mamp/tube). Except when measuring globin the gels were run until the tracker dye had traveled 9-10 cm and the globin had run off the gel.3 For measuring globin the tracker dye was run 6.5 cm and the globin 7 cm. The gels were stained for proteins with Coomassie blue and the relative amounts of protein components determined on a Gilford gel scanner by integration of the areas under each peak. In the range of sample size used, the relative amounts of the components were independent of the amount of protein applied. Bovine serum albumin (69,000 MW) and ovalbumin (43,000 MW) were run simultaneously with each preparation. By this means published apparent molecular weights of membrane polypeptides were confirmed.16 Apparent molecular weights of the new bands appearing on incubation were determined using these known polypeptides as internal molecular weight standards.

Preparation of catalase. Catalase was isolated from 800 ml of bank blood by chromatography,
Table 1. Comparison of the Polypeptide Composition of RBC Membranes

<table>
<thead>
<tr>
<th>Poly-peptide</th>
<th>RBC Hemolyzed in 5 mM Phosphate, pH 8</th>
<th>RBC Hemolyzed in 10 mM Tris, pH 7.4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fresh Normal (n = 10)</td>
<td>Fresh HS (n = 7)</td>
</tr>
<tr>
<td>Band 3</td>
<td>31.3 ± 1.4</td>
<td>27.0 ± 1.4(\dagger)</td>
</tr>
<tr>
<td>Band 4.1</td>
<td>5.09 ± 0.32</td>
<td>5.34 ± 0.39</td>
</tr>
<tr>
<td>Band 4.2</td>
<td>5.24 ± 0.49</td>
<td>5.01 ± 0.48</td>
</tr>
<tr>
<td>Band 4.5</td>
<td>2.91 ± 0.55</td>
<td>4.08 ± 0.59(\dagger)</td>
</tr>
<tr>
<td>Band 6</td>
<td>3.49 ± 0.37</td>
<td>3.65 ± 0.64</td>
</tr>
<tr>
<td>Band 8</td>
<td>1.4 ± 0.9</td>
<td>2.8 ± 0.9(\dagger)</td>
</tr>
<tr>
<td>Hb</td>
<td>3.2 ± 1.9</td>
<td>6.7 ± 4.6</td>
</tr>
</tbody>
</table>

Results are expressed as per cent nonhemoglobin protein ± SD.
*RBC hemolyzed with 1 mM calcium.
†RBC incubated (24 hr, 37°C) in PBS with calcium (10 mg/dl).
‡RBC incubated (24 hr, 37°C) in PBS without calcium.
§p < 0.005 in Student's t test, compared to fresh normal RBC membrane prepared with same buffer.

Table 2. Comparison of the Enzymatic Composition of RBC Membranes

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>RBC Hemolyzed in 5 mM Phosphate, pH 8</th>
<th>RBC Hemolyzed in 10 mM Tris, pH 7.4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fresh Normal (n = 10)</td>
<td>Fresh HS (n = 7)</td>
</tr>
<tr>
<td>Catalase</td>
<td>74 ± 70</td>
<td>353 ± 168(\dagger)</td>
</tr>
<tr>
<td>LDH</td>
<td>0.002 ± 0.002</td>
<td>0.113 ± 0.049(\dagger)</td>
</tr>
<tr>
<td>G3PD</td>
<td>1.21 ± 0.13</td>
<td>1.02 ± 0.05(\dagger)</td>
</tr>
</tbody>
</table>

Results are expressed as IU/mg nonhemoglobin protein ± SD.
*RBC hemolyzed with 1 mM calcium.
†RBC incubated (24 hr, 37°C) in PBS with calcium (10 mg/dl).
‡RBC incubated (24 hr, 37°C) in PBS without calcium.
§p < 0.005 in Student's t test, compared to fresh normal RBC membranes prepared with same buffer.
first on diethylaminoethyl cellulose and then on carboxymethyl (CM) cellulose.\textsuperscript{17} Rabbit antibodies against catalase were prepared as described by Ben-Yoseph and Shapira.\textsuperscript{18} The anticalatalase did not react with hemoglobin or spectrin. The anticalatalase decreased the catalase activity of an equal volume of 1:20 hemolysate to 14\% of control aliquots. The controls were incubated with normal rabbit serum\textsuperscript{18} instead of anticalatalase.

\textit{Enzyme assay.} Glycolytic enzymes were assayed on hemolysates and isolated membranes using a Gilford spectrophotometer with a chart recorder and standard methods at 37°C. No solubilizers were used with membrane-bound enzymes, but freeze-thaw preparations were made as indicated.\textsuperscript{19} Catalase was assayed by the initial rate of decrease in absorbancy of a 0.059 M solution of hydrogen peroxide in 0.05 M phosphate buffer, pH 7, at 25°C,\textsuperscript{20} and was reported as \( \mu \text{mole/min/ml} \) or U/ml. The specific activity of CM cellulose-purified RBC catalase was 43,000 U/mg.

\textbf{RESULTS}

Figure 1 compares the PAGE SDS of equal amounts of membrane polypeptides from fresh RBC, membranes prepared with 1 mM calcium chloride, and those from RBC after a 24-hr sterile incubation without added glucose in PBS with calcium. In both experiments in which the membranes were exposed to calcium there was an increase in bands 4.5 (60,000 MW) and 8 (23,000 MW) and a decrease in band 3 (88,000 MW). Only the membranes from RBC hemolyzed with calcium-containing buffers show increased band 2.3 (180,000 MW) and decreased 4.2 (72,000 MW) as noted by others.\textsuperscript{6} Membrane content of 4.5 reported by us is less than that reported previously\textsuperscript{9} since we now use calcium-free PBS in washing the RBC prior to hemolysis and confine the measurement of 4.5 to those bands of 60,000 ± 2,000, rather than the entire region between 4.2 and 5 as 4.5, as defined by Steck.\textsuperscript{16}

In Fig. 2 the PAGE SDS of the RBC membranes from a patient with HS is compared with control membranes from normal RBC run simultaneously, showing an increase in bands 4.5 and 8 and a decrease in band 3 in HS.

A summary of the quantitative compositional changes in membrane polypeptides in HS and those produced by either calcium in the hemolyzing solution or metabolic stress is shown in Table 1. Note the statistically significant increased percentage of polypeptides 4.5 and 8, and hemoglobin, and the decreased band 3 compared to controls.\textsuperscript{21} No EDTA was used in preparing the RBC membranes, as this chelating agent reverses the calcium-induced changes; this experimental difference may account for the failure of others to observe the effects of calcium.\textsuperscript{5} Measurement of membrane hemoglobin\textsuperscript{15} gave results consistent with measurement of globin on PAGE SDS,\textsuperscript{5} although incubated RBC showed a slight excess of the latter. If the calcium in the PBS on sterile incubation is omitted (Table 1) the polypeptide composition more closely resembles that of fresh RBC. However, band 6 (G3PD) is increased in membranes from RBC incubated in PBS with or without calcium.\textsuperscript{4} Addition of glucose to the PBS and frequent mixing of the RBC suspension likewise prevent most of these changes in polypeptide composition. No difference was observed between band 4.2 of membranes from HS patients and that from controls.\textsuperscript{22}

Because of the limited solubility of calcium phosphate salts, hemolysis of the RBC was repeated in 10 mM Tris buffer, pH 7.4, with or without 1 mM calcium\textsuperscript{6} (Table 1). The content of bands 4.2 and 6 (G3PD) was increased when membranes were prepared with Tris at pH 7.4, compared to phosphate at pH 8.
When 1 mM calcium was added to the Tris, there was a decrease in bands 3, 4.1, and 6 and an increase in band 4.5, band 8, and hemoglobin, analogous to the changes with calcium in phosphate buffer.

Fourteen enzymes were assayed in the RBC membranes in an attempt to equate the membrane-bound polypeptides with specific enzymatic activity. Hexokinase, glucose phosphate isomerase, phosphofructokinase, aldolase, triose phosphate isomerase, G3PD, phosphoglycerokinase, phosphoglyceromutase, enolase, pyruvate kinase (PK), and lactate dehydrogenase (LDH), as well as catalase, glucose-6-phosphate dehydrogenase (G6PD), and 6-phosphogluconic dehydrogenase were assayed in the supernatant and membrane fraction. In the membrane fraction there was significant activity of catalase, G3PD, and LDH. The enzymatic activity appearing in the membranes could be accounted for by a similar decrease in the enzymes in the membrane-free supernatants. In no case was the enzymatic specific activity altered by the concentrations of calcium used. Thus, changes in the activity of membrane enzymes could be ascribed to a redistribution of red cell enzymes, either in the intact RBC or during hemolysis and cell fractionation.

Table 2 shows the enzyme content of fresh normal and HS RBC membranes compared with membranes from normal RBC hemolyzed with calcium-containing hypotonic buffers and with membranes from RBC incubated in PBS with and without calcium. The concentrations of the membrane enzymes have been corrected for the amounts of enzyme present in the retained supernatant, utilizing the results of the hemoglobin concentration in the membranes and the value of enzyme activity per gram of hemoglobin in the supernatant, assuming that hemoglobin absorption to the membranes is nonspecific. G3PD binding to the membranes is decreased in the presence of calcium,23 but catalase and LDH are retained more avidly than hemoglobin to the membrane fraction. It is evident that the pattern of enzymes absorbed by the membranes is similar in HS to that caused by the presence of calcium in the two model systems. Note that omission of calcium from the PBS largely prevents the changes produced by incubation of the RBC. The LDH and catalase are significantly higher in membrane preparations from HS RBC, as in the two model systems. In Tris buffer at pH 7.4 more G3PD is bound and less catalase and LDH than in phosphate, but the effects of calcium in Tris on membrane binding of all three enzymes are similar to the effects in phosphate.

No consistent effect of splenectomy on membrane polypeptide or enzymatic composition was evident in normals or HS patients. RBC were separated depending on in vivo age by centrifugation at 800 g for 1 hr, and eight layers of the successively older, denser cells were carefully removed, exactly as described by Kadlubowski and Harris.24 Adequacy of separation of younger cells was documented by a highly significant increase ($p < 0.005$) of the G6PD, PK, and hexokinase content of younger, top layers of cells. No change in PAGE SDS of older membranes could be demonstrated, failing to confirm the previous report.24 Enzymes more concentrated in younger cells were increased in the total hemolysates of HS RBC, but after correction for the membrane absorbed supernatant, there was no change in membrane binding of these enzymes.
Of the enzymes studied, catalase, with a subunit molecular weight of 60,000,25 may be one component of band 4.5 on PAGE SDS. The experimental relationship between band 4.5 and membrane catalase is shown in Fig. 3; these data were obtained by measuring 4.5 and catalase in RBC membranes after hemolysis with varying calcium concentrations in 5 mM phosphate, pH 8, or 10 mM Tris, pH 7.4. No difference is evident in the buffers used. With no catalase activity, band 4.5 was 2.0% of the nonhemoglobin protein of the membranes, because the baseline of the protein scan was arbitrarily set as the nadir between bands 2 and 3, rather than between bands 4.2 and 4.5. In Fig. 3 the slope of a line predicted by assuming the equivalence of band 4.5 and catalase is 430 units catalase/per cent band 4.5, calculated from the observed specific activity of catalase (1% of 43,000 U/mg). Inspection of Fig. 3 suggests that the points do not lie on this or any other straight line and a test for linearity of regression by analysis of variance confirms this impression.21 One possible explanation for this departure from linearity is that, while pure catalase is bound initially at low levels of intracellular calcium and the slope approaches 430, as intracellular calcium concentrations increase other components of band 4.5 are also bound, and the slope is inversely proportional to the amount of 4.5. Thus

\[
\frac{d(\text{catalase})}{d(\text{band 4.5})} = \frac{b}{(\text{band 4.5})}
\]

or, in integral form,

\[
\text{catalase} = a + b \ln(\text{band 4.5}).
\]

A computer-derived logarithmic curve fit gives values of \(a = -331\), \(b = 479\), \(r^2 = 0.71\), and a considerably closer fit to the data in Fig. 3 than a linear regression. Thus when catalase = 0, band 4.5 = 2, and \(b/(\text{band 4.5}) = 240\), one obtains a closer but not satisfactory estimate of the specific activity (430) than the value obtained by a linear regression (118). An additional explanation for this departure from prediction is that the enzymatic activity of bound catalase is less than free catalase, as suggested by the results of immunodiffusion (see below).
To confirm membrane binding of catalase, six pairs of samples of washed RBC were hemolyzed in 20 volumes of 10 mM Tris, pH 7.4, with and without 1 mM calcium, the hemolysates centrifuged (80,000 g, 30 min), and the catalase measured on the supernatant, after adjustment to equal calcium concentrations. There was a 9% decrease in the catalase activity of the soluble cytoplasm from calcium hemolysates, which was highly significant ($p = 0.005$). Assuming RBC membrane protein content as reported by Weed et al. and RBC catalase content as reported by Ben-Yoseph and Shapira, catalase would account for a 1% increase in membrane protein, rather than the 2.5% increase in band 4.5 actually observed (Table 1). This finding again suggested the presence of additional proteins in band 4.5, by measurements independent of our determination of the activity of catalase.

Figure 4 shows the apparent identity of band 4.5 and catalase on PAGE SDS. Catalase was added to membranes already containing 4.5, and with this internal standard a single peak was observed.

Catalase was also identified with a component of band 4.5 by immuno-diffusion. In Fig. 5 a line of identity is seen between purified catalase and band 4.5 extracted with EDTA from membranes of RBC hemolyzed with calcium or incubated in calcium-containing buffers. Following extraction of the membranes with 10 volumes of 0.1 mM EDTA, the extract was concentrated 50-fold.

Fig. 4. Identity of catalase with band 4.5 of 25 μg membrane protein from RBC incubated 24 hr with calcium-containing Dulbecco's PBS. PAGE SDS of membrane proteins from incubated RBC alone (membranes, ---); membranes electrophoresed with 5 μg of catalase (membranes + catalase, ---); 25 μg catalase alone (catalase, ---). Note that the catalase increases the 4.5 peak, showing the identity of catalase and 4.5 in this system.
Fig. 5. Double immunodiffusion with rabbit anticatalase in the center well. Well 1 contains an EDTA extract of membranes from normal fresh RBC hemolyzed in 1 mM calcium; well 4 contains purified catalase; well 3 contains an EDTA extract of membranes from normal RBC incubated 1 day in calcium-containing PBS; well 2 contains an EDTA extract of normal fresh RBC membranes containing no 4.5. Note the line of identity between catalase and the two extracts of band 4.5. All membrane EDTA extracts contained traces of bands 1 and 2 (spectrin) and hemoglobin, to both of which the anticatalase was shown unreactive (cf. well 2).

by ultrafiltration using Diaflo UM 10 membranes. The additional band of lower molecular weight catalase (closer to the antibody well) seen with the 4.5 extracts probably represented monomeric catalase as observed by others\textsuperscript{18,25} in many catalase preparations, and shown to be enzymatically inactive.\textsuperscript{25} This immunologic identification of a component of 4.5 with catalase confirmed the circumstantial enzymologic evidence cited above and was strengthened by the specificity of the anticatalase. The presence of a large proportion of enzymatically inactive monomeric catalase may also explain why membrane-bound catalase activity was less than that predicted from the 4.5 content.

DISCUSSION

Except for catalase, the changes in membrane binding of enzymes assayed here do not explain to any extent the changes in polypeptide composition observed on PAGE SDS. LDH, with a subunit molecular weight of 35,000, probably represents a minor contaminant of band 6. With calcium in the hemolysate\textsuperscript{59}, it is recovered with the membranes. Assuming an activity of RBC LDH of the same order of magnitude as rabbit muscle LDH (100–1000 U/mg protein), then 0.4 U/mg protein (see Table 2) represents only 0.004–0.0004 of the membrane protein or 0.1–0.01 of band 6. Since G3PD is the principal component of band 6 and is affected oppositely by calcium, it is evident that the LDH changes are not visible on PAGE SDS. Binding of none of the enzymes studied explains the increase in band 8 (23,000 MW). Increased binding of G3PD to the inside of the RBC membrane, which occurs whether or not calcium is present, is apparently related to glucose deprivation and metabolic stress per se,\textsuperscript{4} rather than increased cellular calcium uptake, and illustrates the complexity of the changes occurring.

Enzymes bound to the cytoplasmic surface of the RBC membrane may represent an extension of membrane organization into the cytoplasm.\textsuperscript{8,16,26,27} Non-enzymatic components may also participate in this cytoplasm–membrane interaction. Failure of the calcium pump mechanism with increased intracellular calcium in HS\textsuperscript{3} or after glucose deprivation and adenosine triphosphate deficiency\textsuperscript{1,28} apparently results in binding of band 4.5 (catalase), band 8, and
LDH to the membrane as adaptive or pathologic consequences of metabolic stress. In acatalasemic patients hydrogen peroxide is destroyed by glutathione, requiring the action of glutathione peroxidase and stimulation of the hexose monophosphate shunt. Thus, catalase may represent the first line of defense against this toxic substance. If catalase is bound to membrane in metabolically stressed cells, it may represent an optimal location for this protective enzyme.

Three alternative mechanisms may be advanced for this calcium-related increased membrane binding of cytoplasmic proteins. The enzymes bound have acidic isoelectric points: catalase, $pI = 5.4$ (on exposure to oxygen), $LDH_1$, $pI = 5.2$, and $LDH_2$, $pI = 5.9-6.3$.

Hence ionic binding to the membrane through calcium of these anionic proteins may play a role. Like hemoglobin, both contain cysteine and demonstrate microheterogeneity, possibly related to sulfhydryl oxidation, so that membrane binding by sulfhydryl groups represents a second possibility. Finally, the calcium may alter the properties of a membrane protein, such as spectrin, so as to increase its tendency to bind this particular pattern of proteins. All three proposed mechanisms should be susceptible to experimental study.

REFERENCES

Increased membrane binding of erythrocyte catalase in hereditary spherocytosis and in metabolically stressed normal cells

DW Allen, S Cadman, SR McCann and B Finkel