Decreased Pyrimidine Nucleoside Monophosphate Kinase Activity in Sickle Erythrocytes


We have previously shown that physiologic concentrations of hemin cause marked inhibition of several red blood cell (RBC) enzymes. Because endogenous heme content is elevated in sickle RBCs, we have examined the activity of hemin-sensitive enzymes in these RBCs. One of the hemin-sensitive enzymes, pyrimidine nucleoside monophosphate kinase (PNMK), was shown to have decreased activity in sickle RBCs relative to RBCs of equivalent cell age. The other hemin-sensitive enzymes, including adenylate kinase (AK), pyrimidine 5'-nucleotidase (P5N), 6-phosphogluconate dehydrogenase (6PGD), and aldolase, had activities that were appropriate for cell age. We have also examined the affinity of the hemin-sensitive enzymes to hemin. Using two different methods, PNMK was shown to have the highest binding affinity to hemin. The exquisite sensitivity of PNMK to inhibition by hemin, coupled with the enzyme's high affinity to hemin, may account for the decrease in PNMK activity and the lack of significant decrease in the other hemin-sensitive enzymes in sickle RBCs. These results suggest that the increased endogenous heme content in sickle RBCs may be responsible for the decrease in PNMK activity. Whether the increased endogenous heme content of sickle RBCs can cause hemolysis indirectly by inhibiting RBC enzymes remains to be determined.

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ERYTHROCYTES from patients with sickle cell disease are characterized by increased oxidant sensitivity both in vivo and in vitro. This is manifested by increased membrane lipid peroxidation, decreased membrane protein thiols, decreased reduced glutathione content, decreased antioxidant enzyme activities, including catalase and glutathione reductase, impaired pentose phosphate shunt activity, increased hydrogen peroxide hemolysis, increased incubated Heinz body formation, and decreased pyridine nucleotide redox potential. The increased oxidant sensitivity in sickle red blood cells (RBCs) has been postulated to be a cause of hemolysis in sickle cell disease. Current evidence strongly implicates hemoglobin (Hb) S instability as the primary cause of oxidant sensitivity and its many manifestations. This inherent instability leads ultimately to denaturation of Hb S with concomitant heme release, which, in turn, leads to increases in free (cytoplasmic) heme, membrane-associated heme, and membrane-associated nonheme iron. It is these latter chemical species that actually mediate the many oxidative changes in RBC membranes and cause the other manifestations of oxidative damage in sickle cells. In addition to being the primary candidate for causing oxidant damage, the endogenous release of heme from sickle RBCs has been shown to cause hemolysis through a direct mechanism. Hemin (ferriprotoporphyrin IX chloride), which is the chloride form of heme, has been shown to cause marked hemolysis of dilute suspensions of mouse and rat RBCs at micromolar concentrations. Although hemin also causes hemolysis of dilute human RBC suspensions, supraphysiologic concentrations of hemin (28 to 70 μmol/L) are required. The concentrations of free heme in normal and sickle RBCs were estimated to be 0.1 to 0.15 and 0.4 to 0.75 μmol/L, respectively, which are too low to cause direct hemolysis.

Recently, we have shown that physiologic concentrations of hemin cause potent inhibition of several RBC enzymes in vitro, which led us to propose the hypothesis that endogenous free heme may cause hemolysis indirectly by inhibiting key RBC enzymes. Because the hereditary deficiency of some of these enzymes is a known cause of hemolytic anemia, enzyme inhibition by heme could potentially constitute another heme-mediated mechanism for hemolysis in sickle RBCs. Five hemin-sensitive enzymes, including pyrimidine nucleoside monophosphate kinase (PNMK; EC 2.7.4.14), adenylate kinase (AK; EC 2.7.4.3), pyrimidine 5'-nucleotidase (P5N; EC 3.1.3.5), 6-phosphogluconate dehydrogenase (6PGD; EC 1.1.1.44), and aldolase (EC 4.1.2.13) have previously been shown to be inhibited by hemin at submicromolar concentrations in vitro. We examine here the activity of hemin-sensitive enzymes in sickle RBCs and show that one of these enzymes, PNMK, has a significantly decreased activity. We also show that PNMK has higher affinity to heme than the other hemin-sensitive enzymes. Thus, the high affinity of PNMK to heme, together with the exquisite sensitivity of PNMK to inhibition by heme, may account for the decrease in PNMK activity and the lack of significant decrease in the other hemin-sensitive enzymes in sickle RBCs. These results suggest that the increased endogenous heme content in sickle RBCs may be responsible for the decrease in PNMK activity.

MATERIALS AND METHODS

Procurement of blood samples. After obtaining informed consent, blood was obtained from individuals with sickle cell disease (nine patients with Hb SS, three patients with Hb SC, and one...
patient with Hb S-β*-thalassemia) by routine venipuncture using heparin-coated tubes (15 U heparin/mL whole blood) to prevent coagulation. Blood samples from normal white and black subjects served as controls; individuals with Hb A and autoimmune hemolytic anemia or hemolytic anemia of unknown etiology were used as high reticulocyte controls.

Isolation of erythrocytes and preparation of hemolysates. An RBC-enriched fraction was prepared by passing whole blood through a column of α-cellulose and microcrystalline cellulose to deplete white blood cells and platelets as described by Beutler.26 RBCs were washed three times with 0.15 mol/L NaCl by repeated centrifugation and resuspended in 0.15 mol/L NaCl at a final concentration of 6 to 10 g Hb/dL. Crude hemolysates were prepared by freezing RBC suspensions in an acetone-dry-ice bath and thawing in a room temperature bath three times. Stroma-free hemolysates were prepared by centrifuging crude hemolysates at 30,000g for 20 minutes and discarding the translucent pellets. Hb was determined using the cyanmethemoglobin method.26

Separation of erythrocytes on density gradients. Erythrocytes were separated using Percoll (Pharmacia LKB Biotechnology, Inc, Piscataway, NJ) and meglumine diatrizoate (ER Squibb & Sons, Inc, Princeton, NJ) density gradients as described by Vettore et al.7 RBCs to be separated on the density gradients were prepared by passing whole blood through the cellulose column (see above) and then centrifuging to pack the cells without washing with 0.15 mol/L NaCl. After density separation, RBC fractions were washed four times with at least 20 vol of 0.15 mol/L NaCl using centrifugation.

Enzyme assays. Hexokinase (EC 2.7.1.1), pyruvate kinase (PK; EC 2.7.1.40), AK, 6PGD, and aldolase activities were determined spectrophotometrically as described by Beutler.26 PSN activity was determined using the continuous spectrophotometric method of Zerez and Tanaka.28 PNMK activity was determined spectrophotometrically using a direct system in which pyrimidine nucleoside diphosphate formation was coupled to PK and lactate dehydrogenase (LDH) as described by Lachant et al. PNMK activity was determined using the method of Hunt et al.20 Hemin did not affect either PK or LDH activity in the PNMK assay system. PSN and PNMK activity were determined using uridine monophosphate (UMP) and cytidine monophosphate (CMP) as substrates.

Preparation of hemin solutions. A 10 mmol/L stock solution of hemin was prepared fresh daily using the method of Hunt et al.20 Briefly, stock solutions of hemin were prepared by dissolving equine hemin (Sigma Chemical Company, St Louis, MO) in 0.2 mL 1.0 mol/L NaOH, and then adding 1.0 mL of 0.20 mol/L Tris-HCl buffer (pH 7.8) and 2.6 mL of water. The pH of this solution was carefully adjusted to 7.8 by adding 1.0 mol/L HCl and then enough water was added to bring the final concentration of hemin to 2.0 mmol/L. This stock solution was diluted to the concentration needed with distilled water. Hemin solutions were kept dark, in foil-wrapped test tubes, at 0°C.

Statistical analysis. All data are expressed as the mean ± 1 SD. The Student's t-test was performed using standard methods.

RESULTS

Enzyme activity in crude hemolysate. We have determined the activity of five hemin-sensitive and two hemin-insensitive enzymes in crude hemolysates. Because no differences were found in any of the enzyme activities between normal whites and blacks, these activities were combined into one normal cohort. The activities of all enzymes assayed were higher than normal in the high reticulocyte RBCs due to the younger cell age of these samples (Table 1 and Fig 1). However, the classical cell age-dependent enzymes, hexokinase, PK, and PSN, were increased to a greater extent than the other enzymes (Table 1). Sickle RBC samples in this study had a similar percentage of reticulocytes as high reticulocyte RBC samples. As expected, there was no statistically significant difference in the activity of the hemin-insensitive enzymes between sickle and high reticulocyte RBCs (Table 1). There was no statistically significant difference in the activity of the hemin-sensitive enzymes PSN, AK, 6PGD, and aldolase between sickle and high reticulocyte RBCs (Table 1). However, PNMK activity was significantly decreased in sickle RBCs compared with high reticulocyte RBCs of equivalent cell age when determined using either UMP (Fig 1A) or CMP (Fig 1B) as substrate.

Effect of cell age on PNMK activity. In addition to determining the effect of cell age on PNMK activity by measuring it in high reticulocyte RBCs, we have determined the effect of cell age on PNMK activity by density fractionation of normal, high reticulocyte, and sickle RBCs. Using hexokinase as a marker of cell age, cells from the top of density gradients (low density) consistently had higher hexokinase activity than cells from the bottom (high density) in all RBC samples examined. In Hb A-containing cells from normal and high reticulocyte individuals, there was a significant positive correlation between PNMK activity and hexokinase activity (P < .005 for UMP-dependent PNMK and P < .01 for CMP-dependent PNMK) (Fig 2A and B). Similarly, Hb S-containing cells also had a positive correla-

| Table 1. Activity of Hemin-Sensitive and Hemin-Insensitive Enzymes in Crude Hemolysates From Normal, High Reticulocyte, and Sickle RBCs |
|---|---|---|---|---|---|---|---|---|---|
| Cohort | Reticulocytes (%) | Hemin-Insensitive Enzyme Activity (μmol/min · mL RBC) | Hemin-Sensitive Enzyme Activity (μmol/h · mL RBC) |
| | | Hexokinase | PK | PSN |
| | | | | UMP | CMP | AK | 6PGD | Aldolase |
| Normal (n = 13) | Mean ± 1 SD | 1.2 ± 0.4 | 0.42 ± 0.06 | 4.87 ± 0.81 | 4.96 ± 1.75 | 4.84 ± 1.47 | 85.3 ± 12.5 | 2.44 ± 0.44 | 0.820 ± 0.144 |
| | Range | (0.4-1.9) | (0.34-0.51) | (3.77-6.30) | (2.73-7.66) | (2.70-8.26) | (64.7-106) | (1.51-3.35) | (0.640-1.05) |
| High reticulocytes (n = 6) | Mean ± 1 SD | 17 ± 16 | 0.95 ± 0.39 | 8.54 ± 3.04 | 11.2 ± 7.2 | 10.3 ± 6.6 | 93.3 ± 21.4 | 3.65 ± 0.88 | 1.22 ± 0.30 |
| | Range | (3.2-45) | (0.53-1.45) | (4.16-12.4) | (4.80-24.4) | (4.20-20.8) | (74.6-126) | (2.42-4.79) | (0.83-1.62) |
| Sickle (n = 13) | Mean ± 1 SD | 17 ± 12 | 1.09 ± 0.28* | 9.18 ± 2.09* | 9.12 ± 4.92* | 9.04 ± 5.27* | 108 ± 33* | 3.76 ± 0.73* | 1.48 ± 0.41* |
| | Range | (2.6-42) | (0.69-1.59) | (6.28-13.0) | (3.28-20.1) | (2.54-19.9) | (50.2-166) | (2.40-5.11) | (0.80-2.28) |

*Student's t-tests show no statistically significant difference compared with the high reticulocyte cohorts.
tion between PNMK and hexokinase activity. More importantly, at any given cell age indicated by hexokinase activity, Hb S-containing cells had less PNMK activity than Hb A-containing cells (Fig 2A and B).

**Affinity of hemin for the hemin-sensitive enzymes.** Because PNMK is the only hemin-sensitive enzyme to have decreased activity in sickle RBCs, we estimated the affinity of hemin for the hemin-sensitive enzymes using two different methods. In the first method, dialysis was used to remove hemin from hemolysate after exposure to hemin. Incubation of stroma-free hemolysate with hemin resulted in decreased activity of P5N, 6PGD, and aldolase, and, more importantly, resulted in the obliteration of PNMK and AK activity (Table 2), the two hemin-sensitive enzymes most susceptible to inhibition by hemin.25 Subsequent dialysis to remove the hemin resulted in increased activity of P5N, 6PGD, and aldolase and partial restoration of AK activity (Table 2). In contrast, PNMK activity remained undetectable after dialysis, suggesting a higher binding affinity of hemin to PNMK.

In the second method to estimate hemin affinity of the hemin-sensitive enzymes, albumin, which binds hemin tightly,31,32 was used for hemin sequestration. The addition of hemin directly to assay mixtures resulted in the total obliteration of PNMK and P5N activities, the near total obliteration of AK, and substantial decreases in 6PGD and aldolase activities (Table 3). The addition of albumin to the same mixtures resulted in partial restoration of AK, P5N, and 6PGD and full restoration of aldolase activity (Table 3). In contrast, albumin failed to restore PNMK activity, which remained undetectable.

**DISCUSSION**

We have examined the activity of five hemin-sensitive enzymes in sickle RBCs to determine whether they are affected by the higher endogenous free heme concentration in these RBCs. Only one of the hemin-sensitive enzymes, PNMK, had significantly decreased activity in crude hemolysates from sickle RBCs relative to cells of equivalent cell age (Fig 1A and B). The finding of a significant positive correlation between hexokinase and PNMK activity indi-

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**Fig 1.** Activity of the hemin-sensitive enzyme PNMK in crude hemolysates from normal, high reticulocyte, and sickle RBCs. (A) UMP was used as substrate. (B) CMP was used as substrate.

**Fig 2.** Effect of cell age on PNMK activity in RBCs containing Hb A (○ and △) and Hb S (□). (A) UMP was used as substrate in the PNMK assay. (B) CMP was used as substrate in the PNMK assay. RBCs from a normal volunteer (○), a patient with autoimmune hemolytic anemia (△), and a patient with sickle cell anemia (□) were separated into equal top, middle, and bottom fractions using density gradient centrifugation (see Materials and Methods). RBC fractions were washed and hexokinase and PNMK activity were determined in crude hemolysates. Best-fit lines were calculated using the method of least squares; correlation coefficients (r) are shown. (In [A], activities from only the bottom and middle fractions of Hb A-containing normal RBCs are shown.)
DECREASED PNMK ACTIVITY IN SICKLE RBC

Table 2. Effect of Dialysis on the Activity of Hemin-Sensitive Enzymes in Normal Stroma-Free Hemolysate After Exposure to Hemin

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>None</th>
<th>Hemin Exposure</th>
<th>None</th>
<th>Hemin Exposure</th>
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</thead>
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<td>4.29</td>
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<tr>
<td>AK</td>
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<td>&lt;0.3</td>
<td>120</td>
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<tr>
<td></td>
<td>1.03</td>
<td>0.309</td>
<td>1.11</td>
<td>0.384</td>
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</table>

Hemin exposure was performed by adding the appropriate volume of a stock solution of 2.0 mmol/L hemin to the stroma-free hemolysate to obtain a 400 μmol/L final concentration of hemin and incubating for 10 minutes at room temperature. An aliquot of the hemin-treated hemolysate was immediately diluted and appropriate volumes were added to premixed cuvettes for the spectrophotometric determination of the indicated enzymes. The remainder of the hemin-treated hemolysate was dialyzed against 50 vol of 0.10 mol/L Tris-HCl, pH 7.8, for 4 hours at 4°C; dialysis buffer was changed every hour. PNMK and 6PGD activities were determined with UMP as substrate. Activities are expressed in pmol/min · mL RBC, except for 5SN, which is expressed in pmol/h · mL RBC.

The decrease in PNMK activity in sickle RBCs and the lack of decrease in the activity of the other hemin-sensitive enzymes prompted an investigation into the mechanism responsible for this phenomenon. The determination of activity of all RBC enzymes requires substantial dilution of crude hemolysate to ensure that activity remains within the “linear range” of the assay system (ie, so that activity would remain proportional to quantity of enzyme added to the assay system). This dilution would lead to a decrease in the endogenous heme concentration of sickle hemolysate in the assay system unless the enzyme being assayed bound heme tightly. Neither dialysis (Table 2) nor the addition of albumin (Table 3), a protein that binds heme tightly, was capable of restoring PNMK activity after exposure to hemin. In contrast, these treatments resulted in partial or complete restoration of the activity of all of the other hemin-sensitive enzymes after exposure to hemin. These results suggest that hemin has a higher binding affinity to PNMK than to the other hemin-sensitive enzymes. Previous studies have shown that PNMK is the most susceptible of the hemin-sensitive enzymes to inhibition by hemin in vitro: the concentration of hemin necessary for 50% inhibition (IC50) of PNMK activity in stroma-free hemolysate is only 0.23 μmol/L when UMP is used as substrate and 0.27 μmol/L when CMP is used as substrate. Thus, we propose that the high affinity of hemin to PNMK and the exquisite sensitivity of PNMK to inhibition by hemin are responsible for the decreased PNMK activity in sickle RBCs. These data suggest that the increased endogenous heme content in sickle RBCs may be responsible for the decrease in PNMK activity.

We have previously shown that sickle RBCs have a relative impairment in pentose phosphate shunt (PPS) activity. In vitro studies in this laboratory have shown that the IC50 for PPS activity in normal stroma-free hemolysate is approximately 1.8 μmol/L hemin (Zerez, Lachter, and Tanaka, unpublished data), which is similar to the IC50 for 6PGD (1.5 μmol/L hemin) in normal stroma-free hemolysate. The finding that sickle RBCs have 6PGD activity that is appropriate for cell age4 (Table 1) is consistent with the lower binding affinity of hemin for 6PGD than for PNMK (Tables 2 and 3). These data, together with our previous demonstration of inhibition of 6PGD by hemin25 and the increased free heme in sickle RBCs, suggest that inhibition of RBC 6PGD by endogenous heme is a mechanism for the relative impairment in PPS activity in intact sickle RBCs.

Hemolysis is a well-recognized manifestation of hereditary RBC enzyme deficiencies. Deficiencies in two of the hemin-sensitive enzymes, P5N and aldolase, are known genetic abnormalities that result in chronic hemolytic anemia. Deficiency of AK, another of the hemin-sensitive enzymes, is associated with chronic hemolytic anemia, but may not be a sufficient cause of hemolysis by itself. To the best of our knowledge, chronic hemolytic anemia due to 6PGD or PNMK deficiency has not been described. Because of this lack of precedent, it is unclear whether the decreased PNMK activity in sickle RBCs, which we have observed, is a contributing mechanism for the hemolytic anemia seen in patients with sickle cell disease.

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REFERENCES


