A Soluble Adenosine Triphosphate–Dependent Proteolytic System in Human Peripheral Red Blood Cells

By R.F. Rieder, A. Ibrahim, and J.D. Etlinger

A soluble adenosine triphosphate (ATP)-dependent proteolytic system has been detected in human peripheral blood erythroid cells. Hemolysates prepared from reticulocyte-rich blood of subjects with autoimmune hemolytic anemia, treated pernicious anemia, and iron deficiency anemia or from pools of red blood cells enriched for reticulocytes by density gradient centrifugation were tested against a radioactive casein standard. Up to 57% of the casein was rendered radioactive by reductive methylation using [14C]-formaldehyde (0.25 mCi in 0.14 mg, New England Nuclear, Boston) followed by treatment with sodium borohydride as described previously.17 The generation of trichloroacetic acid (TCA)-soluble radioactivity was measured, and the degradation of labeled protein was expressed as the percentage of the acid-soluble counts divided by the added acid-precipitable counts × 100.

RESULTS

A 42-year-old woman with an exacerbation of Coomb's positive autoimmune hemolysis (AIHA) had a decrease in the packed red blood cell volume to 19% and an increase in reticulocytes to 21%. Therapy with prednisone was instituted, and over the course of 2 months hemolysis declined, the hemoglobin level rose, and the reticulocyte count fell (Fig 1). During the early portion of the course of therapy when the reticulocyte count varied from 22% to 13%, the 100,000-g supernatant of the red cell lysate hydrolyzed 26% to 37% of a standard preparation of radioactive casein in the presence of 1 mmol/L ATP during 60 minutes of incubation at 37°C (Fig 1). In the absence of ATP, 6.9% to 9.5% of the standard was

PROTEIN BREAKDOWN is a constant process within bacteria and animal cells.1,4 The rates of proteolysis as well as the rates of synthesis determine the concentrations of various proteins, those with abnormal structure being degraded especially rapidly.3 A soluble adenosine triphosphate (ATP)-dependent proteolytic system has been described in cell-free preparations from rabbit reticulocytes.5 This proteolytic activity appears to be especially active against denatured or abnormal proteins. Upon maturation of the reticulocyte, the ATP-dependent proteolysis is gradually lost from the rabbit cells.6 Examination of more primitive erythroid cells, the Friend mouse erythroleukemia (FEL) and the human K562 erythroleukemia cell lines, has also revealed the presence of ATP-dependent proteolytic enzyme systems.9 In the two erythroleukemia cell lines, we found the ATP-dependent activity to be associated with a particulate fraction sedimentable at 100,000 g, although soluble activity also has been reported recently in MEL cells.10

In human erythroid cells there is postsynthetic degradation of certain unstable abnormal hemoglobin as well as unpaired excess globin chains produced in the thalassemias.11-15 The proteolytic mechanism responsible for this phenomenon has not been defined. We have examined human reticulocyte-rich peripheral blood for the presence of an ATP-dependent proteolytic activity. An active system has been demonstrated in cell-free erythrocyte extracts; as in the analogous system in rabbit reticulocytes, the activity in human peripheral red blood cells is soluble and markedly declines with erythrocyte maturation.

MATERIALS AND METHODS

Rabbit reticulocytes were isolated as previously described from phenylhydrazine-treated animals.5 Human peripheral venous blood was anticoagulated with EDTA. The red blood cells were washed three times with 0.1 mmol/L NaCl (Fisher Scientific, Springfield, NJ), 15 mmol/L KCl (Fisher), 25 mmol/L Tris, pH 7.8, and lysed with 1.6 vol of hypotonic buffer (10 mmol/L Tris, pH 7.8, containing 0.5 mmol/L dithiothreitol). Leupeptin (Sigma Chemical Co, St Louis), 10 µg/mL, was added to stabilize ATP-dependent proteolysis. The cells were then homogenized using the A and B pestles of a Potter-Elvehjem homogenizer (Thomas Scientific, Swedesboro, NJ). The hemolysates were centrifuged at 15,600 × g for 15 minutes, followed by centrifugation of the supernatant at 100,000 × g for 60 minutes. Twenty-five microliters of the 100,000-g supernatant were assayed for proteolytic activity in a 50-µL reaction mixture in the presence or absence of 1 mmol/L ATP and an ATP-generating system consisting of 50 µg/mL creatine phosphokinase and 12 mmol/L creatine phosphate as previously described.1 An ATP trap consisting of 10 mmol/L glucose and 0.5 units of hexokinase was added to reaction mixtures lacking added ATP. Hemoglobin Gun Hill16 and hemoglobin Leiden17 were each isolated by diethyl aminoethyl (DEAE)-cellulose chromatography from the blood of subjects heterozygous for these unstable hemoglobins. In each case, hemoglobin A was also prepared from the same blood. The hemoglobins and α-casein were used as proteolytic substrates at a concentration of 5 µmol/L after being rendered radioactive by reductive methylation using [14C]-formaldehyde (0.25 mCi in 0.14 mg, New England Nuclear, Boston) followed by treatment with sodium borohydride as described previously.17 The generation of trichloroacetic acid (TCA)-soluble radioactivity was measured, and the degradation of labeled protein was expressed as the percentage of the acid-soluble counts divided by the added acid-precipitable counts × 100.
rendered TCA soluble. In some preparations stimulation of proteolysis by ATP was as high as sixfold. Similar lysates prepared from two normal control subjects hydrolyzed, respectively, 10.7% and 9.7% of the casein in the presence of ATP and 6.9% and 7.1% without ATP (Table 1). Recovery of the patient from the hemolytic process was associated with a decline in ATP-dependent proteolysis. When the packed cell volume had risen to 34% and the reticulocyte count had fallen to 8.4% per cent, the 100,000-g supernatant of the red cell lysate hydrolyzed 18% of the casein standard with ATP and 10% without ATP (Fig 1). When the reticulocyte count reached 4.6% proteolysis was reduced to 12.0% with ATP and 6.1% without ATP (data not shown). A precise correlation between the number of reticulocytes and the degree of proteolysis was not observed in these studies, nor would it be expected using a nonpurified enzyme preparation derived from heterogeneous, nonsynchronized cell populations.

The time course of in vitro proteolysis was examined when the subject’s blood contained 19.1% reticulocytes. Proteolysis of the casein substrate was assessed at several points during 120 minutes of incubation. In the presence of ATP, hydrolysis of the substrate was most rapid for the first 30 minutes (Fig 2). By 60 minutes, 30% of the substrate had been hydrolyzed with ATP and 11.1% without ATP. Hydrolytic activity declined for the next 60 minutes; by 120 minutes, 34.8% of the substrate had been hydrolyzed in the presence of ATP and 14.1% in the absence of ATP (Fig 2). Exhaustion of some component of the reaction mixture could account for this decline in the rate.

Because the activity of the ATP-dependent proteolytic system in the peripheral blood cells declined with the decrease in reticulocyte count during the course of treatment and because normal subjects with low reticulocyte counts showed little ATP-dependent activity, an attempt was made to fractionate circulating red blood cells by age to examine changes in ATP-dependent proteolytic activity with erythrocyte maturation.

During the process of aging, peripheral red blood cells are known to become increasingly dense. Blood from the patient with AIHA was obtained when the reticulocyte count was 19.1%. The red blood cells were adjusted to a hematocrit value of 50% in plasma and mixed with 20 vol of a solution containing colloidal silica (Percoll, Pharmacia Fine Chemicals, Piscataway, NJ), 60% meglumine diatrizoate (Hypaque, Winthrop Laboratories, New York), H2O, and 0.9% NaCl in the proportion of 3.5:1.7:4.1:0.7. The mixture was centrifuged at 32,000 g for 20 minutes at room temperature. The resultant multiple erythrocyte bands were collected in three pools, top, middle, and bottom, that contained 67%, 5.5%, and 0% reticulocytes, respectively. Lysates prepared from the three erythrocyte fractions were assayed for proteolytic activity against the radioactive casein standard after 60 minutes of incubation at 37 °C (Fig 3). The least dense fraction of red cells, containing the bulk of the reticulocytes, demonstrated marked (55.7%) ATP-dependent proteolytic activity (Fig 3). Stimulation of proteolysis by ATP was 4.4-fold. Much less ATP-dependent proteolysis (13.3%) was observed in the bottom, dense erythrocyte fraction containing 0% reticulocytes (Fig 3). When normal red blood cells were subjected to density gradient centrifugation, the top fraction exhibited 15.6% proteolysis in the presence and 13.0% in the absence of ATP.

Several enzyme inhibitors were studied for their effect on the ATP-dependent proteolytic system (Table 2). Hemin, the serine protease inhibitor phenylmethylsulfonyl fluoride (PMSF), N-ethylmaleimide (NEM, a sulfhydryl reagent), and the ATPase inhibitor sodium vanadate were all markedly depressed ATP-dependent proteolysis by a hemolysate from the subject with AIHA. Except for the observed inhibitory

### Table 1. ATP-Dependent Proteolysis in Some Subjects With Reticulocytosis

<table>
<thead>
<tr>
<th>Subject</th>
<th>Reticulocytes (%)</th>
<th>+ ATP</th>
<th>- ATP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treated iron deficiency anemia</td>
<td>10.0</td>
<td>15.9</td>
<td>9.8</td>
</tr>
<tr>
<td>Treated pernicious anemia</td>
<td>28.5</td>
<td>28.6</td>
<td>11.5</td>
</tr>
<tr>
<td>Normal</td>
<td>&lt;1.0</td>
<td>10.7</td>
<td>6.9</td>
</tr>
<tr>
<td>Normal</td>
<td>&lt;1.0</td>
<td>9.7</td>
<td>7.1</td>
</tr>
</tbody>
</table>

Fig 1. Proteolysis by erythrocyte lysates during the course of recovery from an episode of AIHA. The hemoglobin and reticulocyte levels as well as the proportion of casein digested in the presence and absence of 1.0 mmol/L ATP are shown for a 49-day period.

Fig 2. Time course of proteolysis in vitro by a peripheral red blood cell lysate. The proportion of casein digested for a period of 120 minutes in the presence (---) and absence (A-A) of 1.0 mmol/L ATP is shown for a lysate prepared from the blood of the patient with AIHA when the reticulocyte count was 19.1%.

Fig 3. Time course of proteolysis in vitro by a peripheral red blood cell lysate. The proportion of casein digested for a period of 120 minutes in the presence (---) and absence (A-A) of 1.0 mmol/L ATP is shown for a lysate prepared from the blood of the patient with AIHA when the reticulocyte count was 19.1%.

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Fig 3. Proteolysis by erythrocytes of different densities. Red blood cells were collected from the subject with AIHA at a time when the reticulocyte count was 24.7%. The erythrocytes were centrifuged at 32,000 g for 20 minutes in a mixture of colloidal silica, 60% meglumine diatrizoate, water, and 0.9% NaCl (see text for details). Three pools of erythrocytes were collected from the top, middle, and bottom portions of the resultant gradient and contained 67%, 5.5%, and 0% reticulocytes, respectively. The digestion of the casein standard in the presence and absence of 1.0 mmol/L ATP is shown for the lysates prepared from the three pools of red blood cells.

Fig 4. Hydrolysis of hemoglobins A, Leiden, and Gun Hill by rabbit reticulocyte lysates. In the presence of ATP there was enhanced proteolysis of the unstable hemoglobin Leiden (A) and hemoglobin Gun Hill (B) compared with normal hemoglobin A. The decreased amount of proteolysis of the unstable hemoglobins in the absence of ATP as well as the inhibition of ATP-enhanced proteolysis by hemin is shown.

Table 2. Effect of Various Enzyme Inhibitors on ATP-Dependent Proteolysis by a Hemolysate From a Subject With AIHA

<table>
<thead>
<tr>
<th>Inhibitor (mmol/L)</th>
<th>Proteolysis (%)</th>
<th>+ ATP</th>
<th>− ATP</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>19.7</td>
<td>7.3</td>
</tr>
<tr>
<td>Hemin 0.05</td>
<td></td>
<td>7.9</td>
<td>5.4</td>
</tr>
<tr>
<td>PMSF 1.0</td>
<td></td>
<td>10.0</td>
<td>5.9</td>
</tr>
<tr>
<td>NEM 5.0</td>
<td></td>
<td>3.0</td>
<td>2.1</td>
</tr>
<tr>
<td>Na vanadate 5.0</td>
<td></td>
<td>6.1</td>
<td>5.9</td>
</tr>
</tbody>
</table>

PMSF, phenylmethylsulfonyl fluoride; NEM, N-ethylmaleimide.
of the ATP-dependent proteolytic activity that declines with a decrease in the degree of reticulocytosis. Little activity is demonstrable in normal reticulocyte-poor blood. Density gradient separation of peripheral erythrocytes also clearly indicated that the enzyme system is most active in the young reticulocyte-rich cell fractions and essentially absent in dense, older red blood cells. The enzyme is inhibited by hemin, PMSF, and the sulphydryl reagent, NEM. Hydrolysis of ATP is probably required for enzyme activity because proteolysis is markedly reduced by the ATPase inhibitor sodium vanadate.

Previous studies of hemoglobin synthesis in vitro have demonstrated that the intact human erythroid cell is able to degrade abnormal unstable hemoglobins as well as the unpaired globin chains that occur in the thalassemia syndromes. A proteolytic mechanism has been suspected of being responsible for this activity. In red blood cells from patients with β-thalassemia, in vitro turnover of excess α-globin chains has been shown to decline when the cells are deprived of energy and the ATP levels fall. The ATP-dependent proteolytic system derived from rabbit erythrocytes has been shown previously to be capable of distinguishing between artificially induced abnormal and normal hemoglobin. Although we have not yet studied this capacity in the human enzyme system, the present experiments with rabbit reticulocyte lysates demonstrated that the unstable human hemoglobin Gun Hill and Leiden were preferentially hydrolyzed compared with hemoglobin A.

The enzyme system described here in human reticulocytes seems to be analogous to the activity found in rabbit reticulocytes. The human system is sensitive to the same series of enzyme inhibitors and is also most active in reticulocytes, declining in older red blood cells. The activity demonstrated in the human reticulocytes also is limited to the soluble portion of the cell lysate. In this characteristic it differs from a previously described ATP-dependent proteolytic system found in certain more primitive erythroid cells. In both MEL and K562 cells an ATP-dependent proteolytic system with characteristics otherwise quite similar to the soluble system in reticulocytes has been found associated with a subcellular fraction sedimentable at 100,000 g. Recently Waxman and associates found soluble ATP-dependent activity in MEL cells. The relationship of the soluble to the insoluble activity is not understood at present. It is conceivable that the activity in young nucleated erythroid precursors is associated initially with a membrane or particle fraction and during cell differentiation becomes solubilized. Finally, upon further cell maturation the activity might be lost entirely. Further studies are planned to test this hypothesis. A similar change in physical state has been previously suggested for the erythrocyte NADH–cytochrome b5 reductase (methemoglobin reductase).

There are several earlier reports of other proteolytic activity detected in extracts of human erythroid cells. Lewis and Harris described a group of peptidases in lysates of peripheral erythrocytes that were separable by electrophoresis. These enzymes exhibited differences in substrate specificity; genetic variants were also observed. Hanash and Rucknagel reported that lysates prepared from thalassemia marrow and peripheral blood normoblasts could, upon addition to radioactive lysates of β-thalassemia erythrocytes, reduce the level of excess α-globin radioactivity. Vettore et al also reported that after incubation of peripheral blood from both thalassemic and nonthalassemic subjects with tritiated leucine a further 24-hour incubation of crude hemolysates resulted in the loss of approximately 30% of the α-globin radioactivity. This loss of radioactivity was blocked by an inhibitor of chymotrypsinlike proteases, tosyl-amido-phenylethyl-chloromethyl-ketone (TPCK). In that report the extensive loss of the α-globin radioactivity even from hemolysates of normal blood is surprising since presumably the radioactive α-globin was incorporated into completed hemoglobin tetramers that are relatively stable.

A proteolytic activity associated with the erythrocyte membrane was described by Ballas and Burka. Melloni and associates have extensively investigated several distinct proteolytic activities found in human red blood cells. None of the enzymes studied required energy, and there was only a relatively modest decline in activity during the period of the reticulocyte-erythrocyte transition. A calcium-dependent neutral protease was detected in erythrocyte cytosol that was inhibited by hemin, but unlike the ATP-dependent activity described in the present paper, it was also markedly inhibited by leupeptin. Membrane-bound acid proteases optimally active at approximately pH 2.5 were also found. In the present experiments some proteolytic activity was always detected even in the absence of ATP. However, especially in lysates prepared from cell samples containing high proportions of reticulocytes, the activity present without ATP was only a fraction of the ATP-dependent activity (Fig 2).

In rabbit reticulocytes, ubiquitin, a heat-stable polypeptide of 76 amino acids, becomes covalently attached to proteins through the action of activating and conjugating enzymes. The activation of ubiquitin requires ATP and Hershko and associates have proposed that ubiquitin conjugation to substrate proteins acts as a tag for protease recognition. In contrast, Speiser and Etlinger have provided evidence that ubiquitin acts to release the protease from an endogenous inhibitor. The role of ubiquitin in human reticulocytes has not been studied.

Several other ATP-dependent proteolytic systems have been described in bacteria, eukaryotic cytosol preparations, and mitochondria. The function of these enzymes in the economy of the various cells is not clearly understood, but it has been suggested that they could play a role in such functions as regulation of the activity of other short-lived enzymes in growth and development in addition to providing a pathway for removal of polypeptides with abnormal structures. In rabbit reticulocytes the ATP-dependent system is also thought to be responsible for the programmed degradation of proteins that is associated with the loss of mitochondria, ribosomes, and cytosolic enzymes during cell development.

The role of the ATP-dependent proteolytic system in human red blood cells requires further study. Unstable hemoglobins and free unpaired globin chains tend to precipi-
tate within erythroid cells to form Heinz bodies. These cellular inclusion bodies may attach to the band 3 component of the cell membrane causing damage and stimulating erythropagocytosis by macrophages. An enzyme system capable of removing precipitated hemoglobin might have a salutary effect on red cell survival. Thus the ATP-dependent system could modulate disease severity in both the thalassemias and the unstable hemoglobinopathies.

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REFERENCES

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