Red Blood Cells Contain a Pathway for the Degradation of Oxidant-damaged Hemoglobin That Does Not Require ATP or Ubiquitin*

Julie M. Fagan†, Lloyd Waxman, and Alfred L. Goldberg

From the Department of Physiology and Biophysics, Harvard Medical School, Boston, Massachusetts 02115

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It is generally accepted that ATP is required for intracellular protein breakdown. Reticulocytes contain a soluble ATP-dependent pathway for the degradation of highly abnormal proteins and for the elimination of certain proteins during cell maturation. Reticulocytes and erythrocytes also selectively degrade proteins damaged by oxidation. When these cells were exposed to oxidants, such as phenylhydrazine or nitrite, they showed a large increase in protein breakdown. This oxidant-induced proteolysis was not inhibited in cells depleted of ATP. However, ATP depletion did prevent the degradation of pre-existent cell proteins. In reticulocyte extracts, phenylhydrazine-treated hemoglobin is also degraded rapidly by an ATP-independent process, unlike endogenous proteins and many exogenous polypeptides. This lack of an ATP requirement means that the degradation of oxidant-damaged proteins does not require ligation to ubiquitin (even though phenylhydrazine treatment does make hemoglobin a very good substrate for ubiquitin conjugation).

In many respects, the pathway for breakdown of oxidant-treated hemoglobin differs from the ATP-dependent process. The latter has a much higher activation energy than the degradation of oxidized proteins. The ATP-dependent process is inhibited by hemin, 3,4-dichloroisocoumarin, diisopropylfluorophosphate and N-ethylmaleimide. The ATP-independent pathway is less sensitive to N-ethylmaleimide, hemin, and 3,4-dichloroisocoumarin and is not affected by diisopropylfluorophosphate. In addition, only the ATP-dependent proteolytic process is inactivated by dilution or incubation at 37 °C in the absence of nucleotides.

Reticulocytes thus contain multiple soluble systems for degrading proteins and can rapidly hydrolyze certain types of abnormal proteins by either an ATP-independent or ATP-dependent process. Erythrocytes lack the ATP-dependent process present in reticulocytes; however, erythrocytes retain the capacity to degrade oxidant-damaged hemoglobin. These two processes probably are active in the elimination of different types of abnormal proteins.

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† Postdoctoral Fellow of the Muscular Dystrophy Association and the National Institutes of Health.

An important function of protein degradation within animal and bacterial cells is to remove proteins with highly abnormal structures as might arise by mutation or errors in biosynthesis (1-3). For example, although hemoglobin normally exists for the life-span of the erythrocyte, several hemoglobin variants have been found which are rapidly hydrolyzed, presumably because of their inability to form the usual tetrameric structure (1). Similarly, when reticulocytes are exposed to amino acid analogs, they synthesize large amounts of highly abnormal globins which are degraded at rates 10- to 20-fold higher than normal proteins (4, 5). Cell proteins with altered structures may also arise from postsynthetic modifications (6) including nonenzymatic glycosylation, spontaneous deamination, or reaction with free radicals and oxidants. For example, activated neutrophils generate sufficient hydrogen peroxide to oxidize the methionine residues in intracellular proteins and to partially inactivate several enzymes (7). However, the fate of proteins damaged by oxidants has not been extensively studied. Clearly, the cellular mechanisms which prevent the accumulation of such inactive and potentially toxic proteins are of both clinical and biochemical interest.

The red blood cell is particularly attractive for studying the mechanisms by which cells cope with damage to proteins by oxidants and free radicals. Greater than 95% of the soluble protein of the erythrocyte is hemoglobin, and like other iron-rich substances, this protein may promote the generation of highly reactive oxygen species, including superoxide (8) and the hydroxyl radical (9). In addition, several recent reports from this laboratory have shown that treatment of red blood cells with nitrite (10), phenylhydrazine (10), hydrogen peroxide (11), or xanthine/xanthine oxidase (11, 12) cause damage to intracellular proteins and a large increase in their rate of degradation (10-12). The present studies were initiated to define further the properties of the degradative system responsible for the breakdown of oxidant-damaged proteins in the red blood cell.

The breakdown of proteins in mammalian and bacterial cells (1, 13), or in organelles (14-17), has been found to require metabolic energy. Although all mammalian cells show an apparent ATP requirement for intracellular protein breakdown, thus far it has been possible to obtain cell-free preparations in which proteolysis is clearly dependent on ATP only from reticulocytes (4, 13) and murine erythroleukemia cells (18, 19). The soluble ATP-dependent proteolytic system in reticulocytes catalyzes the rapid hydrolysis of abnormal proteins containing amino acid analogs or puromycin (4, 20). This system also is responsible for the programmed elimination of various proteins as reticulocytes mature into erythrocytes (21, 22) and seems to be missing in mature erythrocytes (22, 23).
The biochemical basis for this energy requirement in reticulocytes has received great attention. ATP appears to be required at two steps in this process (13, 24-26). It is essential for the covalent attachment of ubiquitin, a small, heat-stable polypeptide, to amino groups on protein substrates. This modification is thought to serve as a marking reaction rendering the proteins more susceptible to hydrolysis by the cell's ATP-dependent protease (13, 24-26). A second, hemin-sensitive ATP-dependent step has also been identified, although in this case the role of the nucleotide is not known (24-26). We have suggested that the ATP may be utilized directly by a protease (19, 24, 26). ATP-hydrolyzing proteases have been isolated from Escherichia coli (29, 31) and liver mitochondria (32), where they have been shown to play a critical role in protein degradation (14, 29, 30, 32-35). An ATP-dependent protease has also been partially purified from extracts of erythroblasts (19). The studies presented here demonstrate that oxidant-treated hemoglobin, in contrast to other kinds of abnormal proteins, is rapidly degraded in red cells by a process not requiring ATP. Thus, mammalian cells appear to contain multiple proteolytic pathways that degrade different types of abnormal proteins.

**EXPERIMENTAL PROCEDURES**

**Chemicals**—a-Casein, N-ethylmaleimide (NEM), diisopropylfluorophosphate (DFP), alanine dehydrogenase (*Bacillus subtilis*), phenylhydrazine, heparin, and disodium ATP were purchased from Sigma. Poly Glu-Tyr, the random amino acid polymer (M, 39,000) consisting of glutamate and tyrosine residues in a ratio of approximately 1:1 was also obtained from Sigma (Cat. No. P3899). Bovine serum albumin (BSA) and egg white lysozyme were obtained from Miles. Globin was prepared from purified human hemoglobin by extraction of the heme with acidified acetone (36). Homogeneous ubiquitin was prepared from human erythrocytes according to published procedures (37, 38).

Human hemoglobin was obtained from freshly drawn erythrocytes which were lysed in hypotonic medium. Most of the nonhemoglobin proteins were removed by passing the lysates over DEAE-cellulose (Whatman, DE52) equilibrated in 10 mM Tris-HCl (pH 7.1). The flow-through, which contains primarily hemoglobin, was concentrated in an Amicon Corp. pressure cell and applied to a column of AcA34 (LKB, Sweden) equilibrated in 10 mM Tris-HCl (pH 7.1). 20 mM NaCl to remove small molecules.

**Preparation of Cells and Extracts**—Reticulocytes were collected in saline containing heparin from the marginal ear vein of male rabbits treated with phenylhydrazine (4). Since the reticulocytes were collected 3 days after the last phenylhydrazine injection, the amount of hemoglobin damaged by this oxidant still present in the reticulocytes is probably very small. In fact, the visible spectrum of hemoglobin from such reticulocytes is the same as that from untreated rabbits (data not shown). Reticulocytes, as well as fresh human erythrocytes, were washed five times in ice-cold saline to remove plasma components and white blood cells. The cells were then washed twice in Krebs-Ringer bicarbonate buffer containing 5 mM glucose and stored on ice until used (generally within 12 h). Extracts were prepared by lysing the cells in 1.5-2.0 volumes of 1 mM dithiothreitol (DTT) and removing the membranes by centrifugation (30,000 × g for 2 h).

**Extracts were dialyzed against 20 mM Tris-HCl, 20 mM KCl, 1 mM Mg acetate, 0.5 mM DTT, 20% glycerol (pH 7.6) and stored frozen at -70° C. We have found that reticulocyte extracts prepared and stored under these conditions maintain the ATP-dependent degradative system for more than 1 year even with repeated thawing (24).**

**Reticulocyte extracts were fractionated by chromatography on DEAE-cellulose as described previously (24, 39). Under these conditions, hemoglobin and ubiquitin fail to adsorb to this ion exchange resin (Fraction I). Most nonhemoglobin proteins in these extracts bind, including the proteolytic activities and the enzymes required for conjugation of ubiquitin to protein (13, 39), and can be eluted with 0.5 M NaCl (Fraction II). Following elution from DEAE-cellulose, Fraction II was dialyzed to lower the concentration of salt and stored at 4° C until used.**

**Assays of Protein Breakdown**—50-75 μl of reticulocyte or erythrocyte lysate were incubated in a volume of 200 μl containing 50 mM Tris-HCl (pH 8.0), 10 mM Mg acetate, 1 mM DTT, a radiolabeled substrate, and 5 mM neutralized ATP (where indicated). After incubation for 1 h at 37°C, the reaction was terminated by the addition of 25 μl of 10% BSA and 75 μl of 30% trichloroacetic acid, and the acid-soluble radioactivity was measured.

Proteolysis in dialyzed extracts was also measured by monitoring the release of free alanine from endogenous proteins or from exogenously added hemoglobin. Incubations were carried out as described above, except that alanine was quantitated by a sensitive enzymatic assay using alanine dehydrogenase (40). All assays were carried out in duplicate or triplicate. The results presented are similar to those seen in at least three different preparations of red blood cells and lysates.

In suspensions of intact erythrocytes and reticulocytes, protein breakdown was also monitored by the release of free alanine into the medium. Generally, washed cells were incubated in Krebs-Ringer phosphate buffer for up to 2 h, during which time alanine was generated at a linear rate. The increased appearance of alanine in the medium after incubation with these reagents reflects net protein breakdown (11) and is not due to cell lysis. Exposure to 10 mM nitrate or 10 mM phenylhydrazine did not increase significantly the rate of lysis over that in control (untreated) cells (data not shown). Interestingly, 10 mM phenylhydrazine increased lysis by 2% when the cells were at a final concentration of 70%, but at a 10-fold dilution (7%) the cells were almost completely lysed (83.6%). Therefore, we exposed red blood cells to phenylhydrazine concentrations of 1-10 mM.

**Preparation of Oxidant-treated Hemoglobin**—Purified hemoglobin (Hb) was incubated at a concentration of 64 mg/ml (1 mM) in 1 ml containing 50 mM NaHepes (pH 8.0) and 0.1 mM EDTA. Phenylhydrazine (Hb), freshly prepared in H₂O and neutralized with 2 M NaOH, was added to purified hemoglobin at a final concentration of 1-10 mM. Incubations were carried out on ice for 2-3 min. After the addition of hemoglobin was dark brown. This material was then dialyzed at 4°C against 2 liters of 20 mM NaHCO₃, 20 mM NaCl (pH 8.0) with several changes of buffer. After dialysis, any precipitated protein was removed by centrifugation (10,000 × g × 15 min). Removal of this insoluble protein (as much as 20% of the total) had little effect on the rate of alanine production. Therefore, this aggregated material does not appear to be an important substrate for either ATP-independent or ATP-dependent pathways. Upon sodium dodecyl sulfate-polyacrylamide gels, the supernatant and insoluble material migrated as anticipated for hemoglobin; thus, there was no evidence for cross-linking of the polypeptides to the molecular weight standards. As has been reported to occur when erythrocyte membrane proteins are treated with phenylhydrazine (41). In our experiments, the hemoglobin treated with phenylhydrazine is still primarily a tetramer since it has the same Stokes radius upon gel filtration as native hemoglobin.

**Other Analytical Methods**—In some experiments in which proteolysis was to be measured in the absence of ATP, it was necessary to first deplete the cells of ATP by preincubating in the presence of 2-deoxyglucose (5 mM) and 2,4-dinitrophenol (0.2 mM) for 2 h at 25°C. The levels of ATP in the red blood cells before and after depletion were estimated by the firefly luciferase assay (42). The concentration of hemoglobin treated with various oxidizing agents was estimated by the Lowry procedure (43) with hemoglobin as standard. The protein content of Fraction II was determined by the method of Bradford (44) with BSA as standard.

**Ubiquitin-binding Assay**—200 μg of dialyzed reticulocyte Fraction II protein were incubated for 30 min at 37°C in a total volume of 200 μl that contained 50 mM Tris-HCl, 1 mM DTT, 10 mM Mg acetate, 5 mM ATP, and 5 μg of 125I-ubiquitin (300,000 cpm) (24). Hemin (50 μM) was included in the incubation to block ATP-dependent proteolysis (24, 45). A qualitative measure of the incorporation of ubiquitin into oxidant-treated hemoglobin was determined by including 100 μg of this material in the assay and using 20% trichloroacetic acid incorporated into Fraction II proteins (when incubated alone). The reaction was terminated by the addition of 10 mM NEM at room temperature. After 10 min, 5 μl of 2 N NaOH was added to raise the pH to about 10 in order to discharge the intermediate between ubiquitin and the ubiquitin-binding factor (14, 46). The supernatant was applied to a Sephadex G-75 column (1 × 35 cm) equilibrated in 25
mm NH₄HCO₃ (pH 8.0) and 0.1 M NaCl to separate ³⁵S-labeled ubiquitin incorporated into Fraction II proteins (or Fraction II proteins and oxidant-treated hemoglobin) from free ubiquitin (27). 0.5 ml-fractions were collected and counted in a gamma radiation spectrometer.

RESULTS

The Effect of Nitrite and Phenylhydrazine on Protein Breakdown in Intact Cells—As reported previously (10), exposure of erythrocytes to nitrite or phenylhydrazine stimulates protein breakdown and causes the release of amino acids into the incubation medium. This process is linear for at least 2 h under all experimental conditions (data not shown). As shown in Table I, proteolysis increased dramatically in cells treated with 1 mM phenylhydrazine and to a much smaller extent in cells exposed to nitrite (10 mM). A very surprising finding was that these effects were critically dependent on the concentration of red cells present in the incubation mixture. For example, at the lowest hematocrit studied (7%), no effect of nitrite on proteolysis was observed, while the stimulation by phenylhydrazine was greatest at this concentration (Table I).

Reticulocytes show a much higher basal level of proteolysis than erythrocytes (Table I), which probably reflects the programmed elimination of many soluble and organelle proteins as reticulocytes differentiate into erythrocytes (21, 22). Treatment of reticulocytes with these oxidants stimulated protein breakdown in a fashion similar to that observed with erythrocytes. Again, the effect of phenylhydrazine was clearly larger than that of nitrite, and the concentration of cells in the incubation appears to be an important parameter determining the amount of proteolysis (Table I). For example, 1 mM nitrite had no effect on proteolysis in a 7% solution of reticulocytes, while 1 mM phenylhydrazine caused a clear stimulation when cells were maintained at the lower density but was only 25% as effective at the higher density (70% hematocrit).

The reasons for the unexpected dependence of protein breakdown on cell concentration are not understood. Since the reaction of phenylhydrazine with oxyhemoglobin is accompanied by oxygen consumption (47, 48), reduced oxygen tension or competition with mitochondrial respiration in reticulocytes may influence the extent of hemoglobin damage, such that the phenylhydrazine effect could be smaller at high cell densities. Why concentration of cells is an important parameter in damage by nitrite is less clear. The oxidation of hemoglobin by nitrite to met-hemoglobin and to further oxidized forms is a highly complex process involving the generation of several peroxide and superoxide intermediates (49).

It is noteworthy that upon exposure of reticulocytes and erythrocytes to nitrite or phenylhydrazine, the cells changed in color from red to dark brown. Although the oxidation of the hemoglobin to met-hemoglobin or to further oxidation states appeared to coincide with an increase in proteolysis, exposure of cells at low hematocrit to nitrite did not necessarily increase proteolysis even though the cells turned brown. In related studies we found that met-hemoglobin prepared with ferricyanide (50), is not hydrolyzed in erythrocyte or reticulocyte lysates. Therefore, after exposing hemoglobin to oxidants, another product distinct from met-hemoglobin must be the substrate for proteolysis in the treated cells.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Protein degradation</th>
<th>Erythrocytes</th>
<th>Reticulocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Alaminol/mmol Hb/2 h</td>
<td>70%</td>
<td>7%</td>
</tr>
<tr>
<td>None</td>
<td>30</td>
<td>16</td>
<td>184</td>
</tr>
<tr>
<td>Nitrite</td>
<td></td>
<td>40</td>
<td>16</td>
</tr>
<tr>
<td>(1 mM)</td>
<td></td>
<td>46</td>
<td>16</td>
</tr>
<tr>
<td>(10 mM)</td>
<td></td>
<td>84</td>
<td>193</td>
</tr>
<tr>
<td>Phenylhydrazine (1 mM)</td>
<td>298</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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Control erythrocytes showed low rates of alanine production (Tables I and II) which were depressed further by ATP depletion (Table II). This low amount of ATP-dependent proteolysis may reside in contaminating reticulocytes (21-23). The control reticulocytes showed much higher rates of protein breakdown than erythrocytes (Tables I and II), and this process was completely blocked by ATP depletion (Table II). Erythrocytes and reticulocytes treated with 2-deoxyglucose and 2,4-dinitrophenol were then incubated on ice for 10 min with 1 mM phenylhydrazine or 10 mM nitrite. During a subsequent 2-h incubation at 37°C, the cells exposed to oxidants showed greatly enhanced rates of proteolysis, as described above (Tables I and II). However, quite unexpectedly, treatment of ATP-depleted erythrocytes or reticulocytes with phenylhydrazine still led to a high rate of proteolysis (Table II). In fact, ATP depletion seemed to reduce the basal rate of proteolysis in reticulocytes to the same degree in the oxidant-treated and control cells but did not inhibit significantly the increased proteolysis induced by oxidant treatment. In erythrocytes, where breakdown in the control cells is low and largely ATP-independent (Ref. 23, Table II), energy depletion caused little or no inhibition of the proteolysis induced by phenylhydrazine treatment (Table II). Data obtained by incubating erythrocytes with nitrite also support the conclusion that oxidant-treated proteins are degraded primarily by an ATP-independent process, as found with phenylhydrazine treatment. Upon exposure of reticulocytes to nitrite, less definitive results were obtained because nitrite...
Fig. 1. Time course of ATP depletion in reticulocytes and erythrocytes. Fresh rabbit reticulocytes and erythrocytes were washed in saline and resuspended in Krebs-Ringer bicarbonate buffer (pH 7.4) at 20% hematocrit. Cell suspensions were then incubated at 25°C in the presence or absence of 5 mM glucose, 5 mM 2-deoxyglucose, or 5 mM 2-deoxyglucose plus 0.2 mM 2,4-dinitrophenol. At the times indicated, the cells were lysed by addition of an equal volume of 0.2 M perchloric acid, and the amount of ATP was determined.

Table II
Effect of ATP depletion on protein degradation in nitrite- and phenylhydrazine-treated erythrocytes and reticulocytes

Cells were washed in saline and resuspended in Krebs-Ringer bicarbonate buffer (pH 7.4) containing either 5 mM glucose or 5 mM 2-deoxyglucose plus 0.2 mM 2,4-dinitrophenol. After a 2-h incubation at 25°C, the cells were washed and resuspended in the above buffers at 20% hematocrit. Nitrite or phenylhydrazine was added, and the cells were incubated at 37°C for 2 h. The release of alanine from both untreated and treated cells (determined as described in Table I) was linear for 2 h.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Protein degradation</th>
<th>Erythrocytes</th>
<th>Reticulocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>ATP-depleted</td>
<td>Control</td>
</tr>
<tr>
<td>None</td>
<td>27</td>
<td>15</td>
<td>97</td>
</tr>
<tr>
<td>Nitrite (10 mM)</td>
<td>66</td>
<td>46</td>
<td>—</td>
</tr>
<tr>
<td>Phenylhydrazine (1 mM)</td>
<td>124</td>
<td>101</td>
<td>193</td>
</tr>
</tbody>
</table>

seemed to affect the ATP-dependent degradative system (see below).

Activation Energy of Proteolysis in Oxidant-treated Reticulocytes—Recently, Hough and Rechsteiner (53) have measured the temperature dependence of protein degradation in HeLa cells and reticulocyte lysates to learn more about the rate-limiting step in this process. When a protein substrate is hydrolyzed by a typical well-characterized protease (e.g. papain or trypsin), an activation energy of 5–15 kcal/mol is obtained (53). By contrast, the ATP-dependent proteolytic system in reticulocyte lysates gave values of 27 ± 5 kcal/mol (53). Based on these data, they suggested that protein denaturation or cleavage of peptide bonds is not the rate-limiting step in the ATP-dependent proteolytic pathway. We therefore incubated reticulocytes at various temperatures and measured the breakdown of endogenous proteins in control cells and of proteins in phenylhydrazine-treated cells. At each of the temperatures studied, a linear rate of alanine release was seen (data not shown). As shown in Fig. 2, the activation energy calculated for the breakdown of endogenous proteins in untreated reticulocytes was 23.4 kcal/mol while that for oxidant-treated cells was 11.4 kcal/mol. Thus, the rate-limiting step in the degradation of oxidant-damaged proteins in vivo differs from that in the ATP-dependent breakdown of reticulocyte proteins.

Breakdown of Hemoglobin Treated with Oxidants in Red Blood Cell Lysates—Because the breakdown of proteins in intact reticulocytes and erythrocytes treated with oxidants appeared to occur independently of ATP, we attempted to obtain further evidence using cell-free extracts. Initial experiments showed that extracts of cells which had been treated with phenylhydrazine, and then washed and lysed, still degraded proteins at a much faster rate than extracts of untreated cells. As shown in Table III, the addition of phenylhydrazine directly to untreated reticulocyte lysates stimulated protein breakdown in the absence of ATP. Exposure to nitrite also caused a significant increase in ATP-independent proteolysis, even though this reagent actually inhibited ATP-dependent protein breakdown in reticulocyte lysates (Table III). This latter effect is probably on the degradative system itself since the breakdown of 15N-lysyl-lysine was also inhibited, whereas ATP levels were similar to those in untreated cells (data not shown).

Since it is difficult to regulate or limit the oxidative reactions after addition of phenylhydrazine or nitrite to the extract, in subsequent experiments we treated purified hemoglobin with these oxidants and then added known amounts to untreated lysates. The phenylhydrazine-treated hemoglobin had a characteristic dark-brown appearance and appeared to be the same size as native hemoglobin upon gel filtration and sodium dodecyl sulfate-gel electrophoresis (see above). Addition of this oxidant-damaged hemoglobin to reticulocyte or
TABLE III
ATP requirement for protein degradation in reticulocyte extracts exposed to nitrite and phenylhydrazine

75 μl of reticulocyte lysate were preincubated for 10 min at 0 °C in a final volume of 200 μl containing 50 mM Tris (pH 8), 10 mM Mg acetate and 1 mM DTT. In the experiments indicated, 10 mM sodium nitrite or 1 mM phenylhydrazine was also present. The mixtures were then incubated for 2 h at 57 °C with or without 5 mM ATP, and proteolysis was measured by the production of alanine from protein.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Proteolysis</th>
<th>−ATP</th>
<th>+ATP</th>
<th>Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>0.8</td>
<td>9.5</td>
<td>8.7</td>
</tr>
<tr>
<td>+Nitrite</td>
<td></td>
<td>2.9</td>
<td>8.2</td>
<td>5.3</td>
</tr>
<tr>
<td>+Phenylhydrazine</td>
<td></td>
<td>19.6</td>
<td>28.8</td>
<td>9.2</td>
</tr>
</tbody>
</table>

In related experiments, we found that hydrogen peroxide-treated hemoglobin was also degraded in both reticulocyte and erythrocyte extracts in an ATP-independent manner.

**Effects of Dilution and Incubation on Proteolysis in Reticulocyte Extracts**—The ATP-dependent ubiquitin-requiring degradative system in reticulocytes is known to require the interaction of multiple protein components (13). Therefore, we compared the effects of increasing dilution on the ATP-dependent breakdown of 125I-lysozyme or phenylhydrazine-treated hemoglobin. As shown in Table V, a 6-fold dilution of the lysate, relative to the standard assay volume, resulted in a 45% inhibition of the ATP-dependent breakdown of lysozyme while the degradation of phenylhydrazine-treated hemoglobin was not affected. Additional dilution (from 6- to 15-fold) resulted in the total loss of detectable ATP-dependent breakdown of lysozyme but had much less effect on the breakdown of the hemoglobin exposed to oxidants (Table V).

In the presence of 20% trichloroacetic acid was added to terminate the reaction, and denatured proteins were removed by centrifugation. 300 μl of the supernatant was neutralized, and the free alanine was determined.

The addition of native hemoglobin generated no alanine above that measured in the control. Phenylhydrazine-treated hemoglobin incubated in the absence of lysate also did not generate free alanine.

**Effect of dilution of reticulocyte lysates on the degradation of 125I-lysozyme, endogenous proteins and phenylhydrazine-treated hemoglobin**

40 μl of reticulocyte lysate were incubated in a final volume of either 120, 750, or 1,850 μl containing 50 mM Tris-HCl (pH 8), 1 mM DTT, 10 mM Mg acetate, 6.7% glycerol, 5 mM ATP where indicated, and 267 μg of 125I-lysozyme (498,000 cpm). The final concentration of lysozyme in each incubation was at least five times the Kₚ for this substrate. Assays were carried out for 1.5 h at 37 °C, and acid-soluble radioactivity was determined. The breakdown of phenylhydrazine-treated hemoglobin was determined under similar conditions except that the assay mixtures contained oxidant-treated hemoglobin instead of 125I-lysozyme. After 2 h at 37 °C, the protein was acid-precipitated, and alanine was measured in an aliquot of the supernatant. ATP-dependent activity at each condition was calculated by subtracting that seen in the absence of ATP. To obtain the breakdown of phenylhydrazine-treated hemoglobin, we subtracted the alanine production observed in the absence of added protein or ATP.

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**Table III**

| AtP requirement for protein degradation in reticulocyte extracts exposed to nitrite and phenylhydrazine |
| 75 μl of reticulocyte lysate were preincubated for 10 min at 0 °C in a final volume of 200 μl containing 50 mM Tris (pH 8), 10 mM Mg acetate and 1 mM DTT. In the experiments indicated, 10 mM sodium nitrite or 1 mM phenylhydrazine was also present. The mixtures were then incubated for 2 h at 57 °C with or without 5 mM ATP, and proteolysis was measured by the production of alanine from protein. |

**Table IV**

The degradation of phenylhydrazine-treated hemoglobin by reticulocyte and erythrocyte lysates

75 μl of reticulocyte or erythrocyte lysates were incubated in a final volume of 200 μl containing 50 mM Tris (pH 8), 10 mM Mg acetate, 1 mM DTT, and 5 mM ATP where indicated. Hemoglobin (1 mg) treated with 1 or 10 mM phenylhydrazine was added to the assay mixtures which were then incubated for 2 h at 57 °C. An equal volume of 20% trichloroacetic acid was added to terminate the reaction, and denatured proteins were removed by centrifugation. 300 μl of the supernatant were neutralized, and the free alanine was determined.

The addition of native hemoglobin generated no alanine above that measured in the control. Phenylhydrazine-treated hemoglobin incubated in the absence of lysate also did not generate free alanine.

**Table V**

Effect of dilution of reticulocyte lysates on the degradation of 125I-lysozyme, endogenous proteins and phenylhydrazine-treated hemoglobin

40 μl of reticulocyte lysate were incubated in a final volume of either 120, 750, or 1,850 μl containing 50 mM Tris-HCl (pH 8), 1 mM DTT, 10 mM Mg acetate, 6.7% glycerol, 5 mM ATP where indicated, and 267 μg of 125I-lysozyme (498,000 cpm). The final concentration of lysozyme in each incubation was at least five times the Kₚ for this substrate. Assays were carried out for 1.5 h at 37 °C, and acid-soluble radioactivity was determined. The breakdown of phenylhydrazine-treated hemoglobin was determined under similar conditions except that the assay mixtures contained oxidant-treated hemoglobin instead of 125I-lysozyme. After 2 h at 37 °C, the protein was acid-precipitated, and alanine was measured in an aliquot of the supernatant. ATP-dependent activity at each condition was calculated by subtracting that seen in the absence of ATP. (With 125I-lysozyme, but not with endogenous proteins or phenylhydrazine-treated hemoglobin, this ATP-dependent proteolysis increased with dilution.) To obtain the breakdown of phenylhydrazine-treated hemoglobin, we subtracted the alanine production observed in the absence of added protein or ATP.
Effect of preincubation of reticulocyte lysates without ATP on their ability to degrade \(^{125}\text{I}-\text{lysozyme},\) endogenous proteins and phenylhydrazine-treated hemoglobin

<table>
<thead>
<tr>
<th>Additions</th>
<th>(^{125}\text{I}-\text{lysozyme} ) degradation</th>
<th>Endogenous proteins</th>
<th>Phenylhydrazine-treated hemoglobin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \mu \text{m} / \text{h} )</td>
<td>nmol Ala/2 h</td>
<td>nmol Ala/2 h</td>
</tr>
<tr>
<td>None</td>
<td>0.2</td>
<td>0.2</td>
<td>0.8</td>
</tr>
<tr>
<td>ATP</td>
<td>3.5</td>
<td>1.0</td>
<td>4.9</td>
</tr>
</tbody>
</table>

The effect of preincubation of reticulocyte lysates was done at 0 or 37 °C for 1 h with 160 mM Tris-HCl (pH 8). 10 mM Mg acetate and 1 mM DTT. Substrate (20 \( \mu \)g of \(^{125}\text{I}-\text{lysozyme} \) or 60 \( \mu \)g of phenylhydrazine-treated Hb) was then added in the presence or absence of 5 mM ATP, and the mixtures were incubated at 37 °C for 1.5 h (lysozyme) or 2 h (phenylhydrazine-treated Hb). \(^{125}\text{I}-\text{Lysozyme} \) breakdown was measured by acid-soluble radioactivity, and the degradation of endogenous proteins or phenylhydrazine-treated Hb was measured by the release of alanine. The values for the degradation of phenylhydrazine-treated Hb were determined after subtracting alanine production from endogenous protein breakdown.

The Effect of Inhibitors on the Two Proteolytic Processes—

We also tested several compounds known to block the ATP-dependent proteolytic pathway in reticulocyte lysates. Both NEM (4, 22) and hemin (24, 45, 55) inhibit the ATP-dependent breakdown of lysozyme and endogenous substrates (Table VII) in a dose-dependent manner. In contrast, NEM and hemin had little effect on the breakdown of oxidant-treated hemoglobin in these same extracts (Table VII). Similarly, the ATP-dependent breakdown of endogenous proteins is inhibited by high concentrations of diisopropylfluorophosphate, an inhibitor of serine proteases (56), while the ATP-independent degradation of oxidant-damaged hemoglobin is unaffected by this reagent (Fig. 3). Another inhibitor of serine proteases, 3,4-dichloroisocoumarin (57), also appears to block the ATP-dependent breakdown of endogenous protein but has little effect on the ATP-independent degradation of oxidized hemoglobin (Table VII). Thus, NEM, DFP, hemin, and 3,4-dichloroisocoumarin are all able to block the ATP-dependent process without significantly affecting the degradation of oxidant-treated hemoglobin.

We have also found that certain random copolymers of amino acids are good protease inhibitors. Incubation of reticulocyte lysates with poly Glu-Tyr strongly inhibited both the breakdown of endogenous proteins and the degradation of oxidant-treated hemoglobin, but these processes clearly differed in their sensitivity to this inhibitor (Table VII). A concentration of 0.01 mg/ml inhibited the ATP-dependent degradative process by 50%, while 0.1 mg/ml was needed to obtain a similar degree of inhibition of the oxidant-treated hemoglobin.

Ubiquitin Conjugation Is Not Required for Breakdown of Oxidant-treated Hemoglobin—A recent report by Chin et al. (58) suggested that the rate of breakdown of denatured proteins was proportional to the amount of covalently bound ubiquitin (28). Since the results presented in our study indicate that ATP is not required for the hydrolysis of oxidant-treated hemoglobin, we investigated whether this protein was in fact a substrate for ubiquitin incorporation. When hemoglobin oxidized by either phenylhydrazine or \( \text{H}_2\text{O}_2 \) treatment was incubated in the presence of \(^{125}\text{I}-\text{ubiquitin}, \) ATP, and Fraction II, we found that both proteins were ubiquitinated, while native hemoglobin was not (Table VIII). These results confirm the observation (58) that oxidant-damaged hemoglo-
The incorporation of $^{125}$I-ubiquitin into Fraction II proteins and oxidant-damaged hemoglobin

The incorporation of $^{125}$I-ubiquitin into Fraction II protein (i.e., the material that was bound to DEAE-cellulose) or Fraction II protein plus hemoglobin was measured as described under "Experimental Procedures." In the absence of ATP, 5.5 $\times$ 10$^3$ cpm were bound to DEAE and this value has been subtracted. In the absence of ATP, no effect of the added hemoglobin was seen.

<table>
<thead>
<tr>
<th>Protein added</th>
<th>$^{125}$I-Ubiquitin incorporation (cpm $\times$ 10$^3$)</th>
<th>Stimulation by added protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>42.9</td>
<td>1</td>
</tr>
<tr>
<td>Hb</td>
<td>48.1</td>
<td>5.2</td>
</tr>
<tr>
<td>$H_2O_2$-treated Hb</td>
<td>58.7</td>
<td>15.8</td>
</tr>
<tr>
<td>Phenylhydrazine-treated Hb</td>
<td>68.3</td>
<td>25.4</td>
</tr>
</tbody>
</table>

**TABLE IX**

Effect of ubiquitin on the degradation of $^{125}$I-lysozyme and phenylhydrazine-treated hemoglobin by Fraction II

The degradation of $^{125}$I-lysozyme by reticulocyte Fraction II (300 µg) was measured in a final volume of 200 µl containing 50 mM Tris- HCl (pH 8), 10 mM Mg-acetate, 1 mM DTT, 5 mM ATP and 7.5 µg of ubiquitin were added where indicated. Incubations were carried out for 1 h at 37°C, and soluble radioactivity was determined. The ability of Fraction II to degrade endogenous proteins or phenylhydrazine-treated Hb (60 µg) was determined in a similar assay, except that breakdown was monitored by the release of alanine. The values obtained for the degradation of phenylhydrazine-treated Hb were determined after correcting for the degradation of endogenous proteins.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Degradation of $^{125}$I-Lysozyme</th>
<th>Degradation of Endogenous proteins</th>
<th>Phenylhydrazine-Hb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µg/h</td>
<td>nmol Ala/2 h</td>
<td>11.8</td>
</tr>
<tr>
<td>None</td>
<td>0.3</td>
<td>1.0</td>
<td>11.8</td>
</tr>
<tr>
<td>ATP</td>
<td>0.6</td>
<td>2.2</td>
<td>11.8</td>
</tr>
<tr>
<td>ATP + ubiquitin</td>
<td>1.6</td>
<td>4.9</td>
<td>11.4</td>
</tr>
</tbody>
</table>

DISCUSSION

It is widely believed that all intracellular protein breakdown, both lysosomal and nonlysosomal, requires metabolic energy. The basis for this ATP requirement has been extensively studied for nonlysosomal proteolysis in mammalian and bacterial cells (1, 13). For example, a continuous supply of ATP is required for the degradation of abnormal and normal proteins in bacteria (59) as well as in mitochondria (14, 16, 17) and chloroplasts (15, 60). Similarly, inhibitors of ATP production prevent the degradation of analog-containing and puromycyl polypeptides in reticulocytes (4, 20), of short- and long-lived normal proteins in growing fibroblasts (61), of various short-lived enzymes in hepatocytes (62), and of normal proteins during reticulocyte maturation (21, 22). However, the present studies clearly show that the rapid breakdown of proteins damaged by oxidizing agents in red blood cells does not require ATP. This conclusion is supported by our observations on intact cells, on reticulocyte or erythrocyte extracts exposed to phenylhydrazine or nitrite (Tables II–IV), and on lysates of normal reticulocytes to which phenylhydrazine-treated hemoglobin was added. This lack of an ATP requirement in reticulocytes is particularly convincing since in these same cells or extracts, the degradation of endogenous proteins (Tables III and IV) and of various exogenous polypeptides, such as lysozyme (Table VI) requires high-energy phosphates.

It is noteworthy that erythrocytes from rabbit and man appear to have lost the ATP-dependent degradative system found in reticulocytes (Table IV, Refs. 22 and 23). Normally, erythrocytes show only very low levels of proteolysis; but this process is clearly enhanced upon exposure to oxidants (Refs. 10–12, Table I). Previously, this rapid degradation of oxidant-treated proteins in erythrocytes was suggested to require ATP (10) in contrast to the present results. The prior conclusion was based on misleading observations apparently resulting from nonspecific effects of reagents used (e.g. arsenate and fluoride) to prevent glycolysis and the complex effects of nitrite-induced damage.

A variety of observations indicate the existence in these cells of two distinct degradative systems, an ATP-dependent system and an ATP-independent apparatus for degradation of oxidant-damaged hemoglobin. For example, the ATP-dependent process is much more labile in extracts. Dilution of the lysates (Table V) or incubation at 37°C in the absence of ATP (Table VI) resulted in a rapid loss of the ATP-stimulated process, but these treatments had little or no effect on the degradation of oxidant-treated hemoglobin. This sensitivity of the ATP-dependent process to dilution may result because this pathway requires the interaction of multiple components, including at least three enzymes involved in ubiquitin conjugation (13). The rapid loss of this process at 37°C is consistent with the observation of Hershko et al. (54) that some unidentified factor is labile at 42°C in the absence of ATP. By contrast, our experiments indicate that multiple interacting proteins or unstable factors are not essential for the degradation of oxidant-damaged hemoglobin.

Further evidence that the breakdown of endogenous reticulocyte proteins and the oxidant-treated hemoglobin occur by distinct mechanisms comes from their different energies of activation (Fig. 3). Hough and Rechsteiner (53) have noted the large activation energy for ATP-dependent proteolysis and interpreted this property as evidence that the rate-limiting step in this process is not the action of a simple proteolytic enzyme. This high energy of activation may be related to the involvement of an ATP-dependent protease or perhaps to the ubiquitin conjugation process, neither of which seems to play a role in the degradation of proteins damaged by oxidants. The low activation energy for the breakdown of proteins exposed to oxidants would be consistent with involvement of a proteolytic enzyme as the rate-limiting step, and evidence for this conclusion will be presented elsewhere.

More direct evidence for two distinct processes was obtained with a variety of inhibitors which clearly discriminate between these two pathways. The inhibitors of serine proteases, DFP and 3,4-dichloroisocoumarin, the sulfhydryl reagent NEM, and hemin, an inhibitor of the ATP-dependent pathway (45), have little effect on the degradation of phenyl-

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hydrazine-treated hemoglobin at concentrations which greatly reduce the ATP-dependent process (Table VII, Fig. 2). Thus, the initial steps in the degradation of oxidant-damaged proteins do not seem to involve a typical serine or sulfhydryl protease, or one sensitive to hemin. In the ATP-dependent pathway, NEM could react either with a protease or one of the components of the ubiquitin cycle, which contain essential sulfhydryl groups (46, 63). Although these two degradative processes differ in many biochemical properties, they may still utilize some of the same cytosolic enzymes (e.g., exopeptidases).

It has been generally assumed that eukaryotic cells contain a single cytosolic pathway for the breakdown of "abnormal proteins." The present studies demonstrate an additional pathway for the breakdown of specific types of abnormal hemoglobin. Damage to hemoglobin by oxidants can occur under many conditions in vivo. Like phenylhydrazine, hydrogen peroxide promotes protein breakdown (11) and makes hemoglobin more susceptible to ATP-independent proteolysis.2 In vivo, this ATP-independent degradative system may be involved in the breakdown of other types of damaged polypeptides or native proteins. Recently, Woods and Lazarides (64) have presented an elegant model for regulation of the assembly of the erythrocyte membrane cytoskeleton. This process involves the selective degradation of free β-spectrin, which also was shown not to require ATP.

What structural features distinguish these oxidant-damaged proteins and lead to their selective breakdown are unclear. Damage to amino acid side chains could result in changes in the secondary and tertiary conformation of a protein. It has been proposed that oxidation of only a single histidine residue on bacterial glutamine synthetase may be a specific marking reagent that renders this enzyme susceptible to proteolytic attack in vivo (65, 66). Although we have not investigated thoroughly changes in amino acid residues in hemoglobin, treatment with phenylhydrazine and nitrite appear to extensively alter the structure of this molecule making it much more susceptible to digestion by a variety of well-characterized proteases.3 It is also unclear what conformational properties of other abnormal polypeptides lead to their degradation by the ATP-dependent pathway. Ubiquitin conjugation appears important in the recognition of potential substrates (13) and has been proposed to tag them for degradation. An observation that had been used to support this role for ubiquitin conjugation (58) is that phenylhydrazine-treated hemoglobin is more susceptible to ubiquitin conjugation than normal hemoglobin and is degraded more rapidly in reticulocytes (Table IV) or in fibroblasts (58, 67). However, the present findings show that proteins treated with phenylhydrazine as well as other oxidants2,3 are degraded by a pathway not requiring ATP or ubiquitin. These observations emphasize that ubiquitin conjugation cannot be equated with ATP-dependent proteolysis and may simply reflect the unfolding of these modified proteins.

It remains to be established whether other cells also contain this ATP-independent soluble proteolytic system. In the red cell, elimination of proteins damaged by oxidants may be particularly important because these cells are exposed continually to high oxygen tensions and contain high levels of a single iron-containing protein, and because HbO2 generates superoxide continually. There has been much interest in the effect of oxidants on proteins within red cells and the possible involvement of the cell membrane in this process. However, our results and the related studies of Davies and Goldberg (68) have shown that oxidants can damage hemoglobin directly. Recently, it has also been proposed that membrane-bound proteases may play a critical role in the breakdown of globin chains or hemoglobin β-chains in erythrocytes (69, 70). However, rapid hydrolysis of hemoglobin was observed when soluble extracts were treated with oxidants or when damaged hemoglobin itself was added to soluble lysates (Tables III and IV). Therefore, membrane-bound components are not necessary for the initiation of protein damage or for the elimination of proteins damaged by oxidants.

We are presently characterizing the components of the ATP-dependent and ATP-independent proteolytic pathways in red cells to identify the critical proteases involved in the degradation of different types of damaged proteins. Red cells contain a variety of endoproteases that can function in the absence of ATP or ubiquitin, including two insulin-degrading endoproteases (71), a calcium-activated thiol protease (69, 70), and a high molecular weight alkaline protease (72, 73). The latter enzyme from liver (74, 75) has recently been shown capable of degrading oxidant-damaged E. coli glutamine synthetase (75). Elsewhere,2 we will present evidence that this high molecular weight protease is involved in the degradation of the oxidant-damaged polypeptides in red cells.

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REFERENCES