Oxidized Proteins in Erythrocytes Are Rapidly Degraded by the Adenosine Triphosphate–Dependent Proteolytic System

Abstract. *The rate of protein degradation in rabbit erythrocytes is normally very low. However, when cells were exposed to agents that oxidize cell proteins (nitrite or phenylhydrazine), the degradation of erythrocyte proteins to amino acids increased 7- to 33-fold. This effect was inhibited by the reducing agent methylene blue. Stimulation of proteolysis also occurred in cell extracts and resulted from the production of substrates (damaged proteins) rather than from activation of proteases. Inhibitors of glycolysis and of the soluble adenosine triphosphate–dependent proteolytic pathway decreased the protein degradation induced by nitrite, whereas inhibitors of lysosomal proteolysis had no effect. Thus, the adenosine triphosphate–dependent proteolytic system is present in mature red cells where it may help protect against the accumulation of proteins damaged by oxidation or other means.*

In mammalian and bacterial cells proteins with highly abnormal structures are hydrolyzed very rapidly (*1, 2*). This process helps to prevent the accumulation of aberrant and potentially harmful polypeptides that may arise by biosynthetic errors or mutation. In extracts of reticulocytes and *Escherichia coli*, nonlysosomal, adenosine triphosphate (ATP)–dependent proteolytic systems selectively degrade abnormal proteins containing amino acid analogs (*3–8*) or puromycin (*5*) or incomplete proteins resulting from nonsense mutations (*7*). Proteins with highly abnormal structures may also arise in cells by postsynthetic damage; polypeptides in vitro are subject to denaturing influences, such as oxidation, thermal denaturation, and free radical damage, that must also be present in vivo. Although complex mechanisms (for example, the methemoglobin reductase and superoxide dismutase systems) reduce such damage to cellular proteins, many postsynthetic covalent modifications have been observed on intracellular proteins (*9*), including nonenzymatic glycosylation, cross-linking, deamidation, and proteolytic cleavages.

We studied the fate of oxidized proteins in rabbit erythrocytes to determine whether postsynthetic damage to a protein may lead to its rapid degradation by the soluble ATP-dependent pathway. We oxidized cell proteins by treating erythrocytes with phenylhydrazine or sodium nitrite, which enter red blood cells and produce methemoglobinemia (*10, 11*). Exposure to phenylhydrazine also results in the formation of intracellular aggregates of oxidized protein that can alter cell shape and shorten cell lifespan in vivo (*12*). In isolated reticulocytes, proteolysis can be easily assayed by measuring the production of tyrosine from cell protein (*13*), but this process occurs very slowly in mature erythrocytes under normal conditions [figure 1 in (*13*)].

When sodium nitrite (10 m*M*) was added to a suspension of erythrocytes, the rate of tyrosine generation from proteins increased within 10 minutes, 7- to 25-fold, in different experiments (Fig. 1). The addition of phenylhydrazine (1 m*M*) stimulated proteolysis by 15- to 33-fold (Fig. 1). Both agents induced methemoglobin production in the cells, but neither induced cell lysis, altered the *p*H of the medium, or affected the sensitivity of the tyrosine assay. Most of the tyrosine produced was recovered in the incubation medium, although the intracellular tyrosine pool also increased slightly (~20 percent). These treatments also stimulated the appearance of 20 other amino acids in the medium, as measured by amino acid analysis (data not shown); thus mature red cells contain enzymes for the complete hydrolysis of proteins.

Table 1. Effects of inhibitors of glycolysis, of the ATP-dependent proteolytic system, and of lysosomal proteolysis on nitrite-induced protein degradation. Cell suspensions were prepared and incubated for 1 hour in the presence of sodium nitrite (10 m*M*) and inhibitors (Fig. 1). For experiments with fluoride and arsenate, the cells were incubated for 1 hour with these agents and nitrite to deplete cellular ATP stores. Protein degradation was measured during the subsequent hour. Erythrocyte suspensions that were not exposed to nitrite generated tyrosine at rates of 0.4 (experiment 1), 3 (experiment 2), and 0.5 nmole/ml-hour (experiment 3). Similar effects were observed in at least three independent experiments.

Inhibitor	Tyrosine production (nmole/ml-hour)	Inhibition (%)
Experiment 1		
None	23.6	0
Sodium fluoride (20 m*M*)	7.4	69
Sodium arsenate (10 m*M*)	8.5	65
Experiment 2		
None	33.9	0
N-Ethylmaleimide (5 m*M*)	6.4	81
o-Phenanthroline (1 m*M*)	15.9	53
Experiment 3		
None	22.3	0
Leupeptin (30 μ*M*)	21.0	5
Chloroquine (10 μ*M*)	20.0	10

After repeated washings of the cell and removal of the buffy coat, most of the erythrocyte preparations from normal rabbits contained less than 1 percent reticulocytes and no white blood cells. Similar results were obtained in preparations in which no reticulocytes were evident on microscopy. In samples obtained from anemic animals with large numbers of reticulocytes (40 to 80 percent), a high basal rate of proteolysis was evident (*13*), but addition of nitrite or phenylhydrazine further increased protein degradation (data not shown). Precise comparisons of the degree of stimulation of proteolysis in reticulocytes and erythrocytes are difficult because reticulocyte preparations also contain many erythrocytes and because the untreated reticulocytes are in negative protein balance (*13*). Nevertheless these experiments demonstrate that reticulocytes show a similar, or perhaps a slightly smaller, response to nitrite and phenylhydrazine than erythrocytes do.

In red cells, methemoglobin, and probably other oxidized proteins, can be reduced by an NADPH-dependent methemoglobin reductase (NADPH, nicotinamide adenine dinucleotide phosphate, reduced form), and this process is greatly stimulated by methylene blue (*14*). Addition of $10^{-5}M$ methylene blue to nitrite-treated erythrocytes resulted in an 85 percent reduction in proteolysis (Fig. 1). In contrast, methylene blue had no effect on the degradation of protein that occurs in reticulocytes in the absence of oxidizing agents. Methylene blue probably prevents the increase in proteolysis by stimulating cellular mechanisms for reducing the oxidized protein.

We tested whether the soluble ATP-dependent pathway, which degrades abnormal proteins in reticulocytes (*3–6*), catalyzes this process. During treatment with nitrite, the cells were incubated with inhibitors of glycolysis or inhibitors of this cytoplasmic ATP-dependent system. The stimulation of protein degradation by nitrite was reduced markedly when ATP production was inhibited with fluoride or arsenate (Table 1). In reticulocytes and reticulocyte extracts, *N*-ethylmaleimide and *o*-phenanthroline are potent inhibitors of the ATP-dependent breakdown of analog-containing proteins (*3*) and normal proteins during reticulocyte maturation (*13*); and in erythrocytes, these agents markedly decreased

0036-8075/82/0226-1107$01.00/0

Table 2. Effects of treating erythrocytes with oxidizing agents on proteolytic activity in cell extracts. Cells were suspended in the presence or absence of 10 m*M* sodium nitrite or 1 m*M* phenylhydrazine and incubated at 37°C for 45 minutes. The cells were then collected by centrifugation and lysed with 1.5 volumes of deionized water. After the suspensions were centrifuged at 100,000*g* for 2 hours, the supernatants were incubated (*13*) for 1 hour in the presence of 2 m*M* ATP. The degradation of endogenous cell proteins was measured by the appearance of tyrosine in acid-soluble form and expressed as nanomoles of tyrosine per hour (see Fig. 1). Hydrolysis of [^{3}H]methyl casein (*26*) to acid-soluble material was measured in 100 μl of the lysates (*3*).

Cell lysates	Endogenous protein		^{3}H-Labeled casein	
	Nanomoles per hour	Percent of control	Percent per hour	Percent of control
Control	0.68	100	11	100
Nitrite-treated	4.29	630	5.1	47
Phenylhydrazine-treated	4.75	698	2.7	25

nitrate-induced proteolysis (Table 1). Nitrate-induced proteolysis was not affected by chloroquine, which raises intralysosomal *p*H and thereby inhibits intralysosomal proteolysis (*15*), or by leupeptin, an inhibitor of several lysosomal proteases (*16*) and of the soluble Ca^{2+}-activated protease in red cells (*17*).

These experiments indicate that an ATP-dependent proteolytic pathway, similar to that responsible for the breakdown of abnormal proteins in reticulocytes (*3*), is active in erythrocytes exposed to oxidants. This pathway has not been found in erythrocyte lysates in some studies (*4, 18*). We have observed, however, that extracts of erythrocytes hydrolyze ^{14}C-labeled globin and ^{3}H-labeled casein at alkaline *p*H by an ATP-stimulated process (*19, 20*), although the stimulation by ATP (20 to 100 percent) is smaller than that routinely found for reticulocyte lysates (*3, 4*). Furthermore, we have purified from these cells an ATP-stimulated alkaline protease similar to that in reticulocytes, and there is no other alkaline protease with the appropriate specificity and inhibitor sensitivity in these cells (*19, 20*).

The proteolysis induced by oxidizing agents in erythrocytes reflects either the activation of the ATP-dependent proteolytic system or the production of new substrates for degradation. Experiments with erythrocyte lysates supported the latter hypothesis (Table 2). Alkaline (*p*H 7.8) extracts from nitrite- and phenylhydrazine-treated cells generated tyrosine from endogenous protein eight times faster than control lysates did, in accord with findings on intact cells (Fig. 1). If this effect were due to activation of proteases, then the lysates from nitrite or phenylhydrazine-treated cells should also degrade an exogenous substrate (^{3}H-labeled casein) at an increased rate. On the contrary, the hydrolysis of ^{3}H-labeled casein by lysates from oxidant-treated cells was 50 to 70 percent lower than that produced by control lysates (Table 2). This result suggests that exposure to the oxidizing agents generates proteins that compete with the ^{3}H-labeled casein as substrates for the proteolytic enzymes.

Besides catalyzing the breakdown of abnormal proteins resulting from mutation or biosynthetic errors (*3–6, 8*) and of reticulocyte proteins lost during maturation (*13, 21*), the soluble ATP-dependent proteolytic system also appears to degrade proteins damaged postsynthetically by oxidation or by other mechanisms such as free radical damage (*22*). There are a number of intracellular systems that help to keep proteins reduced or to prevent structural damage. When these systems are overwhelmed by the quantity of aberrant proteins (for example, after treatment with 10 m*M* nitrite), or when no mechanism exists to repair a particular type of damage (for example, denaturation), the ATP-dependent proteolytic system can reduce the buildup of such abnormal proteins. The accumulation of these damaged proteins could interfere with cell viability, especially in long-lived cells, such as erythrocytes (*12*). In various other cells, the nonlysosomal ATP-dependent system degrades analog-containing proteins (*3–6*) and may also help protect these cells against postsynthetic damage to cell proteins. In rat skeletal muscle, nitrite promotes protein degradation, as it does in erythrocytes (*23*).

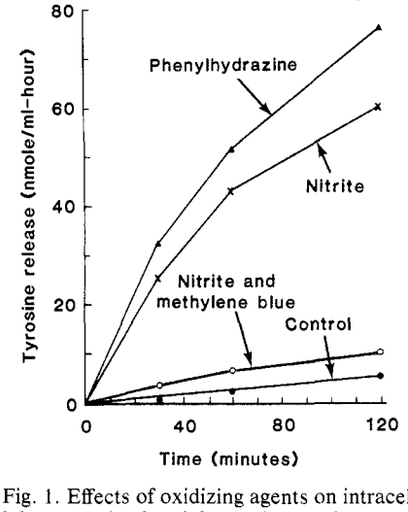

Fig. 1. Effects of oxidizing agents on intracellular protein breakdown in erythrocytes. Erythrocyte suspensions (5 to 7 ml) were incubated as described (*13*) in the presence of (▲) 1 m*M* phenylhydrazine, (x) 10 m*M* sodium nitrite, or (○) 10 m*M* sodium nitrite plus 10^{-5}*M* methylene blue, or (●) in the absence of these agents. The net accumulation of tyrosine in the cells and in the medium was measured (*13, 25*). In this experiment and the others reported here, tyrosine determinations on duplicate flasks agreed within 5 percent. These effects of nitrite and phenylhydrazine were observed in at least 12 similar experiments, and the inhibitory effects of methylene blue were observed in three separate experiments.

It remains to be established what other postsynthetic alterations to proteins lead to their rapid intracellular degradation. Phosphorylation, glycosylation, acetylation, or methylation of proteins, which are usually associated with specific physiological modifications, may also occur nonenzymatically or by random enzymatic errors, and thus may represent a form of damage causing degradation of the affected proteins. Intracellular denaturation has often been suggested as the rate-limiting step in degradation of most cell proteins (*1, 24*). Thus, the selective hydrolysis of damaged or partially denatured proteins by the soluble ATP-dependent system may account for much of the diversity of protein half-lives in vivo.

ALFRED L. GOLDBERG
FRANCEE S. BOCHES*
Department of Physiology,
Harvard Medical School,
Boston, Massachusetts 02115

References and Notes

1. A. L. Goldberg and J. F. Dice, *Annu. Rev. Biochem.* **43**, 835 (1974).
2. A. L. Goldberg and A. C. St. John, *ibid.* **45**, 747 (1976).
3. J. D. Etlinger and A. L. Goldberg, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 54 (1977).
4. A. Hershko, H. Heller, D. Ganoth, A. Ciechanover, in *Protein Turnover and Lysosome Function*, H. L. Segal and D. J. Doyle, Eds. (Academic Press, New York, 1978), pp. 149–169; H. Hershko, A. Ciechanover, H. Heller, A. L. Haas, A. Hershko, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 1783 (1980); A. Ciechanover *et al.*, *ibid.*, p. 1365.
5. J. D. Etlinger and A. L. Goldberg, *J. Biol. Chem.* **255**, 4563 (1980).
6. A. L. Goldberg, J. Kowit, J. Etlinger, Y. Klemes, in *Protein Turnover and Lysosome Function*, H. C. Segal and D. J. Doyle, Eds. (Academic Press, New York, 1978), pp. 171–196.
7. K. Murakami, R. W. Voellmy, A. L. Goldberg, *J. Biol. Chem.* **254**, 8194 (1979); R. W. Voellmy and A. L. Goldberg, *Nature (London)* **290**, 419 (1981); C. H. Chung and A. L. Goldberg, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 4931 (1981).

8. C. S. Chandler and F. J. Ballard, *Biochem. J.* **176**, 151 (1978); M. Rabinowitz and J. M. Fisher, *Biochim. Biophys. Acta* **91**, 313 (1969); Y. Klemes, J. D. Etlinger, A. L. Goldberg, *J. Biol. Chem.* **256**, 8436 (1981).
9. R. Uy and F. Wold, *Science* **198**, 890 (1977).
10. H. M. Ranney and H. Lehmann, in *The Red Blood Cell*, D. M. Surgenor, Ed. (Academic Press, New York, ed. 2, 1975), pp. 873–908.
11. E. Beutler, *Pharmacol. Rev.* **21**, 73 (1969).
12. C. C. Winterbaum and R. W. Carrel, *Nature (London)* **240**, 150 (1972).
13. F. S. Boches and A. L. Goldberg, *Science* **215**, 978 (1982).
14. H. Hsieh and E. R. Jaffe, in *The Red Blood Cell*, D. M. Surgenor, Ed. (Academic Press, New York, ed. 2, 1975), pp. 799–824.
15. B. Poole, S. Ohkuma, M. Warburton, in *Protein Turnover and Lysosome Function*, H. L. Segal and D. J. Doyle, Eds. (Academic Press, New York, 1978), pp. 43–58; P. Libby, S. Burstajn, A. L. Goldberg, *Cell* **19**, 481 (1980).
16. T. Aoyagi and H. Umezawa, in *Proteases and Biological Control*, E. Reich, D. B. Rifkin, E. Shor, Eds. (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1975), p. 429; P. Libby and A. L. Goldberg, *Science* **199**, 534 (1978).
17. L. Waxman, in *Protein Turnover and Lysosome Function*, H. L. Segal and D. J. Doyle, Eds. (Academic Press, New York, 1978), pp. 363–377.
18. S. Speiser, M. J. Glucksman, J. D. Etlinger, *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **39**, 1682 (Abstr.) (1980).
19. F. S. Boches, Y. Klemes, A. L. Goldberg, *ibid.*, p. 1682.
20. F. S. Boches, L. Waxman, A. L. Goldberg, in preparation.
21. M. Muller, W. Dubiel, J. Rathmann, S. Rapoport, *Eur. J. Biochem.* **109**, 405 (1980).
22. R. Kirschner and A. L. Goldberg, *Fed. Proc. Fed. Am. Soc. Exp. Biol.*, in press.
23. A. L. Goldberg and F. S. Boches, unpublished observations.
24. G. McLendon and E. Radany, *J. Biol. Chem.* **253**, 6335 (1978).
25. T. P. Waalkes and S. Udenfriend, *J. Lab. Clin. Med.* **50**, 733 (1957).
26. R. H. Rice and G. E. Means, *J. Biol. Chem.* **246**, 831 (1971).
27. Supported by grants from the National Institute of Neurological and Communicative Disorders and Stroke and from the Muscular Dystrophy Association to A.L.G. and by fellowships from the Massachusetts Heart Association and the Muscular Dystrophy Association to F.S.B. We thank J. Goldberg for technical assistance and R. Levine and M. Rush for help in preparing the manuscript.
* Present address: Clinical Assays, 600 Memorial Drive, Cambridge, Mass. 02139.

22 June 1981; revised 21 September 1981

Tonicity Effects on Intact Single Muscle Fibers: Relation Between Force and Cell Volume

Abstract. *Contraction of isolated, intact frog muscle fibers under increasing tonicity of the external solution was studied by adding (i) effectively impermeant sodium chloride and sucrose and (ii) permeant potassium chloride. Force of isometric contraction decreased as a function of tonicity, independent of the permeability of the solute. In contrast, cell volume changed with tonicity in impermeant solutes and was constant with potassium chloride. The results are evidence that ionic strength in the sarcoplasm directly influences the contraction mechanism. Also, the findings show that force development is unaffected by changes in fiber volume, suggesting that the force per cross-bridge is constant at different distances between the thin and thick myofilaments. Finally, in light of the length-force relation, the results support the idea that cross-bridges are independent force generators.*

Muscle force is generated in the cross-bridges that form between the thin and thick myofilaments during contraction. According to the current sliding filament models of muscular contraction, the cross-bridges act independently of each other. The length-force relation found for frog muscle fibers was important in establishing this conclusion because force levels closely corresponded to the filament overlap in the cross-bridge region at sarcomere lengths between 2.0 and 3.5 μm (*1*). However, the lattice volume of the intact muscle is constant with change in length; therefore the lateral (radial) distance between thin and thick filaments decreases with increasing sarcomere length (*2*, *3*). These observations indicate that the contraction mechanism is sensitive to radial separation and that this is a factor in the length-force relation (*1*). The explanation of this relation assumes that the decrease in radial spacing has no effect on force. Several attempts have been made to test this assumption indirectly (*4*–*6*). In more direct tests on demembranated frog muscle fibers, fiber width and the radial separation between the filaments were found to decrease in the presence of polyvinylpyrrolidone (*7*), and these effects were accompanied by changes in force and fiber stiffness (*8*). These results undermine the assumption that the decrease in radial spacing has no effect on force.

In an effort to clarify this issue, we investigated the influence of raising the tonicity of the external bathing medium on force development in isolated frog muscle fibers with the following strategy (*9*). We applied the principles of Boyle and Conway (*10*) to manipulate cell volume and ionic strength by adding (i) effectively impermeant NaCl and sucrose, which change the volume and the concentrations of intracellular solutes, and (ii) permeant KCl, which changes only the ionic concentrations in the cell. The fiber was maximally contracted under both conditions. The results provide direct evidence that force development in frog muscle is independent of changes in fiber volume and presumably, therefore, of changes in radial separation between the myofilaments.

Single intact fibers were isolated from the semitendinosus muscle in the frogs *Rana pipiens pipiens* and *Rana temporaria* (*11*). Resting sarcomere length was 2.2 to 2.3 μm (*12*, *13*). Each fiber was activated by a rapid temperature step in the presence of caffeine (*14*), achieved by transfer of the fiber from a chamber at 25°C to a chamber at 0°C. The transfer was usually made within 2 seconds (*15*), and the temperature step was practically instantaneous. This technique produced fiber activation in all solutions, including KCl, in which muscle fibers are depolarized and therefore electrically inexcitable.

Figure 1A shows two activation-relaxation cycles of one fiber in two solutions. The fiber, which was relaxed at 25°C, contracted quickly at 0°C, the force reaching a stable plateau. The force response in a solution with a tonicity (T, the total molarity of all solutes) of 1.0 (left-hand trace) is compared with a typical record obtained in a hypertonic solution (1.8 T with KCl) (right-hand trace). The response is nearly the same in each case except that the force level is lower in the hypertonic solution. Similar responses were also seen with NaCl and sucrose. It generally was possible to record 15 to 20 isometric contraction cycles in a fiber with a change from the original force level of less than 10 percent and no significant alteration in the laser diffraction pattern. In hypotonic solutions the force plateau was less stable. In the presence of high calcium, the force plateau for fibers from *R. temporaria* was somewhat more stable than that for fibers from *R. pipiens pipiens*.

The force response with NaCl and sucrose is shown in Fig. 1B. Tension measurements were made at the plateau region of the force response in normal and hypertonic solutions and at the response peak in hypotonic solutions. There is a linear decrease in force with increasing tonicity in the range 0.6 to 1.8 T. The average force at 0.6 T is about 1.3 times greater than that at 1.0 T, and at 1.4 T the relative force is 0.75. These results agree with the corresponding mean values for tetanic force levels obtained with electrical stimulation in single fibers up to 1.4 T (*16*). Also, the results extend the effect of tonicity (with sucrose) to 1.8 T; the relative force in this case is 0.45.

Figure 1C shows the force response

0036-8075/82/0226-1109$01.00/0