Vanadate Inhibits the ATP-dependent Degradation of Proteins in Reticulocytes without Affecting Ubiquitin Conjugation*

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Reticulocytes contain a nonlysosomal, ATP-dependent system for degrading abnormal proteins and normal proteins during cell maturation. Vanadate, which inhibits several ATPases including the ATP-dependent proteases in Escherichia coli and liver mitochondria, also markedly reduced the ATP-dependent degradation of proteins in reticulocyte extracts. At low concentrations (K_v = 50 μM), vanadate inhibited the ATP-dependent hydrolysis of [3H]methylcasein and denatured 125I-labeled bovine serum albumin, but it did not reduce the low amount of proteolysis seen in the absence of ATP. This inhibition by vanadate was rapid in onset, reversed by dialysis, and was not mimicked by molybdate.

Vanadate inhibits proteolysis at an ATP-stimulated step which is independent of the ATP requirement for ubiquitin conjugation to protein substrates. When the amino groups on casein and bovine serum albumin were covalently modified so as to prevent their conjugation to ubiquitin, the derivatized proteins were still degraded by an ATP-stimulated process that was inhibited by vanadate. In addition, vanadate did not reduce the ATP-dependent conjugation of 125I-ubiquitin to endogenous reticulocyte proteins, although it markedly inhibited their degradation.

In intact reticulocytes vanadate also inhibited the degradation of endogenous proteins and of abnormal proteins containing amino acid analogs. This effect was rapid and reversible; however, vanadate also reduced protein synthesis and eventually lowered ATP levels in the intact cells. Vanadate (10 mM) has also been reported to decrease intralysosomal proteolysis in hepatocytes. However, in liver extracts this effect on lysosomal proteases required high concentrations of vanadate (K_v = 500 μM) and was also observed with molybdate, unlike the inhibition of ATP-dependent proteolysis in reticulocytes.

Reticulocytes, and probably other mammalian cells, contain a soluble, ATP-dependent proteolytic system (1, 2). This nonlysosomal degradative pathway appears responsible for the selective breakdown of abnormal polypeptides, as may result from mutations (3), biosynthetic errors (3), or postsynthetic damage (4), and also for the selective elimination of many normal proteins during reticulocyte maturation into erythrocytes (5-7). Of particular biochemical interest is the function of ATP in this process (2, 8). Hershko, Rose, and colleagues have proposed that, in this pathway, ATP is required for the covalent linkage of the polypeptide, ubiquitin, to protein substrates, in order to enhance their susceptibility to cytoplasmic proteases (2, 9, 10). In this conjugation reaction, ATP is essential for the enzymatic activation of the carboxyl-terminal glycine on ubiquitin to a form which is then linked by an isopeptide bond to ε-amino groups on lysine residues in proteins. According to this model, free amino groups must be available on proteins for them to be substrates for ATP-dependent proteolysis. However, we recently showed that if the amino groups were covalently modified so as to prevent their conjugation to ubiquitin, these proteins were still degraded by an ATP-stimulated process (8). Thus, in reticulocytes, ATP seems to serve at least two distinct functions in the soluble pathway for protein breakdown, one requiring and one independent of ubiquitin.

When the original ubiquitin model was proposed, there was no precedent for direct involvement of ATP in the function of proteolytic enzymes. However, since that time, ATP-dependent proteases have been purified from Escherichia coli (11-13) and rat liver mitochondria (14), and the ATP requirement for these enzymes has been shown to be responsible for the energy requirement for protein breakdown in intact bacteria (15) and mitochondria (16). These novel proteases contain an ATPase activity that is essential for proteolysis (11, 12). In fact, inhibition of their ATPase activity with vanadate or quercetin reduces proteolysis in parallel with the fall in ATP hydrolysis (13, 17).

If a similar ATP-dependent protease existed in rabbit reticulocytes, it could help account for the ATP requirement for protein breakdown that is independent of ubiquitin conjugation. To test this possibility, we investigated whether the ATPase inhibitor, vanadate (18), also decreased ATP-dependent proteolysis in reticulocyte extracts and intact reticulocytes. These studies demonstrate that vanadate is a very potent inhibitor of this pathway and that it acts on an ATP-requiring step which is independent of ubiquitin.

MATERIALS AND METHODS

RESULTS

Effect of Vanadate on Proteolysis in Reticulocyte Extracts—As shown in Fig. 1, addition of vanadate (100 μM) to reticu-

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‡ Recipient of a career development award from the Eli Lilly Foundation.

1 The "Materials and Methods" are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 83M-1280, cite the authors, and include a check or money order for $1.60 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
Inhibition of ATP-dependent Degradation of Proteins

**Fig. 1** (left). Effect of vanadate on [CH$_3$-$^3$H]casein hydrolysis in reticulocyte extracts with (•—•) or without (O—O) 5 mM ATP. Vanadate (100 mM) was added either initially (+—+), or after the reaction had proceeded in the presence of ATP for 60 min (×—×), as indicated by the arrow.

**Fig. 2** (right). Effects of different concentrations of vanadate on [CH$_3$-$^3$H]casein hydrolysis in reticulocyte extracts. Assays were conducted in the presence (•—•) or absence (O—O) of 5 mM ATP.

**Table I**

<table>
<thead>
<tr>
<th>Effect of vanadate addition and removal on protein breakdown in reticulocyte extracts</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP was added at the final concentration of 5 mM and vanadate at 100 mM. To remove the vanadate, 1 ml of reticulocyte extract was pretreated with vanadate (100 mM) and dialyzed at 4 °C against 50 mM Tris-HCl, pH 7.8, containing 8 mM KCl, 5 mM MgCl$_2$, 1 mM dithiothreitol, and 20% glycerol. 50 μl of the reticulocyte extracts were used to assay for breakdown of radioactive substrates and 0.5 ml in the assay for the breakdown of endogenous protein.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Hydrolysis of</th>
<th>[CH$_3$-$^3$H]Casein</th>
<th>$^{125}$I-BSA</th>
<th>Endogenous proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>%/h</td>
<td>%/h</td>
<td>nmol Tyr/h</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>2.8</td>
<td>13.4</td>
<td>0</td>
</tr>
<tr>
<td>Vanadate</td>
<td>2.7</td>
<td>6.3</td>
<td>0</td>
</tr>
<tr>
<td>Vanadate then dialysis</td>
<td>2.9</td>
<td>13.9</td>
<td>0</td>
</tr>
</tbody>
</table>

Reticulocyte extracts dramatically inhibited the ATP-dependent hydrolysis of [CH$_3$-$^3$H]casein. At this concentration, vanadate reduced proteolysis nearly to the levels seen in the absence of ATP. This inhibition was rapid in onset and was seen when vanadate was added either initially or after the reaction had been allowed to proceed for 60 min. When different concentrations of vanadate were added in the presence of 5 mM ATP, half-maximal inhibition of proteolysis was obtained at a concentration of approximately 50 mM (Fig. 2). By contrast, vanadate did not affect the degradation of [CH$_3$-$^3$H]casein measured in the absence of ATP.

Vanadate caused a similar marked inhibition of the hydrolysis of other proteins, such as denatured $^{125}$I-albumin (Table I). Prior studies indicated that many proteins within the reticulocytes are also substrates for this degradative system when these cells mature into adult erythrocytes (5-7). The net degradation of reticulocyte proteins during reticulocyte maturation can be easily estimated by measuring release of tyrosine from endogenous cell proteins (6). Table I shows that this breakdown of cell proteins in lysates was almost completely dependent on the presence of ATP. Addition of vanadate inhibited this ATP-dependent process by 60%, but it had little or no effect on the very low rate of proteolysis occurring without ATP.

The inhibition by vanadate of certain ATPases, e.g. the (Na$^+$-K$^+$)-ATPase, is reversible (28). To test whether vanadate inhibited protein degradation reversibly, the reticulocyte extracts were treated with vanadate and then were dialyzed overnight and reassayed. As shown in Table I, dialysis to remove vanadate restored almost completely activity against [CH$_3$-$^3$H]casein and most of the activity against $^{125}$I-BSA.

To examine the specificity of this action of vanadate, the effects of molybdate and various other ATPase inhibitors were studied (Table II). Molybdate had no effect on protelysis, even though it is a chemical homolog of vanadate. Ouabain, a specific inhibitor of the membrane (Na$^+$-K$^+$)-ATPase (29), and azide, an inhibitor of the membrane-bound mitochondrial ATPase (30) had little or no effect on protein synthesis with or without ATP. By contrast, quercetin, which, like vanadate, is a potent inhibitor of several soluble ATPases (31), reduced ATP-stimulated proteolysis, although less effectively than vanadate. The two ATPase inhibitors effective against ATP-stimulated proteolysis in reticulocytes, vanadate and quercetin, are also the only ones that inhibit the ATP-hydrolyzing proteases from E. coli (13, 17) and liver mitochondria (14).

By themselves, these findings do not prove involvement of an ATPase in proteolysis, since vanadate can also inhibit many other ATP-utilizing enzymes which involve a phosphorylated intermediate (32). Possibly, vanadate may reduce proteolysis by inhibiting the ATP-dependent conjugation of ubiquitin to protein substrates (2, 9). To test this possibility, we examined whether vanadate also decreased the breakdown of proteins whose amino groups were covalently modified so as to prevent their conjugation to ubiquitin (8). We have recently reported that such proteins are still degraded by an ATP-stimulated process (8). Accordingly, as shown in Table III, after methylation, acetylation, carbamylation, or succinylation of [CH$_3$-$^3$H]casein and denatured $^{125}$I-BSA, their breakdown is still stimulated 2-4-fold by ATP (although the magnitude of this ATP effect was less than that with normal substrates (8)). Vanadate strongly inhibited the ATP-stimulated breakdown of the substrates lacking free amino groups (Table III), just as it affected the breakdown of unmodified [CH$_3$-$^3$H]casein or $^{125}$I-BSA. Thus, vanadate seems to inhibit a ubiquitin-independent, ATP-activated step in protein degradation.

By themselves, these experiments do not rule out the additional possibility that vanadate also decreases the conjugation of ubiquitin to substrates. Subsequent experiments, therefore, measured the incorporation of exogenous $^{125}$I-ubiquitin into reticulocyte proteins after DEAE-chromatography to remove endogenous ubiquitin from the extracts (i.e. to obtain fraction II (9)). As shown in Fig. 3, $^{125}$I-ubiquitin was incorporated covalently into many different reticulocyte pro-

**Table II**

<table>
<thead>
<tr>
<th>Inhibition of hydrolysis rate</th>
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<tr>
<td>Inhibitor (mm)</td>
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TABLE III
Effect of vanadate on the ATP-stimulated degradation of proteins with free or blocked amino groups in reticulocyte extracts

Vanadate was added at the final concentration of 100 μM. These chemical modifications blocked greater than 98% of the amino groups on casein and BSA (8) and prevented ubiquitin stimulation of proteolysis (8). The data represent the ATP-stimulated hydrolysis, which was determined by subtracting the values obtained in the absence of ATP from the activity with ATP. In the absence of ATP, vanadate had no effect on the basal hydrolysis of modified or unmodified proteins.

<table>
<thead>
<tr>
<th>Chemical modification</th>
<th>ATP-stimulated hydrolysis</th>
<th>%/3 h</th>
<th>%/3 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[CH$_4^3$H]Casein</td>
<td>VO$_4$</td>
<td>VO$_4$</td>
</tr>
<tr>
<td>Control</td>
<td>39.9</td>
<td>3.0</td>
<td>35.7</td>
</tr>
<tr>
<td>Methylolation</td>
<td>8.4</td>
<td>0.9</td>
<td>2.1</td>
</tr>
<tr>
<td>Acetylation</td>
<td>22.2</td>
<td>2.1</td>
<td>11.1</td>
</tr>
<tr>
<td>Carbamylation</td>
<td>10.5</td>
<td>0.6</td>
<td>10.2</td>
</tr>
<tr>
<td>Succinylation</td>
<td>9.3</td>
<td>0.3</td>
<td>3.6</td>
</tr>
</tbody>
</table>

Fig. 3. Effect of vanadate on the formation of covalent bonds between $^{125}$I-ubiquitin and endogenous proteins in fraction II. The assay of $^{125}$I-ubiquitin conjugation was performed by densitometry of radioautographs, as described under "Materials and Methods." A, with ATP (5 mM); B, with ATP (5 mM) and vanadate (100 μM). C, without ATP. Pattern of proteins stained with Coomassie brilliant blue. Marker proteins used and their molecular weights: phosphorylase b ($M_r = 94,000$), bovine serum albumin ($M_r = 67,000$); ovalbumin ($M_r = 43,000$); carbonic anhydrase ($M_r = 30,000$); soybean trypsin inhibitor ($M_r = 20,100$), α-lactalbumin ($M_r = 14,400$), trypsin, and chymotrypsin were obtained from Sigma, and the Ca$^{2+}$-activated protease (type II) was purified from bovine lung (42). Purified E. coli protease La (16) and partially purified mitochondrial ATP-dependent protease (13) were used. The assay of protease was performed in the presence or absence of vanadate (100 μM) or molybdate (100 μM).

Proteins

<table>
<thead>
<tr>
<th>Inhibition of hydrolysis</th>
<th>Vanadate</th>
<th>Molybdate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Papain</td>
<td>0.5</td>
<td>0</td>
</tr>
<tr>
<td>Trypsin</td>
<td>6.0</td>
<td>0</td>
</tr>
<tr>
<td>Chymotrypsin</td>
<td>3.0</td>
<td>0</td>
</tr>
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<td>9.5</td>
<td>0</td>
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<tr>
<td>ATP-dependent protease from rat liver mitochondria</td>
<td>45.0</td>
<td>0</td>
</tr>
<tr>
<td>ATP-dependent protease from E. coli (protease La)</td>
<td>68.0</td>
<td>0</td>
</tr>
</tbody>
</table>

TABLE IV
Effect of vanadate on ATP and ubiquitin-dependent proteolysis in fraction II

The assay of proteolysis in fraction II was similar to that with reticulocyte extracts except that 216 μg of fraction II and 1 μg of denatured $^{125}$I-BSA (50,000 cpm) were used for the assay. Ubiquitin was added at 5 μg/assay and ATP at a final concentration of 5 mM.

<table>
<thead>
<tr>
<th>Addition</th>
<th>$^{125}$I-BSA hydrolysis</th>
<th>Inhibition by VO$_4$</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1.11</td>
<td>0.88</td>
</tr>
<tr>
<td>Ubiquitin</td>
<td>0.99</td>
<td>0.88</td>
</tr>
<tr>
<td>ATP</td>
<td>5.29</td>
<td>3.67</td>
</tr>
<tr>
<td>ATP + ubiquitin</td>
<td>20.87</td>
<td>13.38</td>
</tr>
</tbody>
</table>

TABLE V
Effect of vanadate on $^{3}$H-casein hydrolysis by various proteases

Papain, trypsin, and chymotrypsin were obtained from Sigma, and the Ca$^{2+}$-activated protease (type II) was purified from bovine lung (42). Purified E. coli protease La (16) and partially purified mitochondrial ATP-dependent protease (13) were used. The assay of protease was performed in the presence or absence of vanadate (100 μM) or molybdate (100 μM).

Proteases

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Proteins

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<tr>
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</tr>
</tbody>
</table>

well characterized proteases. As shown in Table V, addition of vanadate or molybdate at a final concentration of 100 μM had very little or no effect on the hydrolysis of $^{3}$H-casein by several serine proteases, including trypsin and chymotrypsin, or by typical thiol proteases, such as papain or the mammalian Ca$^{2+}$-activated protease. By contrast, this concentration of vanadate caused a marked inhibition of the ATP-dependent proteases from E. coli and rat liver mitochondria, as reported previously (13, 14, 16, 17), and this effect was not obtained with molybdate.

Recently, Seglen and Gordon (35) reported that a much higher concentration of vanadate (10 mM) reduced protein degradation in isolated rat hepatocytes and suggested that this agent directly inhibited a lysosomal thiol protease. To test this possibility, we investigated the effects of different concentrations of vanadate on the hydrolysis of $^{3}$H-casein in liver extracts. As shown in Fig. 4, proteolysis in rat liver extracts was much higher at pH 5.0 where lysosomal hydrolyses are active than at pH 7.9. At pH 5.0, vanadate caused a marked inhibition of proteolysis which was maximal (75%) at 1 mM. At concentrations below 100 μM, vanadate had no effect on this process, although these low concentrations did inhibit ATP-dependent proteolysis in reticulocytes (Fig. 2). At pH 7.9 in liver extracts, proteolysis was not stimulatable by ATP (data not shown), and vanadate did not inhibit this process, even at very high concentrations (10 mM).

Molybdate, a homolog of vanadate, also inhibited hepatic proteolysis at pH 5.0, in a similar fashion as vanadate. A specific inhibitor of lysosomal thiol proteases (Ep-475) (36), also markedly inhibited hydrolysis of $^{3}$H-casein at pH 5.0 but not at pH 7.9, and the addition of vanadate and Ep-475 together did not produce any greater inhibition than did either...
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Fig. 4. Effects of vanadate and molybdate on [3H]casein hydrolysis in liver extracts. A 10% homogenate of rat liver was made with 0.25 M sucrose, 20 mM KCl, and 10 mM Tris-HCl, pH 7.4, in a Dounce type A homogenizer and centrifuged at 1000 x g for 10 min. The postnuclear supernatant was frozen and thawed twice, and used as the liver extract. Proteolytic activity was determined by incubating extracts (100 μg of protein) in a volume of 200 μl, which contained 75 mM Tris-HCl, pH 7.9 (———x) or Na-acetate, pH 5.0 (□——□), 0.1 mM EDTA, 0.5 mM dithiothreitol, and 20 μg of [3H]casein (30,000 cpm). After incubation for 1 h, acid-soluble radioactivity was determined as described under "Materials and Methods." Left, concentration-dependent effect of vanadate; right, concentration-dependent effect of molybdate.

agent alone (data not shown). Thus, at high concentrations, vanadate and molybdate probably are affecting the lysosomal enzymes cathepsin B, H, or L, as suggested earlier. However, this effect clearly differs in many ways from the specific action of vanadate on the ATP-dependent proteolytic pathway in reticulocytes.

Effect of Vanadate on Intact Cells—In reticulocytes, the nonlysosomal ATP-dependent pathway is involved in the elimination of various proteins as these cells become erythrocytes (5–7), and also in the rapid degradation of highly abnormal proteins (1–3, 37). Additional experiments tested whether vanadate can inhibit these processes in intact cells. As shown in Fig. 5, the breakdown of endogenous proteins in reticulocytes was linear for about 3 h (as measured by the net production of free tyrosine). The addition of vanadate (0.5 mM) caused a strong inhibition of this process. In cell extracts, the effect of vanadate on proteolysis was found to be reversible (Table I). When cells exposed to vanadate for 1 h were washed and resuspended in vanadate-free medium, the rate of protein degradation returned almost completely after a single lag period. This rapid restoration of proteolysis indicates that vanadate is not decreasing proteolysis by irreversibly damaging the cells.

The inhibition in the intact cells required higher concentrations of vanadate than in the reticulocyte extract (Fig. 6), perhaps because it must compete for entry into the cell via the anion carrier (18, 32). This effect was maximal at 1 mM. Several observations further indicated that vanadate reduced proteolysis by its effect on the soluble ATP-dependent system, and not on the lysosomal route. In the reticulocytes, the maximal inhibition obtained with vanadate (80–90%) was similar to that seen when ATP production was blocked with an uncoupler of oxidative phosphorylation, 2,4-dinitrophenol, and an inhibitor of glycolysis, 2-deoxyglucose (Fig. 6). Furthermore, addition of vanadate to cells already depleted of ATP caused no further reduction in the breakdown of cell proteins. Finally, Ep-475, a highly selective inhibitor of lysosomal thiol proteases which can enter intact cells (36), had no effect on protein breakdown in the reticulocytes (Fig. 6), in accord with previous conclusions (6).

To test whether vanadate also could inhibit the selective breakdown of abnormal proteins in intact cells, the reticulo-

cytes were incubated with the valine analog 2-amino-3-chlorobutyrate. The newly synthesized analog-containing proteins were rapidly hydrolyzed in accord with earlier findings (1), but within 15 min, vanadate (0.5 mM) dramatically this process (Fig. 7). Thus, vanadate seems to be a useful reagent for studies of the ATP-dependent proteolytic pathway in intact reticulocytes.

Additional experiments were undertaken to determine whether treatment with vanadate was highly toxic to the cells and whether the fall in proteolysis might be secondary to a depletion of cellular ATP levels (Fig. 8). Exposure to vanadate for 1 h did promote a small degree of hemolysis (4%) of the reticulocytes (Fig. 8C), but this effect was not evident until 30 min of treatment, when proteolysis was already signifi-
Inhibition of ATP-dependent Degradation of Proteins

FIG. 7. The effect of vanadate on the degradation of proteins containing 2-amino-3-chlorobutyrate in place of valine in reticulocytes. Breakdown of proteins synthesized previously in the presence of the valine analog was measured in the absence (O—O) or presence (■—■) of vanadate (0.5 mM).

FIG. 8. Effect of vanadate on ATP content, protein synthesis, and hemolysis in reticulocytes. A, effect of vanadate on the intracellular ATP concentration. Cells were incubated at 37 °C without (O—O) or with (■—■) vanadate (0.5 mM). At the indicated times, trichloracetic acid was added at a final concentration of 10%, and ATP concentration in the acid-soluble fraction was analyzed as described under "Materials and Methods." The data are the mean ± S.D. of 6 determinations. B, effect of vanadate on protein synthesis in reticulocytes. C, effect of vanadate on the lysis of reticulocytes.

cantly reduced. Addition of vanadate also did not significantly affect the ATP content of the cells initially, but after 45 min of exposure, ATP levels gradually decreased (Fig. 8A). Thus, the rapid inhibition of protein breakdown in vivo (Figs. 5 and 7) did not result from either cell lysis or a reduction of intracellular ATP concentration. One interesting finding was that vanadate dramatically reduced protein synthesis in these cells (Fig. 8). Whatever the mechanism of this effect, it cannot be responsible for the fall in proteolysis, since complete inhibition of protein synthesis does not reduce the breakdown of normal or abnormal proteins in reticulocytes (1, 3, 6). These experiments indicate that although vanadate may affect multiple cellular processes, the rapid, reversible inhibition of proteolysis in these cells probably involves the same mechanism observed in the cell-free system.

DISCUSSION

Specific inhibitors of the soluble ATP-dependent pathway for protein breakdown should prove very useful in attempts to study this pathway and to clarify its physiological significance. The present findings indicate that vanadate, at low concentration (K<sub>I</sub> = 50 μM), is a potent inhibitor of this process in reticulocyte extracts. This effect is not due to an inhibition of ubiquitin conjugation (Tables III and IV and Fig. 3), and vanadate therefore seems to be affecting another ATP-activated process (8). A major goal for future research will be to identify the precise enzymatic step that vanadate blocks in this pathway (see below). In addition, vanadate may be useful in short term studies with intact cells, but caution seems appropriate in such experiments because of the multiple effects of vanadate in vivo (Fig. 8) including its ability to inhibit protein synthesis, and with time, to reduce ATP levels and eventually cause some cell lysis.

The marked inhibition of proteolysis in the reticulocyte extracts by vanadate resembles the inhibition found with the ATP-dependent proteases from E. coli (11–13) and rat liver mitochondria (14). For example, protease Lα from E. coli shows a similar K<sub>I</sub> (50 μM) and also responds to quercetin but not to molybdate (Table II). Also, in intact mitochondria, vanadate at low concentrations prevents energy-dependent proteolysis (16), apparently by inhibiting the ATP-hydrolyzing enzyme found in the mitochondrial matrix (14). Thus, the ATP-dependent pathways for protein degradation in reticulocytes and those in prokaryotic cells and mitochondria may involve similar mechanisms. However, in the mammalian cytosol, unlike in E. coli (17) or mitochondria (16), this process seems to involve ubiquitin (2, 9, 26), and it has been argued (2) that ubiquitin conjugation accounts for the energy requirement for proteolysis in reticulocytes. However, our recent studies indicate that in reticulocytes ATP acts at multiple steps in this pathway (8) and that an energy-requiring reaction, which is sensitive to hemin is necessary for the breakdown of protein substrates, whether or not they contain ubiquitin (8).

By analogy to protein breakdown in bacteria and mitochondria, this unidentified step could be an ATP-hydrolyzing protease. Additional evidence for this conclusion is the present finding that the ATPase inhibitors, vanadate and to a lesser extent quercetin, inhibit this process (Table II) in a similar fashion as hemin (8, 33, 34). Despite much effort, however, we have thus far failed to isolate an ATP-hydrolyzing protease from these cells, although we have purified several novel proteases that appear to be involved in this pathway (38, 39). Recently we have succeeded in isolating from these extracts a fraction capable of carrying out ATP-dependent proteolysis independent of ubiquitin. In this fraction, both

1 Since this manuscript was completed Ranu has reported that vanadate inhibits peptide chain initiation in reticulocyte extracts (Ramu, R. S. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 3148-3152).


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...proteolysis and ATP hydrolysis are inhibited by vanadate as well as by hemin. However, real proof that these inhibitors are acting on an ATP-hydrolyzing protease will require its complete purification and characterization.

Whatever its precise site of action, this effect of vanadate on the reticulocyte degradative system differs qualitatively from its effect on hepatocytes. Our findings with liver homogenates confirm the original suggestion of Seglen and Gordon (35) that in hepatocytes, vanadate at high concentrations can inhibit protein breakdown within lysosomes. Here we have shown that at millimolar concentrations, vanadate and molybdate inhibit the acid-optimal proteases. These agents seem to be acting on thiol proteases, since a similar inhibition was observed below 100 μM, the concentration which caused a strong inhibition of the nonlysosomal pathway in the reticulocyte extracts. In the liver extracts, protein synthesis and ATP production were not affected by inhibitors of lysosomal function and proteins with abnormal structures requires energy and is not affected by inhibitors of lysosomal function (2, 40, 41). Although this process thus seems to resemble the ATP-dependent pathway in reticulocytes, we and others have not succeeded in establishing soluble ATP-dependent proteolytic systems from liver or other mammalian cells. It therefore remains unclear whether the nonlysosomal process in cells other than reticulocytes is sensitive to low concentrations of vanadate and whether this inhibitor will be of general use in studies of intracellular protein breakdown.

REFERENCES

Supplementary Material to
"Vonatec Inhibits the ATP-Dependent Pathway for Protein Degradation in Reticulocytes"
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MATERIALS AND METHODS

Chemicals

Reticulocytes, RNA, sodium thiosulfate, and ATP (stanom cell) were purchased from Sigma Chemical Company, and sodium vonatec from Fisher Scientific Company. Na2[S2O3]2, [3H]formaldehyde, [3H]Histidine, and 1-(4,5-3H)uracil (90.2 Ci/mmol) were obtained from New England Nuclear Corporation.

Stock solutions (2.1 M) of vonatec were dissolved in water, adjusted to pH 10 with 5 N HCl and then boiled to elute polymeric species of [3H]vonatec, which are yellow in color. The concentration of vonatec was determined spectrophotometrically (ε295 = 2985 M⁻¹ cm⁻¹ (19)).

Preparation of Reticulocyte Extracts

Rabbit reticulocytes were obtained after phenylhydrazine treatment of male rabbits, as described previously (1). The washed cells were lysed by the addition of 1.5 volumes of water containing 1 M ethanolamine. The lysate was centrifuged at 40,000 g for 10 min. The supernatant was dialyzed overnight against 50 mM Tris-Cl pH 7.8 containing 0.1 M KCl, 0.5 M 2-mercaptoethanol and 20% glycerol, and stored at −20°C until used.

Assay of Proteolytic Activity in Extracts

Proteolytic activity was determined by incubating 50 μl of vonatec extracts in a volume of 200 μl, which contained 50 mM Tris-Cl pH 8.0, 5 mM MgCl2, 2 mM ATP, 3 mM 2-mercaptoethanol, and 20 μg [3H]MECA (50,000 cpn) or 10 μg [3H]SA (50,000 cpn). After incubation for 3 hr at 37°C, the reactions were terminated by addition of 0.5 ml 10% trichloroacetic acid containing BSA (1 mg/ml) as a carrier, and acid-insoluble radioactivity was measured.

Preparation ofvonatec extracts was performed by incubating 0.25 ml of a 20% suspension of reticulocytes which contained 10 mM glucose, plasma concentrations of amino acids, and 5 μg/ml of [3H]leucine in the absence or presence of vonatec (100 μl). At the indicated times, the reaction was terminated by addition of 0.25 ml of 20% trichloroacetic acid. The acid-insoluble material was washed four times with 30% trichloroacetic acid, dissolved in 3 ml of NaOH containing 0.1% SDS, and the radioactivity of the insoluble proteins was determined.

For the assay of hemoglobin, the cells were incubated under the same conditions, but at the indicated times they were pelleted by centrifugation (1000 g for 10 min at 4°C), and the concentration of hemoglobin in the medium was determined from the absorbance at 550 nm.

All values shown are the average of at least three determinations which agreed within 10% per cent. Similar results were seen in at least three separate experiments using different preparations of reticulocytes.
Vanadate inhibits the ATP-dependent degradation of proteins in reticulocytes without affecting ubiquitin conjugation.

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