Hemin Inhibits Ubiquitin-Dependent Proteolysis in both a Higher Plant and Yeast†

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ABSTRACT: In eukaryotes, a major route for ATP-dependent protein breakdown proceeds through covalent intermediates of target proteins destined for degradation and the highly conserved, 76 amino acid protein ubiquitin. In rabbit reticulocytes, it has been shown that hemin effectively inhibits this pathway by blocking the catabolism of ubiquitin–protein conjugates [K_I = 25 μM (Haas, A. L., & Rose, I. A. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 6845–6848)]. Here, we demonstrate that hemin is also an effective inhibitor of the ubiquitin-dependent proteolytic pathway in both a higher plant, oats (*Avena sativa*), and yeast (*Saccharomyces cerevisiae*). Hemin inhibits all stages of the pathway in vitro, including ATP-dependent formation of ubiquitin–protein conjugates, disassembly of conjugates by ubiquitin–protein lyase(s) (or isopeptidases), and degradation of ubiquitin–protein conjugates by ATP-dependent protease(s). Using ubiquitin–^{125}I-lysozyme conjugates synthesized in vitro as substrates, we determined the specific effects of hemin on the rates of disassembly and degradation separately. The concentration of hemin required for half-maximal inhibition of both processes was identical in each species, ~60 μM in oats and ~50 μM in yeast. Similar inhibitory effects were observed when two hemin analogues, mesoheme or protoporphyrin IX, were employed. These results demonstrate that the effect of hemin on ubiquitin-dependent proteolysis is not restricted to erythroid cells and as a result hemin may be a useful tool in studies of this pathway in *all* eukaryotic cells. These results also question models where hemin serves as a specific negative modulator of proteolysis in erythroid cells.

In the last few years, important insights into the mechanisms of intracellular protein degradation have been made through the identification of a major proteolytic pathway involving the small, highly conserved protein ubiquitin [for reviews, see Finley and Varshavsky (1985) and Hershko and Ciechanover (1986)]. In this pathway, the main function of ubiquitin is to become covalently linked to proteins destined for catabolism, forming an intermediate now committed for degradation. Ligation occurs via an unusual peptide linkage between the terminal glycine carboxyl group of ubiquitin and free amino groups (both N-terminal and internal lysl) on the target protein (Hershko et al., 1984b). Conjugation requires ATP and is performed by a multienzyme system first reconstituted in rabbit reticulocytes (Hershko et al., 1983). Once a protein is tagged with ubiquitin, it has two possible fates. It can be degraded to amino acids by ATP-dependent protease(s) specific for ubiquitin–protein conjugates with the concomitant release of free, functional ubiquitin (Hough et al., 1986, 1987; Waxman et al., 1987). Alternatively, it can be disassembled by ubiquitin–protein lyase(s) (or isopeptidases) which cleave only the peptide bond between ubiquitin and the target protein, regenerating both proteins intact (Anderson et al., 1981; Matsui et al., 1982; Hershko et al., 1984a). Recent evidence that ubiquitin itself has proteolytic activity suggests that this protein may also have a more direct role in proteolysis in addition to serving as a reusable tag for selective degradation by other proteases (Fried et al., 1987).

Lysates from rabbit reticulocytes contain a highly active ubiquitin-dependent proteolytic pathway and as a result represent a rich source for purifying the various components involved (Hershko et al., 1983; Hough et al., 1987). Previously, it has been shown that hemin is an effective inhibitor of protein degradation in these lysates (Etlinger & Goldberg, 1980) and more specifically that this inhibition results from a direct effect on ubiquitin-dependent proteolysis (Haas & Rose, 1981). In this role, hemin has proven useful in the dissection of the pathway (Haas & Rose, 1981; Tanaka et al., 1983; Hough et al., 1986, 1987; Waxman et al., 1987; Fagan et al., 1987) and in the generation of ubiquitin–protein conjugates in vitro for further biochemical analyses (Hough & Rechsteiner, 1986). Here, we document that hemin inhibits ubiquitin-dependent proteolysis in nonerythroid cells as well, being effective in extracts from a higher plant, oats (*Avena sativa*), and yeast (*Saccharomyces cerevisiae*). As a result, hemin may be of general use in studies on ubiquitin-dependent proteolysis in *all* eukaryotic cells.

Materials and Methods

Reagents. Human ubiquitin was purified from erythrocytes according to the method of Haas and Wilkinson (1985). Egg white lysozyme was obtained from Sigma and purified as previously described (Hough & Rechsteiner, 1986). Both ubiquitin and lysozyme were radiolabeled with ^{125}I by the chloramine T method (Ciechanover et al., 1980) using carrier-free Na^{125}I (5.6×10^6 Bq/μg) purchased from Amersham. The initial specific radioactivities for ^{125}I-ubiquitin preparations were $(3.9–14) \times 10^3$ cpm/pmol, and those for ^{125}I-lysozyme preparations were $(1.6–3.1) \times 10^4$ cpm/pmol. Hemin, protoporphyrin IX, ATP, leupeptin, hexokinase, and phosphocreatine kinase were purchased from Sigma. Mesohemin was a product of Porphyrin Products, Logan, UT. Stock solutions (1 mM) of hemin and mesohemin were dissolved in 50 mM

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tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl),[1] pH 7.5 (25 °C). For protoporphyrin IX, saturated stock solutions in 50 mM Tris-HCl, pH 7.5 (25 °C), were prepared by stirring overnight, and undissolved material was removed by centrifugation. The concentration of protoporphyrin XI in solution (approximately 150 μM) was determined spectrophotometrically by using an extinction coefficient at 408 nm of 2.62×10^5 L/(mol·cm) when dissolved in 2.7 N HCl.

Preparation of Ubiquitin–Lysozyme Conjugates. Ubiquitin–lysozyme conjugates were prepared by a modification of the methods of Hershko et al. (1984) and Hough and Rechsteiner (1986). A crude extract capable of ubiquitin conjugation was prepared from wheat germ as described (Hatfield & Vierstra, 1988). To 2.6 mL of this wheat germ extract was added 800 μL of ^{125}I-lysozyme (30 μg or approximately 1×10^8 cpm), 200 μL of unlabeled human ubiquitin (1 mg), 20 μL of phosphocreatine kinase (1 unit/μL), and 400 μL of 2 mM ATP, 5 mM $MgCl_2$, 1 mM DTT, 10 mM phosphocreatine, and 50 mM Tris-HCl, pH 7.5 (25 °C). The mixture was incubated for 90 min at 30 °C. The reaction was terminated by the addition of 700 μL of freshly prepared 200 mM NEM followed by a 30-min incubation at 30 °C. The extract was then chromatographed at room temperature on a 2.6×90 cm Fractogel-TSK HW 55 (F) column in 50 mM Tris-HCl–0.5 M KCl, pH 7.5 (25 °C). Fractions containing high molecular weight ubiquitin–lysozyme conjugates were pooled and dialyzed at 4 °C against 10 mM ammonium acetate (pH 7.5). Precipitated protein was removed from the dialysate by centrifugation and the supernatant lyophilized to dryness. The conjugate pool was redissolved in water and additional precipitated protein removed by centrifugation. This clarified supernatant containing $(3.5–4.1) \times 10^3$ cpm/μL ubiquitin–^{125}I-lysozyme conjugates was used for subsequent analyses.

Preparation of Extracts from Oats and Yeast. Etiolated oat (*Avena sativa* [*L.*], cv Garry) seedlings were grown in darkness for 5 days at 24 °C in near-saturating humidity. The apical portions of the seedlings were homogenized at ice temperatures in 2 mL/g fresh weight of 50 mM Tris-HCl, 1 mM Na_4EDTA, and 14 mM 2-mercaptoethanol, pH 8.0 (4 °C) (Vierstra, 1987). Yeast (*Saccharomyces cerevisiae*, strain S288C) in exponential growth were collected by centrifugation and resuspended in 1 mL/g packed cells of 10 mM Tris-HCl, 1 mM Na_4EDTA, 10% (v/v) glycerol, and 14 mM 2-mercaptoethanol, pH 7.5. The cells were lysed at ice temperatures using glass beads and a Brahn Mill. Both crude extracts were clarified by centrifugation at 50000*g*, made 200 μM in the protease inhibitor leupeptin, and used directly in all subsequent assays.

Assay for Ubiquitin Conjugation. ATP-dependent ubiquitin conjugation in the presence or absence of hemin was assayed as described previously (Ozkaynak et al., 1984; Vierstra, 1987). Reaction mixtures contained 30 μL of either a yeast or an oat crude extract, 5 μL of ^{125}I-ubiquitin, and either 10 μL of various concentrations of hemin dissolved in 50 mM Tris-HCl (pH 7.5, 25 °C) or buffer alone. For ATP-stimulated activity, ATP and an ATP-regenerating system, which consisted of 5 μL of 2 mM ATP, 5 mM $MgCl_2$, 1 mM DTT, 10 mM phosphocreatine, and 50 mM Tris-HCl, pH 7.6 (20 °C), and 1 μL of phosphocreatine kinase (1 unit/μL), were added. For ATP-independent activity, an ATP-depleting system, which consisted of 5 μL of 5 mM $MgCl_2$, 1 mM DTT, 10 mM deoxyglucose, and 50 mM Tris-HCl, pH 7.6 (20 °C), and 1 μL of hexokinase (1 unit/μL), was added. Reactions were incubated for various lengths of time at 30 °C and terminated by boiling in an equal volume of $NaDodSO_4$–PAGE sample buffer (Vierstra, 1987). Samples were subjected to discontinuous $NaDodSO_4$–PAGE (Laemmli, 1970) on 13.5% acrylamide gels [acrylamide:bis(acrylamide) ratio of 30:0.8]. The gels were stained with Coomassie Blue R and dried between two sheets of cellophane, and radioactive proteins were visualized by autoradiography. Quantitation of ubiquitin conjugates formed during each reaction was accomplished by liquid scintillation counting of slices from the individual gel lanes after removing the lower portion of the gel containing free ubiquitin (determined by autoradiography) (Vierstra, 1987).

Assays for Disassembly and Degradation of Ubiquitin Conjugates. Loss of ubiquitin conjugates formed between purified ubiquitin and proteins endogenous to either oat or yeast extracts was determined as described previously (Vierstra, 1987). Ubiquitin conjugates were generated in vitro as described above by adding ^{125}I-ubiquitin and 2 mM ATP (without the regenerating system) to either an oat or a yeast crude extract and incubating the reaction mixture at 30 °C. When maximal conjugate levels were achieved [30 min for oats and 7.5 min for yeast (Figure 1)], various amounts of hemin and the ATP-depleting system (20 mM deoxyglucose and 20 units/mL hexokinase final concentrations) were added to aliquots of the reaction mixture. These ATP-depleted samples were incubated further at 30 °C. At various times, samples were removed and boiled in $NaDodSO_4$–PAGE sample buffer. The amount of ubiquitin conjugates lost after ATP depletion was determined as described above.

Assays for disassembly and degradation of ubiquitin–protein conjugates were similar to those described by Hough and Rechsteiner (1986) using partially purified ubiquitin–^{125}I-lysozyme conjugates as the substrate. Reaction mixtures contained 30 μL of crude oat or yeast extract, 6 μL of either ATP and the ATP-regenerating system or the ATP-depleting system, 4 μL of ubiquitin–^{125}I-lysozyme conjugates, and 10 μL of various concentrations of hemin. At various times after a 30 °C incubation, aliquots were removed and either boiled in an equal volume of $NaDodSO_4$–PAGE sample buffer or mixed with 3 volumes of 10% (w/v) TCA and incubated on ice. Disassembly, as defined by the conversion of high molecular mass ubiquitin–^{125}I-lysozyme conjugates into free ^{125}I-lysozyme, was determined by subjecting the $NaDodSO_4$-boiled samples to $NaDodSO_4$–PAGE followed by scintillation counting slices of the individual gel lanes. Data were expressed as the percentage of total ^{125}I present in the region of the gel containing free lysozyme (determined by autoradiography). Degradation, as defined by the conversion of ubiquitin–^{125}I-lysozyme into acid-soluble ^{125}I, was measured by scintillation counting the supernatants following TCA precipitation and centrifugation. Acid-precipitable radioactivity was determined by scintillation counting the TCA pellets following resuspension in 1 N NaOH.

Results

The ATP-dependent conjugation of ^{125}I-ubiquitin to endogenous proteins can be observed in crude extracts from both etiolated oat seedlings and yeast [see Figure 1 and Ozkaynak et al. (1984) and Vierstra (1987)]. The maximal steady-state levels at 30 °C were achieved 30 and 7.5 min after the addition of ubiquitin for oats and yeast, respectively. In such extracts,

[1] Abbreviations: $NaDodSO_4$, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; DTT, dithiothreitol; NEM, *N*-ethylmaleimide; EDTA, ethylenediaminetetraacetic acid; TCA, trichloroacetic acid; kDa, kilodalton(s).

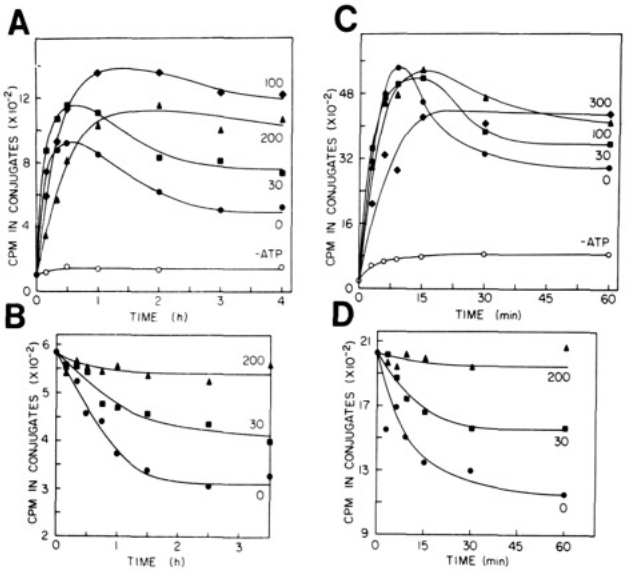

FIGURE 1: Effect of hemin on the synthesis and loss of ubiquitin–protein conjugates generated in crude extracts from either etiolated oat seedlings or yeast. (A and C) Synthesis of ubiquitin–protein conjugates. ^{125}I-Ubiquitin, various concentrations of hemin, and either ATP and an ATP-regenerating system (closed symbols) or an ATP-depleting system (open symbols) were added to crude extract from oats (A) or yeast (C). The reaction mixtures were incubated at 30 °C, and at various times, aliquots were removed and subjected to NaDodSO$_4$–PAGE. The amount of free ubiquitin converted into the conjugated form was then determined by scintillation counting of slices of the individual gels lanes. Final concentrations (micromolar) of hemin were as indicated. (B and D) Loss of ubiquitin–protein conjugates. Ubiquitin–protein conjugates were generated in vitro after the addition of ^{125}I-ubiquitin and ATP to crude extracts from either oats (B) or yeast (D). At $t = 0$, various concentrations of hemin and an ATP-depleting system containing hexokinase and deoxyglucose were added to the extract. The extract was incubated at 30 °C, and at various times, aliquots were removed and subjected to NaDodSO$_4$–PAGE. The amount of ubiquitin–protein conjugates lost during the incubation after ATP depletion was determined by scintillation counting slices of the individual gel lanes. Final concentrations (micromolar) of hemin were as indicated.

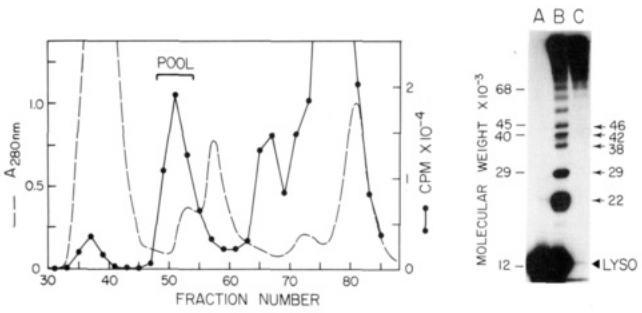

FIGURE 2: Preparation of ubiquitin–^{125}I-lysozyme conjugates. Ubiquitin–^{125}I-lysozyme conjugates were synthesized in a wheat germ extract and partially purified as described under Materials and Methods. (Right panel) Autoradiograms of samples from various stages during the preparation of ubiquitin–^{125}I-lysozyme conjugates subjected to NaDodSO$_4$–PAGE: (lane A) ^{125}I-lysozyme; (lane B) ubiquitin–^{125}I-lysozyme conjugates synthesized in a wheat germ extract; (lane C) high molecular mass ubiquitin–^{125}I-lysozyme conjugates partially purified by gel filtration of a Fractogel TSK HW (F) column. (Left panel) Elution profile on a 2.6 × 90 cm Fractogel TSK HW column of ubiquitin–^{125}I-lysozyme conjugates synthesized in a wheat germ extract. Column fractions were 5 mL. Radioactivity was determined in 100-μL samples from each fraction. Fractions 48–53 were pooled and analyzed by NaDodSO$_4$–PAGE in lane C.

hemin was effective in reducing the initial rate of ^{125}I-ubiquitin conjugate synthesis while simultaneously increasing the final steady-state level of conjugates (Figure 1A,C). Under the assay conditions, the initial rate of conjugate synthesis was reduced by 65–70% with 200 and 300 μM hemin for oats and yeast, respectively. The profiles of oat and yeast conjugates synthesized in the absence and presence of hemin were identical, indicating that the effect of hemin was not restricted to a specific class of target proteins or conjugates (data not shown). For the maximum final accumulation of conjugates in both oat and yeast extracts, 100 μM hemin was optimal.

The ability of hemin to increase net conjugate accumulation was the result of its ability to prevent conjugate loss. When hemin was added to extracts synthesizing ^{125}I-ubiquitin–protein conjugates along with an ATP-depleting system to prevent further ubiquitin conjugation, the subsequent loss of conjugates was substantially inhibited (Figure 1B,D). From a comparison of the initial rates of conjugate loss with and without hemin, K_I values of ~60 and ~50 μM were obtained for the oat and yeast extracts, respectively (Figure 4A,B).

In reticulocytes, ubiquitin conjugates are broken down by at least two routes, degradation involving ATP-dependent protease(s) specific for ubiquitin conjugates or disassembly by ubiquitin–protein lyase(s) (Hershko et al., 1984a; Hough et al., 1986). To identify which routes in oats and yeast were inhibited by hemin, the substrate ubiquitin–^{125}I-lysozyme, was used based on the previous results demonstrating that it is effectively both degraded and disassembled in reticulocyte extracts (Hershko et al., 1984a; Hough & Rechsteiner, 1986). This substrate was synthesized by using a modification of the method of Hough and Rechsteiner (1986). First, an array of ubiquitin–lysozyme conjugates was generated in a wheat germ extract (Hatfield & Vierstra, 1988) in the presence of ATP, purified ubiquitin, and ^{125}I-lysozyme (Figure 2). At steady state, between 20% and 30% of the lysozyme was converted into a series of higher molecular mass conjugates (as observed on NaDodSO$_4$–PAGE), with some species having molecular masses exceeding 100 kDa. Because of their size, the large conjugates were effectively separated from both unincorporated ^{125}I-lysozyme and the bulk of wheat germ proteins by gel exclusion chromatography (Figure 2). Substantial purification was also achieved after lyophilization of the conjugate pool because, in contrast to the ubiquitin–lysozyme conjugates, large amounts of wheat germ protein were insoluble after lyophilization. The conjugates prepared in this manner had molecular masses greater than 68 kDa (Figure 2) and were stable at 30 °C over the assay periods used here (data not shown).

When purified ubiquitin–^{125}I-lysozyme conjugates were added to extracts from oats or yeast, the radiolabel was rapidly converted into either acid-soluble ^{125}I or lower molecular mass species (between 68 and 14 kDa) and finally into free ^{125}I-lysozyme (14.3 kDa) (Figure 3). Loss of conjugates was 3–4 times faster in yeast than in oat extracts, and in each case, the loss was faster in the absence of ATP than in its presence. Because both crude extracts have active conjugation systems, the latter effect was likely the result of ATP-dependent substrate reconjugation. The generation of free lysozyme, which we used as a measure of ubiquitin–protein lyase activity, occurred in the absence and presence of ATP (Figure 3). This lyase was highly active in both oat and yeast extracts, responsible for ~95% of the loss of conjugates. It was extremely stable in oat extracts, retaining full activity for up to 7 h at 30 °C or for 24 h at 4 °C (data not shown).

In contrast to the lyase activity, the proteolytic activity in both oat and yeast extracts responsible for converting the radioactive conjugates into acid-soluble ^{125}I required ATP (Figure 3). This protease was very labile with a half-life of approximately 30 min at 30 °C in oat extracts (data not shown). Because of this instability, acid-soluble products were released for only a short period of time (15 min), and as a

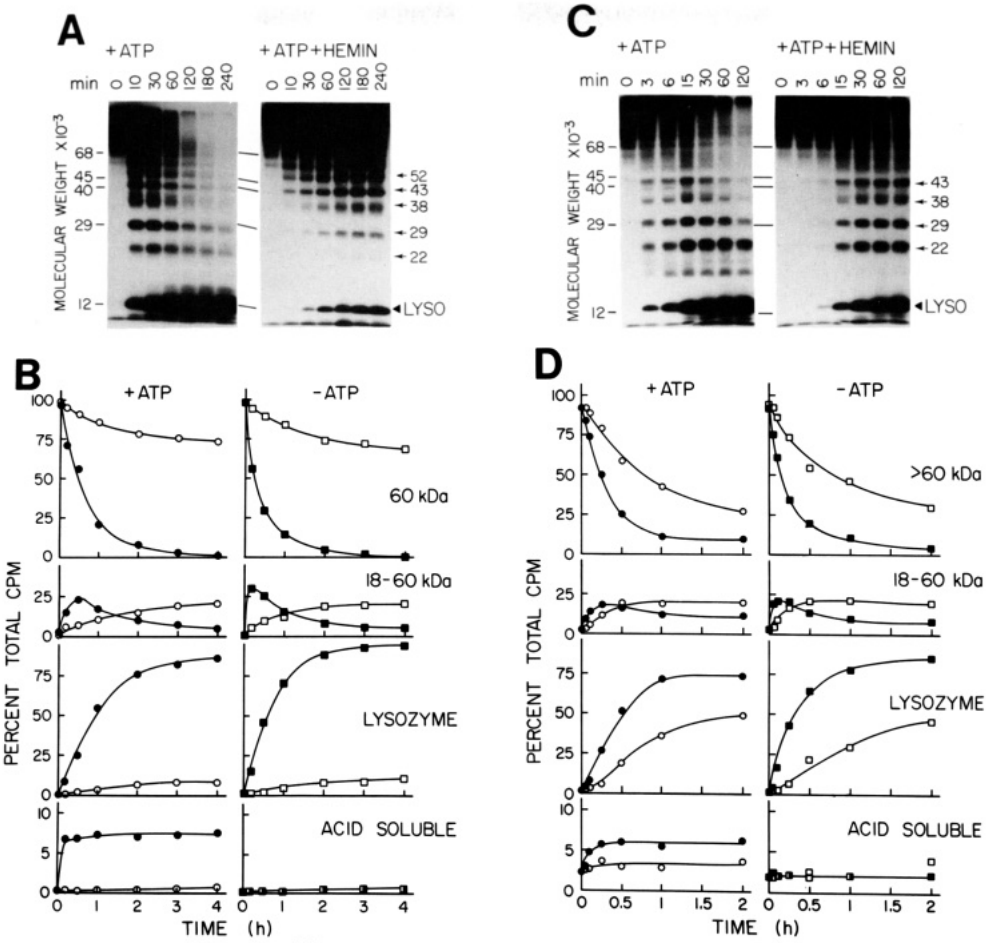

FIGURE 3: Effect of hemin on the loss of ubiquitin–^{125}I-lysozyme conjugates in either oat (A, B) or yeast (C, D) extracts. Ubiquitin–^{125}I-lysozyme conjugates were purified as described in Figure 2. They were added along with either ATP and an ATP-regenerating system (+ATP) or an ATP-depleting system (−ATP) to extracts containing 0 or 200 μM hemin. The reaction mixtures were incubated at 30 °C, and at various times, aliquots were removed and subjected to either TCA precipitation or $NaDodSO_4$–PAGE. (A and C) Electrophoretic analyses of the loss of ubiquitin–^{125}I-lysozyme conjugates by $NaDodSO_4$–PAGE and autoradiography. (B and D) Quantitative analyses of the loss of ubiquitin–^{125}I-lysozyme conjugates. Loss of conjugates and the regeneration of free ^{125}I-lysozyme were determined by cutting the gel lane for each time point [as represented in (A) and (C)] in regions containing bands of given molecular masses (determined from the autoradiograms) and then measuring the amount of ^{125}I in each region by scintillation counting. Degradation to free ^{125}I was followed by the production of acid (TCA)-soluble radioactivity. Data are presented as a percentage of the total radioactivity in each of the four fractions. Open symbols = 200 μM hemin. Closed symbols = 0 μM hemin.

result, the protease was responsible for breaking down only a small percentage of the substrate [3–5% (Figure 3)] as compared with the lyase. A similar ATP-dependent, hemin-sensitive protease which degrades ubiquitin conjugates has been partially purified from reticulocytes and has been shown to require glycerol for stabilization (Hough et al., 1986). However, the addition of glycerol (10% v/v) to oat and yeast extracts failed to provide protection for this proteolytic activity. Both the lyase and protease activities were insensitive to leupeptin (200 μM).

When 200 μM hemin was added to oat and yeast extracts containing ubiquitin–^{125}I-lysozyme conjugates, both the generation of free lysozyme and the ATP-dependent release of acid-soluble ^{125}I were effectively inhibited (Figure 3). Autoradiograms showed that the disassembly products generated in the absence or presence of hemin were qualitatively similar, indicating that hemin affected the disassembly process in general (Figure 3). The concentration dependence for the inhibition of both disassembly and degradation was determined by measuring the amount of free ^{125}I-lysozyme generated and acid-soluble ^{125}I released after a fixed time of incubation in the extracts (90 min for oats and 15 min for yeast). Preliminary studies with oat extracts demonstrated that the final amounts of acid-soluble products released at the end of these incubation periods were linearly related to hemin concentration even though the reaction finished well before the end of the incubation period. From such concentration dependence curves, the K_I's for disassembly and degradation were estimated (Figure 4). The K_I's for both processes were indistinguishable for each species, ~60 and ~50 μM for oat and yeast extracts, respectively. These values were also indistinguishable from the K_I's determined for the inhibition of breakdown of endogenous ubiquitin conjugates synthesized in oat and yeast crude extracts (Figures 1 and 4). It was evident from both Figure 3 and Figure 4 that, unlike oat extracts, sufficient residual lyase activity (15%) remained in yeast extracts even at high hemin concentrations (200 μM). Whether this represents the disassembly of conjugates by a hemin-insensitive component is not known.

Under the same assay conditions, two analogues of hemin, mesohemin and protoporphyrin IX, also were tested for their ability to mimic the effects of hemin. Both analogues at micromolar concentrations effectively inhibited both degradation and disassembly of ubiquitin–^{125}I-lysozyme conjugates (data not shown). Their inhibition in each species was comparable to that of hemin. Estimated K_I's for both processes were approximately 60 μM for mesohemin and 30 μM for protoporphyrin IX.

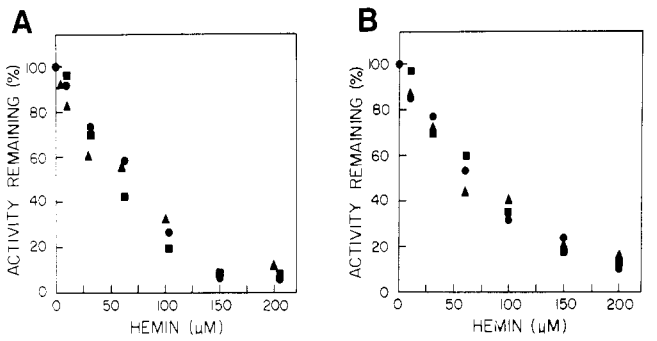

FIGURE 4: Comparison of the effectiveness of hemin on the disassembly and degradation of ubiquitin–protein conjugates in both oat (A) and yeast (B) extracts. (▲) Effect of hemin on the initial rate of loss of ubiquitin–protein conjugates synthesized in oat and yeast crude extracts. Ubiquitin–protein conjugates were synthesized as in Figure 1 (lower panels) using ^{125}I-ubiquitin. Various concentrations of hemin were added to the system along with an ATP-depleting system, and the loss of conjugates was followed over time as in Figure 1. The initial rate of conjugate loss was determined for each hemin concentration and expressed relative to that of the control. (●) Effect of hemin on the disassembly of ubiquitin–lysozyme conjugates. Purified ubiquitin–^{125}I-lysozyme conjugates were added to a crude extract containing various concentrations of hemin and ATP and an ATP-regenerating system. The reactions were incubated at 30 °C for 90 min with oats and for 15 min with yeast and terminated by boiling in an equal volume of $NaDodSO_4$–PAGE sample buffer. The samples were then subjected to $NaDodSO_4$–PAGE, and the amount of free ^{125}I-lysozyme was determined as in Figure 3. The amount of free lysozyme determined for each hemin concentration was expressed relative to that of control. (■) Effect of hemin on the degradation of ubiquitin–lysozyme conjugates. Purified ubiquitin–^{125}I-lysozyme conjugates were added to a crude extract containing ATP and an ATP-regenerating system and various concentrations of hemin. The extracts were incubated at 30 °C for 90 min with oats and for 15 min with yeast and terminated by TCA precipitation. The amount of acid-soluble ^{125}I was determined for each hemin concentration and expressed relative to that of the control.

DISCUSSION

Haas and Rose (1981) previously demonstrated that hemin is a potent reversible inhibitor of ubiquitin-dependent proteolysis in mammalian reticulocyte extracts. Although they did not preclude an effect of hemin on disassembly, they proposed that the major effect of hemin was to inhibit conjugate degradation. The results reported here demonstrate that hemin also is effective at micromolar concentrations in inhibiting ubiquitin-dependent proteolysis in extracts derived from both the higher plant, oats, and yeast. In fact, the estimated K_I's for inhibiting conjugate loss in oat and yeast extracts (50 and 60 μM, respectively) are remarkably similar to that determined for inhibiting the ubiquitin-dependent breakdown of albumin in reticulocyte lysates [25 μM (Haas & Rose, 1981). Using assays able to discriminate between synthesis, disassembly, and degradation of ubiquitin conjugates, we demonstrate that all three processes in yeast and oats are substantially affected. No attempt was made to quantitate the effect of hemin on conjugate synthesis. However, because conjugates accumulate in oat extracts to higher levels in the presence of hemin than in its absence [see this report and Haas and Rose (1981)], we conclude that disassembly and degradation must be more sensitive to hemin than synthesis. From these observations with widely different species, rabbits, yeast, and oats, it is likely that effects of hemin on this proteolytic pathway will be universal to all eukaryotic species and cell types and not restricted to erythroid cells.

In reticulocytes, hemin has a well-documented physiological role in the maintenance of protein synthesis (Zucker & Schulman, 1968). This regulation is accomplished in part through repression of a hemin-sensitive kinase (termed the hemin-controlled repressor) responsible for phosphorylating and hence inactivating the translational factor eIF-2a (Hronis & Traugh, 1986). From the knowledge that hemin stimulates protein synthesis along with the observations that hemin inhibits protein degradation in general and the ubiquitin pathway in particular, models have been proposed where hemin serves two opposing regulatory roles in controlling the accumulation of reticulocyte proteins in vivo (Etlinger & Goldberg, 1980; Haas & Rose, 1981). Our observation with hemin and hemin analogues in nonerythroid cells would certainly question the physiological role of hemin in regulating protein breakdown in vivo. While the intracellular concentration of free hemin in reticulocytes may be high enough to have a regulatory effect in vivo, in both yeast and higher plants its concentration [<1 μM (Granick & Beale, 1978; Schneegurt & Beale, 1986)] is likely to be far below the levels required for physiological relevance. Comparative studies with hemin and hemin analogues indicate that the effect of porphyrins on protein synthesis in reticulocytes is highly specific (Hronis & Traugh, 1986). For example, while hemin and mesohemin (a metalloheme containing ethyl instead of vinyl groups at positions 2 and 4) are effective, porphyrins missing the iron center such as protoporphyrin IX are not. In contrast, our studies with mesohemin and protoporphyrin IX demonstrate that the effect of porphyrins on ubiquitin-dependent proteolysis in oats and yeast is much less structurally restricted, with both metal-containing and metal-deficient porphyrins being active. To date, the effect of protoporphyrin IX specifically on ubiquitin-dependent proteolysis in reticulocytes has not been reported. Tanaka et al. (1983) have shown that while hemin would inhibit protein degradation via nonubiquitinated intermediates in reticulocytes, protoporphyrin IX was ineffective.

The exact nature of hemin's effect(s) on ubiquitin-dependent proteolysis is unknown. The fact that both mesohemin and protoporphyrin IX are as inhibitory as hemin in oat and yeast extracts suggests that the effect is nonspecific. Because porphyrins are strongly hydrophobic, this could involve hydrophobic interactions with one or more of the proteins in the pathway. Given the complexity of reactions involved in conjugate synthesis, degradation, and disassembly (Hershko & Ciechanover, 1986; Hough et al., 1987; Waxman et al. 1987), it appears impossible to ascribe the effects of porphyrins to a particular reaction. However, because ubiquitin is involved in all three sets of reactions, it is possible that hemin interacts with ubiquitin in a way that impairs its recognition by other proteins in the pathway. It is also feasible that the degradation of ubiquitin conjugates requires the action of ubiquitin–protein lyase(s), possibly to expose region(s) masked by ligation. This direct and indirect action of hemin would explain the indistinguishable K_I's for disassembly and degradation observed here.

Regardless of its mechanism(s) of action, the ability of hemin to inhibit ubiquitin-dependent proteolysis should provide a useful method for the further characterization of the pathway in many species. For example, because of the high activity and extreme stability of ubiquitin–protein lyases in crude extracts, the inclusion of hemin may prove to be helpful in protecting ubiquitin–protein conjugates from disassembly during their purification, and as already documented elsewhere (Hough & Rechsteiner, 1986) and in this report, the addition of hemin should improve yields of ubiquitin–protein conjugates synthesized in vitro.

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Dissociation of Yeast Hexokinase by Hydrostatic Pressure†

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ABSTRACT: The pressure-induced dissociation of the isozymes P1 and P2 of hexokinase was investigated by studies of the spectral shift of the intrinsic protein fluorescence and by the fluorescence polarization of dansyl conjugates. The free energy of association of the monomers at atmospheric pressure, K_{atm}, was −14.2 kcal mol^{-1} at 20 °C and −11.4 kcal mol^{-1} at 0 °C. The positive enthalpy indicates that the association of the monomers is entropy-driven, overcoming the negative enthalpy of hydration of the subunit interfaces. At 0 °C and 1 bar, glucose stabilizes the association by −1.1 kcal mol^{-1} and the binding of both adenosine 5′-(β,γ-methylenetriphosphate) (AMPPCP) and glucose by an even larger amount, −1.34 kcal mol^{-1}. Paradoxically, adenosine 5′-triphosphate (ATP), or AMPPCP, in the absence of glucose destabilizes the association by +0.34 kcal mol^{-1}, while adenosine 5′-diphosphate (ADP) stabilizes it by −0.6 kcal mol^{-1}. Comparison of dV^0, the apparent standard volume of association, at different pHs and temperatures indicates that its value (115–160 mL mol^{-1}) is strongly dependent upon the ionization of a group at the subunit interface with a pK near neutrality. Under dissociating pressures, trypsin action results in permanent dissociation of the dimer, confirming earlier observations of Colowick by less direct methods. The P1 and P2 enzymes differ in K_{atm} and dV^0 and markedly so in the effects of salt upon the stability of the dimer. The difference in equilibrium profiles for compression and decompression of both P1 and P2 isozymes and the slow recovery of spectral characteristics and enzyme activity after decompression demonstrate that a process of conformational drift takes place when the subunits are separated. After a cycle of compression and decompression at 0 °C, the protein has much decreased free energy of association (−8.6 kcal mol^{-1}) and association volume (dV^{0*} = 50 mL mol^{-1}).

The effects of hydrostatic pressure upon a number of protein dimers and tetramers have been studied by employing fluorescence techniques [Paladini & Weber, 1981; King & Weber, 1986a,b; Silva et al., 1986; Royer et al., 1986; Verjoski-Almeida et al., 1986; see also review by Weber (1987)]. Pressure observations permit the estimation of the dissociation constant at atmospheric pressure, K_{atm}, and the standard change in volume upon association, dV^0. Additionally, these studies have revealed the existence of a very general phenomenon: the conformational drift of the subunits of an oligomer whenever these became separated, not only through

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