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The Ubiquitin-Mediated Proteolytic Pathway and Mechanisms of Energy-Dependent Intracellular Protein Degradation

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In this review we briefly describe the lysosomal system, consider the evidence for multiplicity of protein degradation pathways in vivo, discuss in detail the ubiquitin-mediated pathway of intracellular ATP-dependent protein degradation, and also the possible significance of ubiquitin-histone conjugates in chromatin. For detailed discussions of the various characteristics and physiological roles of intracellular protein breakdown, the reader is referred to earlier reviews [1–7] and reports of recent symposia [8–10]. Information on the ubiquitin system prior to 1981 was described in an earlier review [11]. Hershko has briefly reviewed more recent information [12].

Key words: ubiquitin, intracellular protein degradation, chromosome function

Cellular proteins are in a state of constant turnover. This process is involved in basic cellular functions such as regulation of levels of key enzymes in metabolic pathways, response to changing energetic and nutritional parameters of the extracellular environment and preferential degradation of defective proteins. Many functionally important proteins have short half-lives in vivo; changes in the rates of synthesis of these proteins, therefore, allow rapid adjustments in the respective protein concentrations in response to specific (eg., hormonal) stimuli. A number of such short-lived proteins play an important role in metabolic regulation. Until recently, studies on protein turnover have been mostly descriptive and phenomenological, while the underlying mechanisms have remained unknown. One crucial property of the highly specific protein degradation in all organisms is its absolute requirement for metabolic energy, which is not expected on purely thermodynamic grounds. This ATP dependence apparently reflects not an unusual pathway of peptide bond hydrolysis, but rather unidentified mechanisms that endow such systems with a high specificity toward their protein substrates.

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It is experimentally feasible and biologically meaningful to classify pathways of intracellular protein degradation as lysosomal and nonlysosomal. While the degradation of endocytosed proteins is a constitutive function of the lysosome, it is primarily under conditions of nutritional deprivation that a significant lysosomal proteolysis of *intracellular* proteins is observed. Nonlysosomal mechanisms are probably responsible for most of the highly selective turnover of intracellular proteins under normal metabolic conditions. The multiplicity of distinct nonlysosomal proteolytic pathways in mammalian cells is not known. A nonlysosomal ATP-dependent proteolytic system has been characterized and partially purified from rabbit reticulocytes. This system, which appears to be responsible for a major proportion of nonlysosomal proteolysis in reticulocytes, consists of several essential components; one of these, a small heat-stable polypeptide, has been identified as ubiquitin, an abundant, 76-residue polypeptide of previously unknown function, which is found in apparently all eukaryotic cells, from yeast to man. Ubiquitin is covalently linked to a large variety of protein substrates in an ATP-requiring reaction, in which one or more ubiquitin polypeptides are conjugated at their C-terminal glycine residues to a substrate polypeptide via isopeptide bonds involving ϵ-amino groups of lysines in the substrate polypeptide. Formation of a ubiquitin-protein conjugate may be the initial event in the degradation of many proteins, since many ubiquitin conjugates are rapidly degraded both in vitro and in vivo. More recently, ATP-dependent proteinases from Escherichia coli and mammalian mitochondria have also been characterized.

In eukaryotic cell nuclei, the major ubiquitin-protein conjugate is ubiquitin-H2A semihistone (uH2A), a protein in which ubiquitin is conjugated to an internal lysine-119 in the H2A histone via an isopeptide bond. uH2A semihistone occurs in a subset of nucleosomes, where it replaces either one or both of the nucleosomal H2A histone molecules. Recent evidence strongly suggests a preferential association of uH2A with transcribed genes: the 5′ end of a transcribed gene appears to be particularly enriched in uH2A. One interpretation of these results is that a locus-specific, ubiquitin-mediated proteolysis of chromosomal proteins may be involved in regulation of gene expression at the level of chromatin structure.

INVOLVEMENT OF LYSOSOMES IN PROTEIN DEGRADATION

That lysosomes may be involved in protein degradation has been apparent from early studies, which have shown that lysosomes contain high concentrations of proteinases of widely different specificities [13]. Lysosomes have been implicated both in proteolysis of endocytosed proteins and in autophagocytosis, which occurs under a variety of pathological metabolic conditions. This work, mainly by Mortimore and co-workers, was previously reviewed in detail [11] and will be briefly summarized here. Four lines of evidence suggest that lysosomes normally participate in the turnover of cytoplasmic proteins.

First, a correlation between structural alterations of the lysosomal system and rates of proteolysis has been demonstrated. Lysosomes prepared from livers perfused with unsupplemented medium (which enhances protein degradation), show increased sensitivity of lysis in hypotonic sucrose, and a changed morphology upon electron microscopic examination [14,15]. These changes in osmotic sensitivity and morphological features of lysosomes were rapidly reversed upon resupplementation of the perfusion medium with insulin and amino acids.

Second, a good correlation is found between the relative rates of the release of free amino acids from lysosome-containing homogenates of livers that had been perfused under various conditions and the rates of protein degradation in identically treated intact livers [16,17]. The amount of endogenous proteolysis in lysosomes incubated in vitro reflects, therefore, the size of the degradable protein pool entrapped within the lysosomes in the intact tissue.

Third, the rates of lysosomal proteolysis where directly compared to the rates of total protein degradation in the perfused liver. The results of these experiments [18,19] strongly suggested that most of deprivation-enhanced degradation of intracellular proteins is carried out by lysosomal autophagy. Furthermore, these results suggested that the lysosomal system might be involved also in basal protein degradation [18,19].

Fourth, in addition to the correlative analyses summarized above, it has been shown that agents that preferentially inhibit *lysosomal* proteolysis also inhibit deprivation-enhanced in vivo protein degradation. For example, Poole and co-workers showed that lysosomotropic agents (weak bases that accumulate preferentially within lysosomes, and thereby increase intralysosomal pH to a value sufficient to inhibit most of the lysosomal acid proteinases) such as chloroquine, inhibit enhanced protein breakdown in the absence of serum, but have much less effect on basal protein degradation in the presence of serum [20–22] (for experiments using other inhibitors, see also [23–29]), suggesting that a nonlysosomal proteolytic system contributes significantly to intracellular protein degradation under basal conditions (see below and [20–22]).

EVIDENCE FOR MULTIPLE PATHWAYS OF CELLULAR PROTEIN DEGRADATION

That several distinct mechanisms operate in intracellular protein degradation has long been clear [4,21]; here we concentrate on the substantial recent progress in this area. For the discussion to follow, we define several classes of cellular proteins with distinct features of degradation: (1) *Long-lived proteins* constitute the bulk of cellular proteins with relatively low turnover rates. (2) *Short-lived proteins* are a class of normal cellular proteins of exceptionally high turnover rate. (3) *Abnormal proteins* are produced in the presence of amino acid analogs or ribosomal drugs, such as puromycin, or as a result of specific mutations; such proteins are degraded in vivo even more rapidly than most of the short-lived proteins. Each "class" consists of many proteins, and may well be heterogeneous in both mechanism and rate of protein degradation.

Most of the available evidence indicates that the degradation of the majority of short-lived and abnormal proteins is nonlysosomal in nature. The rate of degradation of these proteins is not influenced considerably by nutritional deprivation, hormones, or inhibitors of protein synthesis [24,30,31], in contrast to the lysosomal breakdown of long-lived proteins. Decreased temperature affects much more strongly the degradation of long-lived proteins than that of short-lived normal or abnormal proteins [28], suggesting that the rate-limiting steps for these processes are not identical. Somewhat more directly, it was shown that inhibitors of lysosomal proteinases have no influence on the breakdown of either abnormal or short-lived normal cellular proteins [24,28].

With regard to the degradation of long-lived proteins, a distinction can be made between "enhanced" (under conditions of nutritional deprivation) and "basal" states (under conditions of full provision of nutrients, hormones and growth-promoting factors). Little doubt remains that most of the *enhanced* protein degradation in mammalian cells is carried out by the lysosomal system, but there are conflicting reports concerning the involvement of the lysosomal pathway in the basal degradation of long-lived proteins. As noted above, the basal protein degradation is insensitive to agents that inhibit lysosomal proteolysis. The effects of chloroquine on the degradation of endocytosed exogenous proteins and of endogenous cellular proteins were compared in cultured mouse peritoneal macrophages under basal conditions [21]. Since macrophages readily endocytose a variety of proteins, it was possible to compare degradation of endogenous and exogenous proteins in the same cells. It was found that chloroquine inhibits the degradaton of exogenous proteins much more strongly than the degradation of endogenous proteins. A similar difference was seen in experiments in which the source of exogenous material was labeled macrophage proteins, so that the breakdown of similar protein populations could be compared [21]. These results indicate that the degradation of endocytosed proteins, under basal conditions, and the degradation of endogenous proteins under conditions of nutritional deprivation, occur largely within a cell compartment different from that involved in the degradation of cellular proteins in the process of normal protein turnover. In another study, Amenta et al [32] showed that microtubule inhibitors such as vinblastine, vincristine, and colchicine suppress enhanced proteolysis in cultured embryo fibroblasts incubated in serum-free medium, but not the basal protein degradation occurring in the complete medium. These agents are known to inhibit the function of the lysosomal-vacuolar system and to block uptake and digestion of exogenous endocytosed proteins [33]. In apparent conflict with the above findings, it was shown by Dean [34], Ward et al [35], and Neff et al [28] that protein degradation in cultured macrophages, perfused liver and heart, and cultured hepatocytes, respectively, is equally inhibited by pepstatin and leupeptin (inhibitors of lysosomal proteinases) in either the presence or absence of serum.

One possible reason for the conflicting observations mentioned above is that most of the evidence for resistance of the basal protein degradation to lysosomal inhibitors was obtained from in vitro cell cultures where serum deprivation is accompanied by cessation of cell multiplication. It is possible that the relative contribution of the lysosomal pathway to protein degradation is greater in tissues composed largely of noncycling cells, such as the normal liver or muscle. In addition, the definition of the basal state is operational, and thus depends on the conditions employed in a particular study.

Recent experiments by Rechsteiner and co-workers showed that proteins microinjected into cultured cells are degraded mainly in the soluble (nonlysosomal) fraction of the cytoplasm [36], strongly suggesting that nonlysosomal proteolytic pathways are responsible for at least a considerable proportion of the total protein degradation under basal metabolic conditions.

Finally, it appears that the enhanced lysosomal autophagy that is associated with conditions of nutritional deprivation is essentially nonselective, in contrast to a wide variation in the turnover rates of specific proteins under basal metabolic conditions [37,38]. This implies that, if most of the basal protein degradation is carried out by the lysosomal pathway, at least two distinct modes of vacuolar sequestration exist,

namely, a nonselective inducible mode and a selective basal mode. In this regard, the possibility of a selective adsorption of proteins to lysosomal membranes has been suggested [37]; no direct evidence is available on this point.

ATP-DEPENDENT PROTEOLYTIC SYSTEMS

Intracellular Protein Degradation Is Energy-Dependent

The energy requirement for intracellular protein degradation was discovered by Simpson in 1953. He showed that the release of amino acids from labeled proteins is inhibited by either anaerobic conditions or inhibitors of energy metabolism [39]. Numerous studies have since shown that the energy dependence of intracellular protein degradation is an essential feature of the process in both prokaryotes and eukaryotes [40,41]. This energy requirement applies to degradation of both normal [42] and abnormal proteins [43]. Although the effect of energy inhibitors in intact cells may reflect a direct energy requirement for protein degradation, other interpretations are also possible. For example, the process may be linked to protein synthesis [44] and thus affected indirectly by inhibition of ATP synthesis. It was shown, however [42], that inhibitors of protein synthesis can block only the enhanced but not the basal protein degradation, while inhibitors of ATP synthesis block both processes. Energy should be also required for the initial events in autophagy that apparently involve processes of membrane assembly and rearrangement, and for the maintenance of the acidic intralysosomal pH necessary for the action of intralysosomal proteinases [45].

The energy dependence of nonlysosomal intracellular protein degradation may underlie the high specificity of these processes. Several cell-free ATP-dependent proteolytic systems have recently been established; however, the detailed mechanistic basis of the energy requirement is in no case well understood. The system most extensively studied is the ATP-dependent proteolytic system from mammalian reticulocytes, which is described below.

ATP-Dependent Proteolytic System From Mammalian Reticulocytes and Cell Lines: The Ubiquitin-Mediated Pathway

Protein breakdown in intact reticulocytes. The mammalian reticulocyte is a highly specialized cell (an erythrocyte precursor): it synthesizes predominantly one protein, hemoglobin, has few cytoplasmic organelles and no nucleus. In early studies, Rapoport and co-workers have observed a massive release of free amino acids from reticulocytes incubated in a physiological salt solution [46]. No comparable protein breakdown occurred in mature erythrocytes. This breakdown of reticulocyte proteins is energy-dependent, since it can be blocked by anaerobiosis or inhibitors of ATP synthesis. When reticulocytes are labeled with radioactive amino acids, very little degradation of labeled protein takes place, unless abnormal proteins have been synthesized during labeling. This indicates that under normal conditions, most of the proteins that are degraded had been synthesized at an earlier stage of erythroid cell differentiation. Rapoport and co-workers suggest that the major endogenous proteins that are degraded in the reticulocytes are "stromal" proteins, that and that such degradation plays an important role in reticulocyte maturation [47]. In addition, this proteolytic system may have a major role in the disappearance from maturing reticu-

locytes of mitochondria, ribosomes, and of many cellular proteins and enzymes that have no further functions in the mature erythrocyte [48]. The activity of the energy-dependent proteolytic system as assayed in vitro decreases sharply during maturation of the reticulocytes and is virtually absent in mature erythrocytes [43,49,50]. On the other hand, intact mature erythrocytes were shown by Goldberg and Boches [51] to degrade abnormal proteins (produced by phenylhydrazine oxidation) in vivo in an ATP-requiring mode.

The reticulocyte proteolytic system is highly active in the degradation of abnormal globin chains, whereas normal hemoglobin is stable. The degradation of amino acid analog-containing globin chains is blocked by inhibitors of ATP formation [43,50], but not by inhibitors of protein synthesis [50]. Rabinowitz and Fisher [52] first showed that globin chains that incorporate the lysine analog S-(β-aminoethyl)-cysteine, (4-thialysine), or the valine analog, t-α-amino-β-chlorobutyric acid, are degraded extremely rapidly. Reticulocytes rapidly degrade puromycyl peptides as well [53]. In addition, the preferential degradation of certain mutant hemoglobin variants and of excess of normal α-chains in β-thalassemic patients has been noted [reviewed in 3]. Thus a second potential function of the active proteolytic system in reticulocytes may be to remove globin molecules containing biosynthetic errors, or excessive amounts of one species of globin that is not assembled into hemoglobin (under normal conditions the synthesis of α- and β-chains of globin is well balanced [54]).

Hershko and co-workers [50] became interested in the reticulocyte as a model system because of the possibility of following the fate of one predominantly synthesized protein (analog-containing globin) in the presence of a highly active degradative system. Analysis of the size distribution of labeled analog-containing polypeptides on SDS-polyacrylamide gels showed most of the label in complete globin chains, and no significant amounts of cleavage fragments smaller than globin could be detected in the course of a "pulse-chase" experiment. In addition, there was no accumulation of cleavage fragments when degradation was blocked by energy deprivation [50]. It was concluded that the initial reaction(s) in the degradation of globin must be strongly rate-limiting relative to the subsequent rapid proteolysis of intermediate cleavage fragments, and that energy is required at or before the initial cleavage reactions; otherwise such fragments would accumulate under conditions of energy deprivation [50].

Characteristics of the cell-free system. A cell-free ATP-dependent proteolytic system from reticulocyte lysates was first established by Etlinger and Goldberg [43]. The system did not seem to be of lysosomal origin, since it was soluble and had a slightly alkaline pH optimum. Furthermore, the system is not inhibited by peptides that inhibit lysosomal proteinases, such as leupeptin, pepstatin, or chymostatin [55]. ATP is specifically required in the presence of Mg^{+2}. The following evidence indicates that this cell-free system is similar to the one responsible for the degradation of abnormal proteins in vivo: (1) the system requires an energy-rich compound, as does protein degradation in intact cell; (2) the cell-free system degrades abnormal proteins but not normal hemoglobin, as is the case with intact reticulocytes, and (3) the cell-free proteolytic system is inhibited by the same agents that inhibit protein degradation in intact reticulocytes, such as thiol-blocking agents, chloromethyl ketones, metal chelators [43], or hemin [56]. In addition, Botbol and Scornik [57] have used bestatin, an inhibitor of aminopeptidases, to cause accumulation of intermediates in the degradation of abnormal proteins in mouse reticulocytes. They found that the fingerprint pattern of peptides accumulated in cell-free extracts appeared strikingly

similar to that in intact reticulocytes, suggesting that the cell-free system faithfully represents proteolytic events that occur in intact cells.

Resolution of the components of the cell-free system. To address mechanistic aspects of this in vitro system, the resolution and purification of its components proved necessary. Several possibilities for the role of ATP in protein breakdown were considered: (1) ATP may be required for the covalent modification of the substrate protein, which would be a signal for its degradation; (2) covalent modification of a proteolytic enzyme may be required for its activation; (3) ATP hydrolysis (in the absence of protein modificaton) may be required for the action of an unidentified proteinase; (4) there may be an allosteric activation by ATP of an unknown proteinase. In cases 2 and 3, there should be at least two necessary components, the modifying enzyme and the proteinase. Therefore, the initial approach of Ciechanover et al [58] and Hershko et al [59] was to separate the lysate into crude fractions and to search for complementation of activities between the fractions. Initially, lysates from ATP-depleted reticulocytes were separated on diethylaminoethyl (DEAE)-cellulose into two crude fractions: unabsorbed material (fraction I), which contains hemoglobin and a few basic or neutral proteins, and a high-salt eluate (fraction II), which consists of most of the nonhemoglobin proteins that bind to the resin. Neither fraction had a significant ATP-dependent proteolytic activity by itself, but activity was restored upon the combination of the two fractions [58].

The active component in fraction I showed rather unusual features. Although it was stable upon heating to 90°C, it appeared to be a polypeptide by the following criteria: it was nondialyzable, it was precipitable by ammonium sulfate, and its activity was destroyed by treatment with proteinases. By gel filtration on Sephadex G-75, a molecular weight of approximately 9 kilodaltons (kd) was determined [58]. The heat-stable polypeptide, designated as APF-1 (ATP-dependent proteolysis factor I) was purified to apparent homogeneity and characterized [60]. It was found to be present in several tissues of the rat in significant amounts [60]. Subsequently, APF-1 was found to be identical to ubiquitin, a universally occurring polypeptide of previously unknown function [61] (see also below).

Fraction II itself contains several separable components of the ATP-dependent proteolytic system. Ammonium sulfate fractionation yielded two mutually required subfractions, fractions IIA and IIB. Fraction IIA contains a high-molecular-weight component of the proteolytic system that is extremely heat-labile but is strongly stabilized by ATP [59]. However, the requirement of proteolysis for ATP is not due merely to the stabilization of this factor, since the latter was also stabilized by ADP, and by ATP analogs that cannot replace ATP in the stimulation of proteolysis [59] (see also below).

Covalent conjugation of ubiquitin to proteins. A crucial observation was that an ATP-dependent covalent association of the heat-stable polypeptide APF-1 (ubiquitin) with other cellular proteins takes place in the cell-free system. Following incubation of purified, radiolabeled APF-1 with fraction II in the presence of ATP, binding of the polypeptide to high-molecular-weight material was observed, as analyzed by gel filtration on Sephadex G-75 [62] (Fig. 1). The reaction specifically required ATP and Mg^{+2}. The binding was shown to be covalent: bound APF-1 (ubiquitin) was not released by treatments with acid, mild alkali, or heating at 100°C in the presence of sodium dodecyl sulfate (SDS) and 2-mercaptoethanol.

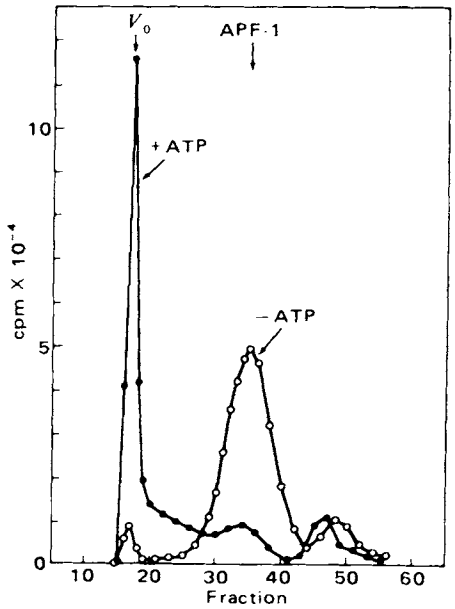

Fig. 1. ATP-dependent binding of APF-1 (ubiquitin) to reticulocyte components 3.2 μg of [^{125}I]-APF-1 (3.8×10^5 cpm/μg) were incubated with 650 μg of fraction II with or without ATP and the reaction mixture was subjected to gel filtration on Sephadex G-75 column (adapted from Ciechanover et al [62]).

Analysis of the reaction products by SDS-polyacrylamide gel electrophoresis revealed the association of the labeled APF-1 (ubiquitin) with numerous high molecular weight proteins [62,63] (Fig. 2, lane 2). It did not seem likely, therefore, that the polypeptide is bound to some specific enzyme(s) involved in the degradative process, but rather that endogeneous substrates of the proteolytic system were the targets of the binding reaction. Indeed, it was found that proteins that are good substrates for ATP-dependent proteolysis in vitro (such as lysozyme, globin, or α-lactalbumin), form covalent conjugates with APF-1 (ubiquitin) in a reaction requiring ATP [63] (Fig. 2, lanes 3–7). Discrete heterogeneity of these conjugates was shown by electrophoretic analysis: the autoradiographic bands formed a periodic array. Apparent molecular weights of lysozyme conjugates (determined by SDS-gel electrophoresis) indicated that the electrophoretic "ladder" represents *increasing* numbers of APF-1 (ubiquitin) molecules bound to *one* molecule of lysozyme [63]. Analysis of the ratio of APF-1 (ubiquitin) to lysozyme radioactivities in the various APF-1-lysozyme conjugates confirmed this conclusion. Examination of the nature of the linkage between AFP-1 (ubiquitin) and proteins suggested that it was an amide bond, since it was stable to prolonged incubation in 0.1 N NaOH or in 1M hydroxylamine at pH 9 [63]. Since poly-L-lysine also could serve as a substrate for conjugation with APF-1 (ubiquitin), it appeared likely that an isopeptide linkage is formed between ϵ-NH_2 groups of lysine residues of the protein and a carboxyl group in the APF-1 (ubiquitin) [63].

Conjugates of the APF-1 (ubiquitin) with proteins formed by reticulocyte fraction II in the presence ATP appeared to be in a state of continual synthesis and degradation [62,63]. When ATP was removed with hexokinase and glucose, rapid

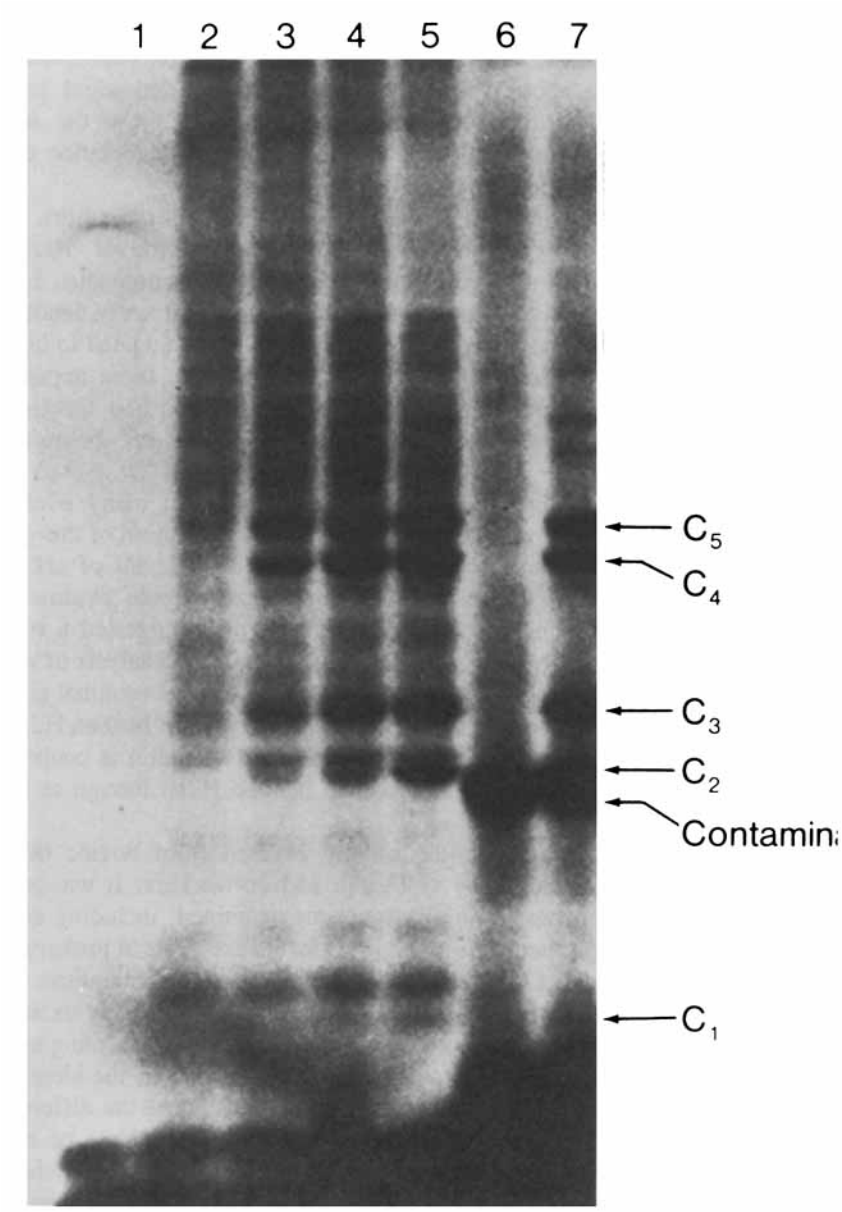

Fig. 2. Formation of covalent conjugates between APF-1 (ubiquitin) and reticulocyte proteins and lysozyme. Reaction mixtures similar to those described in legend to Figure 1 were subjected to SDS-polyacrylamide gel electrophoresis. Lanes 1–5: conjugates formed with [^{125}I]-ubiquitin. Lane 1, without ATP; lane 2, with ATP; lanes 3–5, with 5,10, and 25 μg of unlabeled lysozyme, respectively. Lanes 6 and 7: compounds formed with [^{125}I]-lysozyme and unlabeled ubiquitin. Lane 6, without ATP; lane 7, with ATP (adapted from Hershko et al [63]).

disappearance of the conjugates was observed (with different rates of disappearance for different conjugates) [62]. This was accompanied by the release of free, reusable APF-1 (ubiquitin) as shown by the finding that the released APF-1 (ubiquitin) could be quantitatively converted back to conjugates when incubated again with fraction II and ATP [63]. Based on the above results, a model for ATP-dependent protein degradation was proposed, according to which the covalent linkage of the APF-1 (ubiquitin) to the protein substrate is followed by the proteolytic degradation of the modified substrate [63] (see also below).

Identification of the heat-stable polypeptide (APF-1) as ubiquitin. Wilkinson and associates [61], following the above studies by Ciechanover, Hershko, Rose, and co-workers, noted a marked resemblance of these conjugates to the previously characterized structure of chromosomal protein A24 (currently denoted as ubiquitin-H2A semihistone, uH2A), which consists of ubiquitin conjugated to histone H2A via an isopeptide linkage (see below), and thus research in three apparently unrelated areas (mechanisms of protein degradation, the structure and function of chromosomal proteins, and the isolation of thymic hormones; see below) had converged.

uH2A was discovered by Busch and co-workers as one of many nucleolar proteins resolved by two-dimensional electrophoresis [64,65]. Attention of this group had been focused on this particular protein because the apparent levels of uH2A in rat liver nucleoli were markedly decreased following thioacetamide treatment or during liver regeneration following partial hepatectomy, which suggested a role in regulation of gene expression. Sequencing and computer sequence analysis of uH2A showed it to have a bifurcated primary structure, in which the COOH-terminal glycine of ubiquitin is bound to the ϵ-NH_2 group of the internal lysine-119 of histone H2A via an isopeptide linkage [65,66]. Recently, it was reported that ubiquitin is conjugated in vivo to all subtypes of histone H2A, and also to histone H2B, though at much lower levels [67,68].

Ubiquitin is a 76-residue polypeptide initially isolated from bovine thymus during a search for thymic hormones by Goldstein and co-workers. It was subsequently detected by radioimmunoassay in all organisms examined, including animal cells, plants, yeast, and bacteria; hence its name [69] (for its existence in prokaryotes, see below). Insect [70], bovine [71], trout [72], and human [73] ubiquitins have identical primary structures. With trout ubiquitin, an uncertainty (probably technical) exists in residues 68–71 [72]. This sequence conservation, unparalleled among known proteins, indicated a basic and universally occurring cellular function, the identity of which was, however, unknown. Initial reports that ubiquitin induces the differentiation of lymphocytes [69] and stimulates adenylate cyclase [74] could not be reproduced, and are apparently due to contaminating activities [75,76]. The single clue for the physiological function of ubiquitin was its conjugation to histone H2A, described above. In isolated chromatin, ubiquitin is found not only as a histone conjugate, but also in the free form [72]. The ubiquitin conjugate uH2A was found to be a component of the nucleosome [77], where it replaces either one or both molecules of histone H2A [78–80] (see also below).

The identity of ubiquitin with the proteolytic factor APF-1 was originally suggested on the basis of the following common features: (1) the conjugation of APF-1 with proteins via an isopeptide linkage [63] is the same as the bond between ubiquitin and histone H2A [81]; (2) both APF-1 and ubiquitin are heat-stable proteins

[58,69] of similar molecular size and isoelectric point [60,75]; (3) the amino acid composition of APF-1, determined by Ciechanover et al [60], is very similar to the known composition of ubiquitin [71]. A single detectable difference (see also below) was the presence of six glycine residues in APF-1 [60] versus four in ubiquitin [71]. Ubiquitin from calf thymus migrated identically with rabbit reticulocyte APF-1 in five different polyacrylamide gel elecrophoretic systems, and calf thymus ubiquitin stimulated protein breakdown and formed conjugates with protein in the presence of rabbit reticulocyte fraction II and ATP [61]. In both of the above processes however, thymus ubiquitin was only about 65% as active as APF-1. The carboxyl end of ubiquitin was known to be conjugated to histone H2A via a gly-gly sequence [81], but arginine-74 has been reported as the COOH-terminal amino acid of thymus ubiquitin [71,73,75]. Hershko et al [82] have shown that a COOH-terminal glycine of APF-1 is the specific amino acid residue that is activated by the reticulocyte ubiquitin-activating enzyme (see below). These discrepancies have been resolved by the findings that APF-1 from human [83] or rat [84] erythrocytes contains a COOH-terminal -gly-gly sequence, and that only the species having the -gly-gly terminus is active in the stimulation of ATP-dependent protein degradation [83]. It is not clear whether ubiquitin that lacks the C-terminal -gly-gly is an in vitro proteolytic artefact, or whether this ubiquitin species occurs also in vivo.

Intermediate reactions in the formation of ubiquitin-protein conjugates. Information concerning the enzymatic steps involved in the conjugation of ubiquitin to proteins is still fragmentary. Initially, an enzyme was partially purified from reticulocyte fraction II that catalyzes an ubiquitin-dependent ATP:PP_i and ATP:AMP exchange reactions [85]. A likely explanation of these observations is the following two-step mechanism:

$$E_{SH} + \text{Ubiquitin} + ATP \rightleftarrows E_{SH}\text{. . . Ubiquitin} \sim AMP + PP_i \quad (1)$$

$$E_{SH}\text{. . .ubiquitin} \sim AMP \rightleftarrows \text{E-S} \sim \text{ubiquitin} + AMP \quad (2)$$

In the first step, to account for the ATP:PP_i exchange, an adenylate of the polypeptide was assumed to be formed, with release of pyrophosphate. The ubiquitin adenylate was subsequently isolated by Haas et al [86]. The ubiquitin-dependent ATP:AMP exchange indicates that the activated group is transferred to a secondary acceptor, with the liberation of AMP. The acceptor is a sulfhydryl group of the enzyme, since labeled ubiquitin becomes associated with the enzyme by a linkage that has the characteristics of a thiolester bond [85]. The reaction is specific for ATP (in the presence of Mg^{2+}), and ubiquitin is effective at low concentrations, similar to those required for protein degradation. Although the reaction is unusual for a polypeptide, it is analogous to other known processes such as the activation of fatty acids, activation of amino acids for protein synthesis, and the nonribosomal biosynthesis of peptide antibiotics. In the latter case, the aminoacyl adenylates are transferred to thiolester intermediates on the same enzyme which preserves the activated state of the acyl group, and functions in the formation of the final amide bond [87]. It was subsequently shown by Haas et al [86] that ubiquitin bound to the activating enzyme by the energy-rich thiolester linkage represents a similar reactive intermediate.

Assuming the activation mechanism described above, and using PP_i and AMP to reverse the activation reaction, Ciechanover et al [88] have recently purified the ubiquitin-activating enzyme by covalent affinity chromatography on ubiquitin-Sephar-

ose (Fig. 3). The enzyme is composed of two identical ~ 105-kd subunits. Each subunit has two binding sites for ubiquitin (an adenylate site and a thiolester site) [86]. The enzyme does not catalyze the formation of ubiquitin-protein conjugates by itself but is required as the first enzyme in the reaction of conjugate formation in the presence of two other enzymes designated E_2 and E_3 [89]; (E_1 is the ubiquitin-activating enzyme). E_2 and E_3 were isolated from crude reticulocyte extracts by ubiquitin affinity chromatography. The function of E_2 is the transfer of activated ubiquitin fom E_1 to E_3 in the form of E_2-ubiquitin thiolester intermediate. E_3 is involved in the last step of the ubiquitin-protein conjugation, catalysis of the isopeptide bond formation between ubiquitin and proteins. Each of these three enzymes is required for ATP-dependent proteolysis in the presence of ubiquitin and other components of the system from fraction II [89]. The proposed pathway of ubiquitin-protein conjugation is shown in Fig. 4.

Immunochemical analysis of the turnover of ubiquitin-protein conjugates in intact cells. To address some of the physiological roles of the ubiquitin system in intact cells, a polyclonal antibody that recognizes ubiquitin-protein conjugates was used to isolate such conjugates from cells in different metabolic states [90]. If ubiquitin-protein conjugates are indeed intermediates in protein degradation, their levels should reflect a balance between rates of conjugates formation and breakdown. Rates of conjugates formation may be affected, in turn, by the availability of rapidly degradable cellular proteins. To test these predictions, the rapidly degraded, amino acid analog-containing abnormal proteins and the slowly degraded normal proteins in reticulocytes and in Ehrlich ascites tumor cells were compared.

In reticulocytes, a strong increase in the rate of degradation of newly synthesized proteins can be induced through formation of abnormal proteins. The major product of reticulocyte protein synthesis, hemoglobin, is normally stable, but abnormal globin chains containing lysine or valine analogs (see above) are degraded rapidly [43,50,52]. Reticulocytes were labeled with [^{3}H]-leucine in the presence of the lysine analog, and cell extracts were prepared and subjected to immunoprecipitation. As shown in Table I, in untreated cells (labeled without addition of analogs), about 0.5% of total pulse-labeled proteins was immunoprecipitated. A marked increase in the level of labeled immunoprecipitable protein was observed under conditions of abnormal protein formation: the relative labeling of immunoreactive protein (percentage of total radioactivity) increased almost 10-fold in reticulocytes synthesizing abnormal proteins (Table I, experiment 1).

Since ubiquitin does not contain tryptophan [71,73], labeled tryptophan incorporated into proteins reactive with the antibody represents exclusively the non-ubiquitin portion of ubiquitin-protein conjugates. As shown in Table I, experiment 2, when reticulocytes were preincubated with the lysine analog, and then labeled with [^{3}H]-tryptophan, a strong increase in the labeled immunoreactive protein was observed compared to the analog-free system. This result indicated that under the conditions used, most of the immunoprecipitated label is derived from the nonubiquitin protein moiety of ubiquitin-protein conjugates.

If ubiquitin-protein conjugates are indeed intermediates in protein degradation, it would be expected that the conjugates are in a dynamic state of formation and decay. To examine the decay of ubiquitin-protein conjugates, reticulocytes were labeled with [^{3}H]-leucine in the presence or absence of the lysine analog; further protein synthesis was then blocked by cycloheximide and the incubation continued.

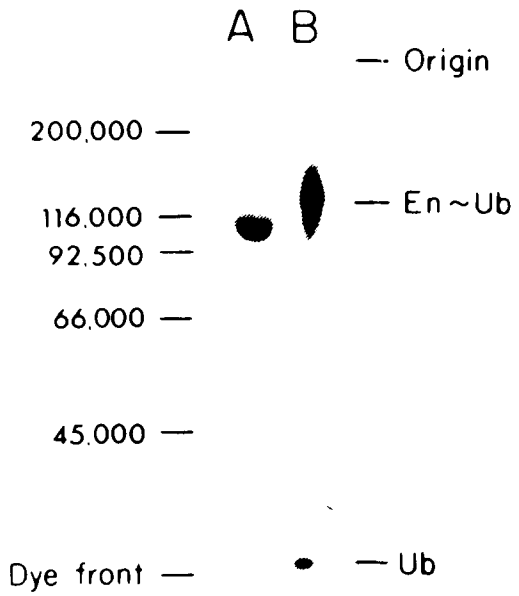

Fig. 3. SDS-polyacrylamide gel electrophoresis of affinity-purified ubiquitin-activating enzyme and its complex with labeled ubiquitin. A. Coomassie blue staining of the electrophoresed purified enzyme (15 μg). B. Autoradiogram of [^{125}I]-ubiquitin-enzyme complex (adapted from Ciechanover et al [88]).

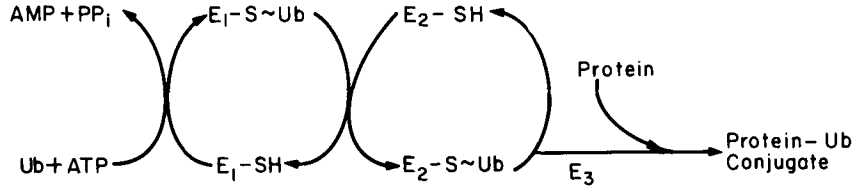

Fig. 4. Activation of ubiquitin and its conjugation to the protein substrate. E_1, ubiquitin-activating enzyme (described in the text). E_2, E_3, additional activating enzymes (see text) (adapted from Hershko et al. [89]).

As observed previously, reticulocyte proteins synthesized in the presence of the analog are degraded at different rates, and following 30–60 min of "chase" incubation, a population of relatively stable labeled cellular proteins remains, accounting for about 50% of the initial radioactivity [90,91]. The decay of labeled ubiquitin conjugates with abnormal proteins was much more extensive than that observed with normal cellular proteins and proceeded in parallel with the degradation of total labeled cellular proteins (Fig. 5). Thus, after one hour, only about 15% of the initial amount of labeled conjugates of abnormal proteins remained, as compared to 45% remaining of total abnormal proteins. Thus, for abnormal proteins, the decay kinetics of ubiquitin-protein conjugates differs from that of total protein, and resembles that of the degraded portion of the labeled protein pool.

TABLE I. Increased Labeling of Protein Reactive With Antibody Directed Against Ubiquitin Under Conditions of Abnormal Protein Formation in Reticulocytes*

		Radioactivity in total protein		Labeled protein adsorbed to protein A-sepharose			
Label	Amino acid analog	15 min (cpm/mg proteins)	60 min (% degradation)	Immune serum (A)	Normal serum (B)	Specific adsorption (A–B)	Immuno-reactive protein (% of total cpm)
Experiment 1							
[^{3}H]-leucine	None	1,230,000	5	7,453	418	7,035	0.57
	4-Thialysine	749,000	44	34,527	380	34,147	4.56
	t-α-amino-β-chlorobutyric acid	529,000	65	27,482	454	27,028	5.11
Experiment 2	None	47,000	0	446	34	412	0.88
[^{3}H]-tryptophan	4-Thialysine	30,000	40	1,558	51	1,507	5.0

*Experiment 1: Reticulocyte suspensions were preincubated at 30°C for 10 min in the presence of the indicated amino acid analogs and were then labeled with [^{3}H]-leucine for 15 min. Radioactivity in total protein was determined and immunoreactive proteins were isolated. To a separate sample of labeled cells, cycloheximide (0.2 mM) was added and incubation was continued for a further 60 min to determine the extent of protein degradation. Experiment 2: the experimental design was similar to that described in experiment 1, except that [^{3}H]-tryptophan was used for labeling (adapted from Hershko et al [90]).

In analog-free reticulocytes as well, the decay of ubiquitin conjugates greatly exceeded that of total labeled proteins. In this experiment, there was an about 10% decrease in total protein label during the first 15 min (Fig. 5A), concomitant with approximately 40% decay in levels of labeled ubiquitin-protein conjugates (Fig. 5B). The rest of the labeled conjugates of normal proteins did not decay further in the course of the chase incubation.

Since the reticulocyte is a highly specialized cell, it was desirable to test the above relationships in a more common type of nucleated animal cell. Ehrlich ascites tumor cells were chosen, since Yushok and Frech [92] had shown that these cells actively degrade abnormal proteins and have a significant proportion of short-lived normal proteins. In these experiments, levels of ubiquitin conjugates varied between 0.8–2.0% of the total protein label in untreated Ehrlich cells and between 1.8–3.5% in cells treated with 2 mM of the lysine analog. The ubiquitination pattern in Ehrlich tumor cells thus resembles the one observed in reticulocytes except that levels of ubiquitin conjugates in analog-free Ehrlich tumor cells are generally higher and the effect of the lysine analog is less pronounced. On the whole, the results of immunochemical analysis are consistent with the interpretation that the degradation of abnormal proteins in Ehrlich tumor cells is carried out by the ubiquitin-mediated pathway.

Independent evidence in support of the notion that ubiquitin conjugation signals proteolysis and that there is a correlation between ubiquitin conjugation and protein degradation comes from the study by Rechsteiner and co-workers [93]. They injected HeLa cells with hemoglobin and [^{125}I]-labeled ubiquitin. When the cells were treated with phenylhydrazine to denature the coinjected hemoglobin, a series of prominent ubiquitin-globin conjugates was formed. These were detected by ^{125}I-autoradiography of SDS gels. At low doses of phenylhydrazine, the intracellular concentration of

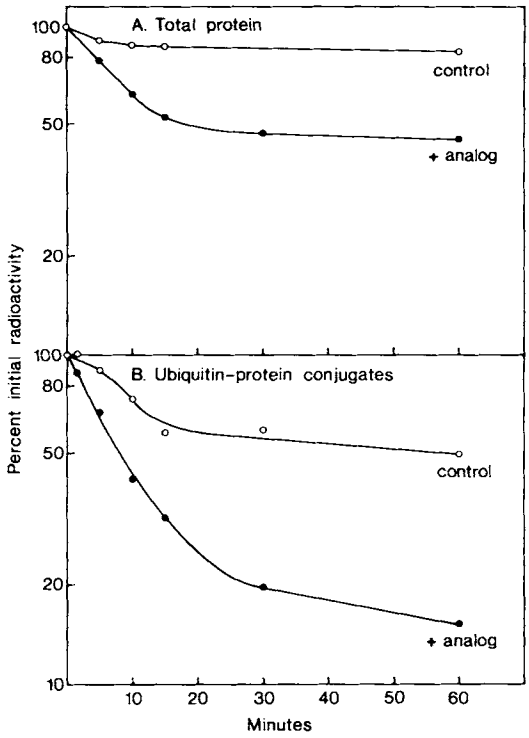

Fig. 5. Time course of decay of labeled proteins and ubiquitin-protein conjugates in reticulocytes . Reticulocytes were labeled for 15 min with [^{3}H]-leucine in the absence (○—○) or presence (●—●) o f a lysine analog. At time zero, cycloheximide was added and the incubation continued. Radioactivity ir 1 total protein (A) and in ubiquitin-immunoreactive proteins (B) is shown as percentage of the correspond - ing radioactivities at the time zero (adapted from Hershko et al [90]).

globin-ubiquitin conjugates was proportional to the rate of degradation of injected hemoglobin. This result, together with the observation that ubiquitin conjugation to globin is markedly enhanced by phenylhydrazine-induced hemoglobin denaturation, indicates that covalent attachment of ubiquitin to protein does signal proteolysis. However, distinctly different possibilities are not excluded by these correlative data. More recently, we have begun to investigate a system which may allow more definitive conclusions to be drawn (see below).

Mouse cell cycle ts mutant with a thermolabile ubiquitin-activating enzyme is temperature-sensitive for protein degradation. The cell line ts85 is a temperature-sensitive mutant derived from FM3A, a cell line established from a spontaneous mouse mammary carcinoma [94]. The wild-type cells were mutagenized and temperature-sensitive cell cycle mutants were selected at 39°C by the [^{3}H]-thymidine suicide technique [95]. When growing, asynchronous cultures of one such mutant line (ts85) were shifted to 39°C, there evolved a stable cell cycle distribution composed of early G2 and (fewer) late S phase cells. The rate of histone H1 phosphorylation in vivo was measured in synchronized G2 and G1/S ts85 cultures, and in both instances ts85 showed a temperature-dependent defect [96]. Histone H1

is stoichiometrically required for certain aspects of supranucleosomal organization in chromatin. H1-chromatin interactions are modulated in particular by phosphorylation, a process which is under cell cycle control [96,97]. Both cytoplasmic and nuclear protein kinases from ts85 cells tested with histone H1 as a substrate were not temperature-sensitive in vitro [96], suggesting that the defect in H1 phosphorylation is secondary to some other, primary defect.

Remarkably, a temperature-sensitive modification of yet another histone, H2A, was identified: the ubiquitin-H2A semihistone (uH2A, protein A24; see above) disappeared from the chromatin at 39°C with a half-life of about 3 hr [97–99]. A temperature-resistant growth revertant, obtained by mutagenesis and selection of ts85 cells at 39°C, displayed a wild-type phenotype of H1 modification, strongly suggesting that the (presumably single) mutation responsible for the cell cycle arrest is also responsible for the histone phosphorylation defects [95]. As discussed below, this mutation is apparently responsible for the second histone modification (uH2A) defect as well.

In our recent in vivo pulse-chase experiments [D. Finley, A. Ciechanover and A. Varshavsky, in preparation], we have found that the disappearance of uH2A at 39°C was due to a reduced rate of ubiquitin-H2A conjugation in vivo. We therefore tested the possibility that the reduced synthesis of uH2A at 39°C was directly due to thermolability (presumably mutational) of some component of the ubiquitin-ligase enzymatic system. We incubated [^{125}I]-ubiquitin with extracts from the mutant (ts85) and the wild type (FM3A) cells. As can be seen in Figure 6, the in vitro conjugation of labeled ubiquitin to protein substrates is indeed temperature-sensitive in the ts85 cells compared to the FM3A cells. By affinity chromatography purification of the components of the ubiquitin ligase system on ubiquitin-Sepharose according to Hershko et al [89], we were able to show that the thermolabile component is E_1 (the ubiquitin-activating enzyme) [D. Finley, A. Ciechanover, and A. Varshavsky, in preparation]. Mixed (mutant plus wild-type) extracts have an intermediate temperature sensitivity; thus loss of activity cannot be due to secondary effects such as control of activity by enzyme modification.

It was then possible to ask whether a defect in the ubiquitin-protein ligase system gives rise to a defect in the degradation of intracellular proteins. We pulse-labeled the wild-type (FM3A) and the mutant (ts85) cells with [^{35}S]-methionine at the permissive and the nonpermissive temperatures during incubation with analogs of the amino acids lysine and valine. As can be seen from Fig. 7A, approximately 70% of the prelabeled abnormal proteins were degraded within 4 hr in both FM3A and ts85 cells at 30.5°C. In striking contrast, only ~15% of the prelabeled abnormal proteins were degraded at 39.5°C in the ts85 cells compared to ~70% in the FM3A cells at the same temperature (39.5°C; Fig. 7B). Analogous experiments with puromycin in ts85 versus FM3A cells produced similar results, and furthermore, lysosomotropic agents had no effect on the degradation of abnormal proteins in these experiments [A. Ciechanover, D. Finley and A. Varshavsky, in preparation]. Direct determination of the ATP concentration in ts85 cells showed no significant differences between permissive and nonpermissive temperatures, making it unlikely that the ts85 mutational lesion affects the general energy metabolism in these cells. The revertant cells mentioned earlier (ts85R-MN3 [96,97]), displayed the wild-type phenotype of intracellular protein degradation (data not shown) strengthening our conclusion that a single mutation in the ubiquitin-activating enzyme is responsible for the complex phenotype of ts85 cells.

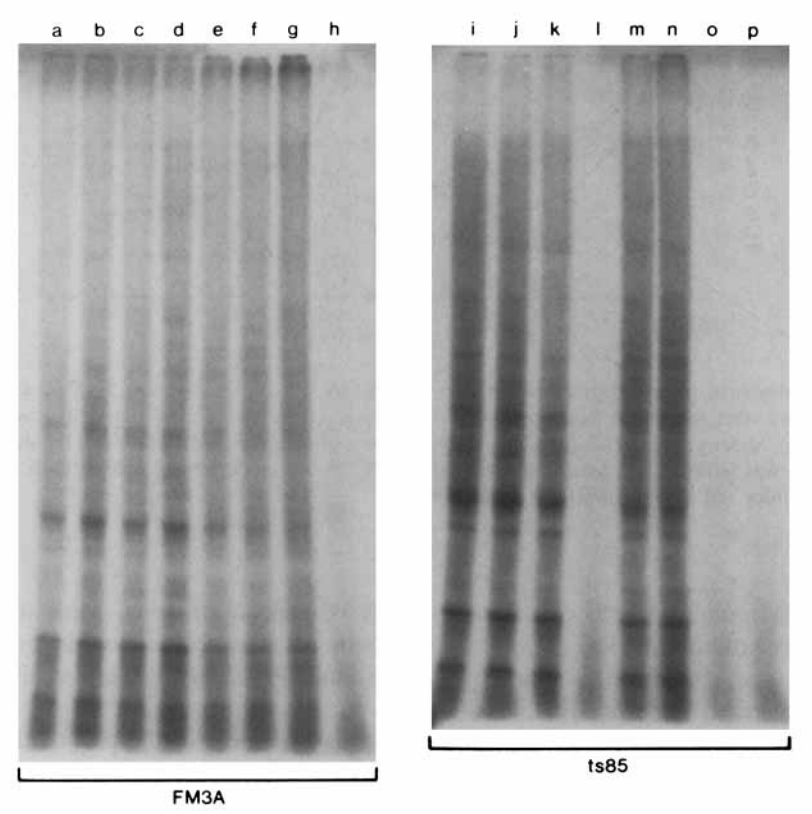

Fig. 6. In vitro ubiquitin conjugation by extracts of FM3A (lanes a–h) and ts85 (lanes i–p) cells. The extracts were prepared and assayed as described [58,62,63] with minor modifications. After preincubation, the assays were initiated by the addition of [^{125}I]-ubiquitin, denatured lysozyme, and an ATP regeneration system. Assays were terminated after 5 min by the addition of SDS. Assays were carried out at 30°C, 35°C, 40°C, and 45°C (lanes a–d and i–l, respectively), with 5-min preincubations at assay temperature. Assays were also carried out at 30°C after 2-hr preincubations at 30°C, 35°C, 40°C, and 45°C (lanes e–h, and m–p for FM3A and ts85 cells, respectively). [D. Finley, A. Ciechanover and A. Varshavsky, in preparation].

Immunochemical analysis of ubiquitin conjugates addressed directly the intermediacy of these adducts in the degradation of abnormal proteins in the ts85 cells. While a very low level of ubiquitin conjugate-specific immunoprecipitate was observed in the ts85 cells at 39.5°C, a high level of immunoreactive proteins that decayed in parallel to the decay of the total pulse-labeled cellular proteins during the chase period, could be demonstrated in the parental FM3A cells at the same (nonpermissive) temperature, and in both cell types at the permissive temperature (not shown).

In another experiment we showed that the degradation of short-lived, *normal* proteins is also inhibited in the ts85 cells at the nonpermissive temperature (not shown). Although this class of normal cellular proteins is distinct from the class of

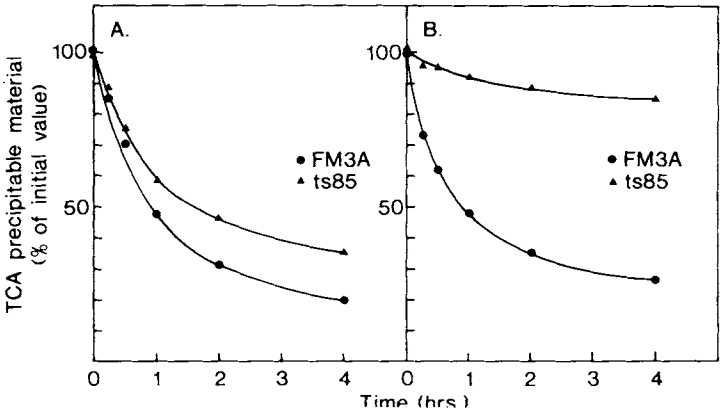

Fig. 7. Abnormal protein degradation in ts85 and FM3A cells at 30.5°C and 39.5°C. Cells were preincubated with the amino acid analogs of lysine and valine and labeled for 6 min with [^{35}S]-methionine. At zero time, a chase was started with excess of unlabeled methionine and the degradation of proteins was followed. A. Assay performed at 30.5°C. B. Assay performed at 39.5°C [A. Ciechanover, D. Finley and A. Varshavsky, in preparation].

long-lived proteins which constitute the bulk of cellular proteins, some important regulatory proteins and key enzymes in metabolic pathways belong to this group. The possible involvement of the ubiquitin pathway in degradation of the long-lived proteins is currently under study.

The ts85 mutant provides extremely strong evidence for the proposed mechanism, in which the modification of proteins by ubiquitin conjugation is a required step in their proteolysis.

The thermolability of the cytoplasmic ubiquitin-activating enzyme (E_1) in ts85 cells (Fig. 6), and the disappearance of uH2A semihistone from ts85 chromatin at the nonpermissive temperature, taken together, strongly suggest that the cytoplasmic and nuclear ubiquitin-protein ligase systems share the thermolabile E_1 enzyme. The ts85 mutant can thus be used to study nuclear functions of ubiquitin-containing proteins.

Proposed pathway for ATP-ubiquitin-dependent protein degradation. The observed relationships between conjugation of ubiquitin to proteins and ubiquitin participation in protein degradation (see above) have led to the scheme in which ubiquitin-protein conjugation is an intermediate step in the degradation of the substrate protein, and in which specific proteolytic enzymes would selectively recognize and degrade ubiquitin-protein conjugates [11]. It appeared likely that the binding of several molecules of ubiquitin to one molecule of the protein substrate produces a marked alteration in the conformation of the substrate protein and thus renders it highly suscepible to attack by such proteases. The proposed sequence of events in this process is shown in Fig. 8:

1. The initial activation of the glycine C-terminus of ubiquitin through formation of an acyl-adenylate [82,88,89], with subsequent transfer of the activated polypeptide to an $-SH$ group of the enzyme (E_1) to yield a thiolester linkage.

2. Transfer of the activated C-terminus of ubiquitin to ϵ-amino groups of lysine residues in the substrate protein [82,89]. This reaction is either partially processive

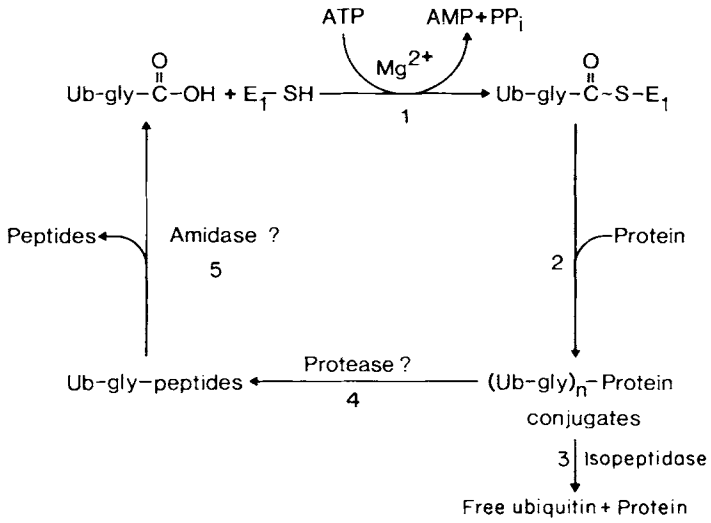

Fig. 8. Proposed pathway of ATP-ubiquitin-dependent protein degradation. See the text for details. 1. Activation of the carboxy-terminal glycine of ubiquitin and its binding to the activating enzyme (E_1). 2. Covalent conjugation of one or more molecules of activated ubiquitin to ϵ-NH_2 lysine groups in the substrate protein. 3. Regeneration of free ubiquitin and the protein substrate by isopeptidase. 4. Breakdown of the conjugates by an endoproteinase(s) acting on the protein substrate moiety. 5. Release of short peptides and free ubiquitin by a specific isopeptidase that cleaves the isopeptide linkage (adapted with modifications from Hershko and Ciechanover [11]).

or has a strong preference for ubiquitin-protein conjugates, since conjugates containing several ubiquitin moieties appear under conditions of excess of nonubiquitin protein substrates [63].

3. In addition, there may exist an isopeptidase (putatively a "correction" enzyme [63]) that would release ubiquitin conjugated to proteins and thus establish a "futile" cycle. The well-characterized isopeptidase that cleaves uH2A semihistone into H2A and free ubiquitin may well provide this more general putative activity [84,100,101].

4. Breakdown of the conjugates by an endoproteolytic attack on the protein substrate moiety, which would produce short peptides still attached to ubiquitin by the isopeptide linkage.

5. Small peptides and free (and reutilizable) ubiquitin may be released by the action of an amidase, presumably the isopeptidase discussed above.

The assumption that short peptides are the products of the ATP-dependent proteolytic system is based on the observations of Botbol and Scornik [56,57] that such peptides accumulate when aminopeptidase action is blocked by bestatin. Steps 4 and 5 are entirely hypothetical at present. It is known that ubiquitin-protein conjugates are broken down [62] with the release of free and reusable ubiquitin [63], but the intermediate reactions of the breakdown process have not yet been investigated. A valuable tool for such studies is hemin, which blocks the breakdown of ubiquitin-protein conjugates [102] and inhibits proteolysis [56]. While the proposed model is consistent with all the presently available data, it obviously requires much further

investigation. For example, the high-molecular-weight component of the system that is stabilized by ATP and some other adenine nucleotides [59] is not required for conjugate formation but appears to copurify with an activity that degrades ubiquitin-protein conjugates [E. Leshinsky, A. Ciechanover, H. Heller and A. Hershko, unpublished data].

An important physiological role for the ubiquitin-mediated proteolytic pathway in cell types other than reticulocytes is now emerging. Conjugation of ubiquitin with proteins has been observed in all rat tissues examined [S. Ferber and A. Hershko, personal communication]. Immunochemical analysis of ubiquitin-protein conjugates suggested that the ubiquitin pathway is involved in abnormal protein degradation in Ehrlich ascites tumor cells. Our recent identification of the mouse cell mutant ts85 as the ubiquitin conjugation mutant (see above) and the results of protein microinjection experiments by Rechsteiner and co-workers [93] (see also above) further support the proposed scheme (Fig. 8), and also show that the characteristics of the reticulocytic ubiquitin system are conserved among various mammalian cell types. The presence of ubiquitin in E coli has been reported by others [69]; however, conventional extracts of E coli are negative in ubiquitin conjugation; radioimmunoassays with mammalian ubiquitin and antiubiquitin serum are also negative with E coli extracts [11; D. Refaeli-Eshkol and A. Hershko, personal communication]. This is evidently an inconclusive result; yet it would not be surprising if the pathway of ubiquitin-mediated proteolysis is minor or nonexistent in prokaryotes, since an important role has been established recently for the proteinase La (a *lon* gene product in E coli [103]), which appears to function efficiently in the absence of ubiquitin [104] (see below).

Possible Significance of Nuclear Ubiquitin-Histone Conjugates

The function(s) of chromatin-associated ubiquitin-histone conjugates is unknown. Although early reports suggested that levels of nuclear uH2A decrease upon stimulation of transcription [105], the results could be also interpreted as an in vitro artefact due to increased activity of a specific isopeptidase that cleaves ubiquitin conjugates at the isopeptide bond, with the liberation of free ubiquitin [100,101]. More recent studies by Varshavsky and co-workers have used a new approach, two-dimensional hybridization mapping of nucleosomes, to compare the structure of mononucleosomes derived from transcribed and nontranscribed regions of the genome [78,79]. It was found that approximately one in two nucleosomes of the transcribed *copia* and heat-shock 70 (hsp70) genes in nonshocked Drosophila melanogaster cultured cells contains uH2A. In striking contrast, less than one in 25 nucleosomes of tandemly repeated, nontranscribed 1.688 satellite DNA contains uH2A, suggesting that most of the nucleosomal uH2A is located in transcribed or potentially transcribed genes [78,79]. Later studies on the distribution of ubiquitinated nucleosomes within the 31-kilobase-long mouse dihydrofolate reductase (DHFR) gene have revealed a striking enrichment in *doubly* ubiquitinated nucleosomes (in which both of the H2A molecules are replaced by uH2A) at the 5′ end of the DHFR gene as compared with the regions of the same gene downstream from the first ~400 base pairs (bp) of the DHFR gene [79] (J. Barsoum and A. Varshavsky, in preparation]. Another recent study showed that the level of nuclear uH2A is much higher in actively transcribing avian erythroid precursor cells than in transcriptionally inactive nucleated erythrocytes [106].

What are the structural consequences of nucleosomal ubiquitination? In the absence of additional modification, ubiquitinated nucleosomes in chromatin do not appear to be preferentially sensitive to staphylococcal nuclease [78,107]. Crystallographic and chemical cross-linking studies strongly suggest that the ubiquitin moiety of uH2A is located on the face of the octameric histone "disk" [108], near the area of histone H1-H2A contact in the H1-containing mononucleosome [109,110]. It is therefore conceivable that the presence of the ubiquitin on either one or both sides (in the doubly uH2A-modified nucleosome) of the disk would weaken or otherwise modify interactions of this nucleosome with either one or more of its neighbors. One possible functional correlate of this hypothetical role for uH2A is that at least local unfolding of higher-order chromosomal structures appears to be a necessary condition for transcription to occur.

The hsp70 genes in nonshocked Drosophila cells are digested by staphylococcal nuclease at a rate similar to, if not lower than, that for bulk chromatin [78]. Upon heat shock, a dramatic increase in the hsp70 transcription rate is observed [111], and this is accompanied (or shortly preceded) by conversion of preferentially ubiquitinated, relatively nuclease-insensitive nucleosomal structure of the hsp70 gene into a structure that is preferentially sensitive to staphylococcal nuclease and no longer possesses nucleosomal organization recognizable with this probe [78,79,111]. What is the structural basis for such a conversion? One interesting possibility is suggested by the proteolytic function of ubiquitin in the cytoplasm [11]. Levinger and Varshavsky [78,79] have previously suggested that this system may operate in the nucleus; proteolytic removal of nucleosomal proteins from the activated chromosomal region may be responsible for the striking changes in chromatin structure observed at very high rates of transcription.

One interpretation of the observed highly preferential ubiquitination of the first one to two nucleosomes in the 31-kilobase-long mouse DHFR gene (see above) is that the degree of ubiquitination in a given region of chromatin fiber may be related to the frequency of RNA polymerase passages through that region. This hypothesis predicts that a significant proportion of initiated RNA polymerase molecules terminate or stall within the first few hundred bp of the DHFR gene, analogous to other known cases of the eukaryotic transcription attenuation [112]. Another possibility is that the heavy ubiquitination of nucleosomes at the 5′ end of the DHFR gene is due to their location proximally to a nucleosome-free "exposed" region of chromatin, such as previously found near 5′ ends of other active genes in both viral and cellular chromosomes [79, and references therein]. It appears that such nucleosome-free structures are present only conditionally in chromatin, and that they can replace preexisting nucleosomal structures. To find ubiquitination directed specifically at or near exposed regions would perhaps suggest that the mechanism by which nucleosome removal may be accomplished involves ubiquitination or, rather, ubiquitin-mediated proteolysis. These hypotheses are now open to experimental testing.

Other ATP-Dependent Proteolytic Systems

In addition to the ATP-ubiquitin proteolytic pathway described in reticulocytes, different, apparently unrelated ATP-dependent proteolytic pathways have been identified in other biological systems, notably in E coli.

Goldberg and co-workers showed that in soluble extracts of E coli, ATP stimulates the breakdown of a nonsense fragment of β-galactosidase and the degradation of [^{14}C]-methyl-globin to acid-soluble material [113,114]. The corresponding purified enzyme, designated proteinase La, has an essentially complete requirement for ATP and Mg^{+2} [115,116]. Nonmetabolizable ATP analogs *cannot* substitute for ATP [116]. More recent work has strongly suggested that proteinase La is identical to the product of the *lon* gene in E coli [104,117,118]. Mutations in the *lon* gene have no effect on the degradation of most normal E coli proteins [3,119,120], but reduce the rate of degradation of nonsense polypeptides [119], missense proteins [103], puromycyl-peptides [2,121] and canavanine-containing abnormal proteins [121]. However, in none of these cases was the process completely inhibited by any of the *lon* mutations; only a partial inhibition of the breakdown of abnormal proteins is consistently observed. The purified *lon* gene product has an ATP-dependent protease activity [118]. Comparison of its properties with protease La showed striking similarity with regard to both nucleotide cofactor and substrate specificities, susceptibility to inhibitors, thermostability, and apparent molecular weight [104,117], strongly suggesting that La is the *lon* gene product. ATP and protein hydrosysis by proteinase La shows the same K_m for ATP (30–40 μM) and are affected similarly by various inhibitors (such as vanadate and serine protease inhibitors [116,122]). ATP hydrolysis by La is stimulated by polypeptides that are substrates for this proteinase, but not by nonsubstrate proteins [122]. Chung and Goldberg showed that both double-stranded and single-stranded DNA stimulate ATP-dependent proteolysis and protein-dependent ATPase activity [123]. They suggested that the association of La proteinase with DNA, and modulation by the DNA of the La activity may play an important role in the regulation of protein turnover in E. coli [see, however, 124,125].

Another well-characterized intracellular proteinase is the *recA* gene product which plays an important role in the SOS repair system of E coli [reviewed in 126]. The *recA* protease is involved in the proteolytic inactivation of the lambda phage repressor and of the *lexA* gene product under conditions leading to an error-prone (SOS) repair response. Roberts and co-workers [127] have found that in cells treated with mitomycin C, the lambda phage repressor (which is an inhibitor of phage genes required for lytic growth, and which is inactivated following treatments affecting DNA, such as mitomycin C and UV irradiation) is cleaved to small fragments. This (irreversible) proteolytic mechanism for repressor inactivation differs fundamentally from the classical case in which induction occurs by the reversible binding of a small molecule to a repressor protein. Purified *recA* protein cleaved the purified repressor in vitro only in the presence of single-stranded DNA or a variety of polynucleotides [128,129]. It was suggested that the formation of a ternary complex between *recA* protein, a polynucleotide and ATP is necessary for activation of the proteolytic function of the enzyme [129–131]. The proteinase function of *recA* protein is involved in the induction of some, if not all of the branches of the SOS response, since the purified *recA* protein cleaves also the *lexA* protein, which is a transcriptional repressor, operating at many loci known to be SOS-inducible, including that of *recA* itself [126,132]. The activation of the proteolytic function of *recA* protein by single-stranded DNA in vitro may represent the physiological mechanism that triggers the expression of SOS functions; it is possible that damage to DNA may expose regions of single-stranded DNA, liberate DNA fragments, or induce synthesis of unusual nucleotides

which may activate the *recA* proteinase activity [129,131]. The exact role of ATP in promoting this activation, and the putative conformational change in the *recA* protein that activates its proteolytic function, remain to be elucidated.

The *lon* and *recA* gene products have several common features. Both are DNA-binding proteins [104,117,133], and both have an ATPase activity that is stimulated by DNA and other polynucleotides [113,118,129]. Proteinase activity of both enzymes is also enhanced by bound DNA [123,126]. It would be interesting to find whether comparable eukaryotic systems share this property. A notable difference between *lon* and *recA* proteins is that, while ATP hydrolysis does not appear to be essential for the *proteolytic* action of *recA* protein, and ATP can be substituted with its nonhydrolyzable analog, adenosine-5′-[γ-thio]-triphosphate (ATP-γ-S) [129], hydrolysis of ATP is essential for the *lon* (La) proteinase activity [104,116–118]; the ATPase activity of the *lon* proteinase is increased in the presence of its protein substrates [116,118,134].

Recently, Desautels and Goldberg [135] described an ATP-dependent, vanadate-sensitive endoproteinase isolated from the matrix of rat liver mitochondria; this enzyme closely resembles the proteinase La from E coli. Both enzymes are endoproteinases that are sensitive to diisopropyl fluorophosphate, phenylmethylsulfonyl fluoride, vanadate, and thiol-blocking agents. They show similar substrate specificities, K_m for ATP and similar molecular weights. Since mitochondria apparently evolved from prokaryotic symbionts of eukaryotic cells, the many similarities between these two proteinases may therefore reflect common ancestry rather than convergent evolution.

Goldberg et al [136–138] and Rose et al [139] have independently described a high molecular weight proteinase from liver and other tissues that appeared to be stimulated by ATP. However, the enzyme is also stimulated by other polyanions such as pyrophosphate [136], creatine phosphate or citrate [139] and does not require Mg^{+2} [136]. The effect of the polyanions appears to be due to the stabilization of the enzyme during the assay at 37°C; at lower temperatures (at which the enzyme is stable), no effects of ATP or other polyanions could be observed [139]. Following several steps of purification, the apparent stimulation by ATP is still only partial [136,139]. This behavior seems distinguishable from the specific and absolute ATP dependence of the ubiquitin-mediated proteolytic pathway and of the *lon* and *recA* proteinases. Nonetheless, the participation of this proteinase in intracellular protein degradation remains a possibility to be explored.

Recently it was reported from the laboratories of Goldberg [137,140], Rechsteiner [93,141], and Kulka [142] that proteins in which the ε-amino groups of lysine residues are chemically blocked still undergo ATP-stimulated proteolysis both in vitro and in vivo. The process is sensitive to hemin [93,140], similarly to both the ubiquitin-mediated pathway [102] and the intracellular protein degradation in general [56]. These data may suggest that there exists another ATP-dependent proteolytic pathway distinct from the ubiquitin-mediated one; however, all these experiments were carried out in systems containing ubiquitin. It is quite possible that ubiquitin may signal proteolysis by conjugation to other free amino groups such as that of asparagine [143], or to free hydroxyl groups of serine, threonine, or tyrosine. Alternatively, cells and crude extracts may contain a specific protein repair activity that regenerates free ε-amino groups of lysine in modified proteins and thereby converts them into substrates of the ubiquitin-mediated proteolytic pathway. The above observations are therefore insufficient for establishing the existence of ATP-dependent, ubiquitin-

independent proteolytic system in eukaryotes; more detailed and direct analyses are required to address this point.

What features of the protein structure determine the specific recognition of a protein by ATP-dependent degradative systems? One interesting possibility is that specific, not necessarily contigious, amino acid sequences in proteins serve as both "recognition" and "regulatory" signals that determine half-lives of normal proteins that are degraded via the ATP-dependent, ubiquitin-dependent proteolytic pathway. An obvious potential analogy is the already characterized system of "signal" amino acid sequences that governs compartmentalization of cellular proteins [144]. The existence of "recognizable" amino acid sequences for intracellular proteolysis has recently been suggested by Dice and co-workers [145] from studies on the degradation of RNase A. On the other hand, systems which preferentially degrade abnormal proteins (eg, the ubiquitin system and the *lon* proteinase) are by definition highly sensitive to substrate conformation, so that only a more complex version of the above hypothesis may be feasible. For example, an internal "signal" sequence could be exposed upon protein denaturation.

CONCLUDING REMARKS

Although our understanding of the mechanisms of intracellular protein degradation has increased significantly in the last few years, a number of important questions remain.

For the lysosomal pathway, a major problem at hand is to identify the mechanisms that regulate formation and regression of autophagic vacuoles. It also remains to be determined whether lysosomes contribute significantly to *selective* protein degradation in nonstressed cells.

The physiological roles of the nonlysosomal ATP-dependent proteolytic systems are presently defined only in broad outlines. In addition, mechanisms that determine the remarkably high selectivity of the intracellular protein degradation remain unknown. For the ubiquitin-mediated, ATP-dependent pathway, extensive evidence discussed above strongly suggests a crucial importance of this pathway for the degradation of abnormal proteins, and apparently also for general nonlysosomal protein turnover in eukaryotic cells. Further analysis of this pathway will concentrate on the detailed characterization of its enzymatic and structural components, and also on a more precise definition of its physiological roles. We have also briefly considered some of the additional, hypothetical functions of the ubiquitin pathway, in particular, its possible involvement in regulation of gene expression via locus-specific, ubiquitin-mediated proteolysis of chromosomal proteins.

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