Natl. Acad. Sci. U.S.A. 78, 838.
Schimmel, P. R., & Söll, D. (1979) *Annu. Rev. Biochem. 48*, 601.
Söll, D., & Schimmel, P. R. (1974) *Enzymes, 3rd Ed. 10*, 489.
Spector, L. B. (1982) *Covalent Catalysis by Enzymes*, p 202, Springer-Verlag, New York.
Zamecnik, P. C. (1983) *Anal. Biochem. 134*, 1.
Zamecnik, P. C., Stephenson, M. L., Janeway, C. M., & Randerath, K. (1966) *Biochem. Biophys. Res. Commun. 24*, 91.
Zamecnik, P. C., Januvay, C. M., Randerath, K., & Stephenson, M. L. (1967) *BBA Libr. 10*, 169.
Zamecnik, P. C., Rapaport, E., & Baril, E. F. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 1791.

Ubiquitin-Dependent Proteolysis of Native and Alkylated Bovine Serum Albumin: Effects of Protein Structure and ATP Concentration on Selectivity†

Anthony C. Evans, Jr., and Keith D. Wilkinson*
Department of Biochemistry, Emory University School of Medicine, Atlanta, Georgia 30322
Received August 7, 1984

ABSTRACT: The susceptibility of bovine serum albumin to degradation by the ubiquitin-dependent system of proteolysis depends on the severity of the iodination conditions [Wilkinson, K. D., & Audhya, T. K. (1981) *J. Biol. Chem. 256*, 9235–9241]. To evaluate if other modifications of the protein changed its susceptibility to degradation, chemically modified derivatives of bovine serum albumin have been synthesized, characterized, and tested as substrates for the ubiquitin-dependent system. Serum albumin was reduced or reduced and alkylated with iodoacetic acid or iodoacetamide. Only the alkylated derivatives exhibit saturation kinetics. Both alkylated proteins competitively inhibit the degradation of the other. These substrates are useful for assay of the intact proteolysis system in crude extracts and in assays for other substrates using competitive alternate substrate inhibition. The physical properties of these proteins suggest that charge, denaturation, or aggregation is not correlated with the degradation rate of these proteins by this system. However, the selectivity of the ubiquitin-dependent proteolysis depends strongly on the ATP concentration. At saturating substrate concentrations, both alkylated substrates are degraded equally. At low ATP concentrations, there is a 2.4-fold difference in the degradation rates of the alkylated proteins. The results presented here indicate that the ubiquitin-dependent protein degradation system is selective and responsive to ATP concentrations and that not all abnormal proteins are equally preferred substrates. Thus, the system may be more selective than previously thought.

The rate of cellular protein turnover varies markedly, depending upon the protein being degraded. The highly selective nature of this process is presumed to arise from the cell's recognition of certain physical and chemical properties of each particular protein (Goldberg & Dice, 1974; Goldberg & St. John, 1976; Hershko & Ciechanover, 1982). For instance, large proteins generally possess shorter half-lives than small ones (Dehlinger & Schimke, 1972; Glass & Doyle, 1972; Dice et al., 1973; Dice & Goldberg, 1975). Also acidic polypeptides are degraded faster than neutral or basic ones (Dice & Goldberg, 1975; Dice et al., 1979; Duncan et al., 1980), indicating that the isoelectric point is related to the half-lives of cellular proteins. Moreover, several proteins which are turned over rapidly in vivo show an increased susceptibility to proteolytic attack by neutral or acidic proteases in vitro (Dice et al., 1973; Goldberg, 1972; Bohley et al., 1972; Segal et al., 1974; Dean, 1975a). Hydrophobicity has also been cited as a property of proteins which leads to an increased rate of breakdown (Dean, 1975b; Segal et al., 1976; Bohley & Reimann, 1977).

Although the distinct pathways by which various classes of proteins are degraded in vivo are not clear, available evidence indicates that, in general, degradation of "long-lived" proteins occurs by lysosomal autophagy, especially in times of nutritional deprivation (Mortimore et al., 1978). In contrast, abnormal proteins produced by the incorporation of amino acid analogues or puromycin are degraded with extreme rapidity by cells, mainly through energy-requiring nonlysosomal systems (Mortimore et al., 1978; Knowles & Ballard, 1976; Neff et al., 1979). Furthermore, it has been shown that most, if not all, cells require ATP for sustained protein catabolism. This energy requirement does not appear to be related to lysosomal integrity, since even cells without lysosomes exhibit the ATP requirement (Goldberg & St. John, 1976). Since proeolysis is an exergonic process, this energy requirement is suspected to impart selectivity to the proteolytic process.

One energy-dependent protein degradation system, which degrades abnormal globins in cell-free extracts of rabbit reticulocytes, has been shown to require ATP (Etlinger & Goldberg, 1977) and a small (8500 daltons) polypeptide cofactor (Ciechanover et al., 1978; Hershko et al., 1979; Ciechanover et al., 1980) identified as ubiquitin (Wilkinson et al., 1980; Wilkinson & Audhya, 1981). In this system, ubiquitin is coupled to the ϵ-amino groups of lysine residues in the substrate protein via an ATP-coupled condensation reaction requiring no less than three separate proteins (Ciechanover et al., 1981; Haas et al., 1982; Haas & Rose, 1982; Hershko et al., 1983). It is presumed that soluble proteases recognize these conjugates and degrade the attached protein (Hershko

† This work was supported by Grant GM30308 from the National Institutes of Health and a grant from the Emory University Research Committee. Portions of this work were submitted by A.C.E. in partial fulfillment of the requirements for the M.S. degree.

0006-2960/85/0424-2915$01.50/0

et al., 1980; Chin et al., 1982).

One of the major unanswered questions about this system, the selectivity of proteolysis, has not been defined. Early work with the partially purified system showed that several amino acid analogue containing reticulocyte proteins and puromycyl polypeptides were degraded by the soluble, cell-free extracts (Etlinger & Goldberg, 1977); however, no kinetic data were reported. More recently, a number of enzymes were examined as possible in vivo substrates for this system (Saus et al., 1982). None of the enzymes tested were hydrolyzed to any significant extent by the ubiquitin-dependent system. Fibroblasts have also been shown to use this system to degrade globin microinjected into the cells and subsequently denatured (Chin et al., 1982).

We have reported (Wilkinson & Audhya, 1981; Wilkinson & Evans, 1983) that iodination of bovine serum albumin (BSA)[1] with the enzymatic technique produced a protein with very little susceptibility to degradation by this system. More severe iodination conditions appear to damage the protein and result in a much better substrate. Thus, BSA can be converted to a substrate by modifying its structure. Unfortunately, the modifications introduced by chemical iodination are not easily defined. These results suggested that it would be instructive to chemically modify BSA, characterize the derivatives, and examine the effect of the modification on the susceptibility to degradation by this system. This amounts to a model study in which specific features of a protein's structure are varied in order to deterine which general features of proteins structure are recognized by the system. It is obvious that the overall selectivity of this complex system is due to a complex interplay of the selectivity of the individual enzymes involved. We can, however, begin to ask questions about the effective selectivity of degradation by examining the rates of degradation of different proteins by this system. The present study demonstrates the usefulness of that approach and expands upon our preliminary report of the preparation of substrates which are rapidly degraded in a kinetically interpretable fashion (Wilkinson & Evans, 1983).

Experimental Procedures

Materials. Ubiquitin was isolated as described earlier (Wilkinson & Audhya, 1981). Fraction II was purified on DEAE-cellulose by the method described previously (Ciechanover et al., 1978; Hershko et al., 1979). Reticulocyte-rich (>80%) whole rabbit blood and bovine serum albumin antiserum (developed in rabbit) were obtained from Pel-Freez Biologicals, Rogers, AK. Carrier-free $Na^{125}I$ was purchased from New England Nuclear. Bovine serum albumin (Cohn fraction V), creatine phosphokinase, and hexokinase were from Sigma Chemical Co., St. Louis, MO. Enzymobead iodination reagent was from Bio-Rad Laboratories, Richmond, CA. Sephacryl S-300 was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. All other chemicals were from commercial sources.

Chemical Modifications. BSA, 67.5 mg in 7.5 mL of 50 mM Tris-HCl, pH 7.6, was incubated with 7.5 mL of 0.1 M DL-dithiothreitol to reduce the disulfide bonds to free sulfhydryl groups. After 1 h at 37 °C, 1.10 mL of either 1.35 M iodoacetate (equimolar amounts of iodoacetic acid and sodium hydroxide) or iodoacetamide was added to alkylate the protein. This step was omitted in the preparation of reduced BSA. After incubation for 1 h at 37 °C, the reaction mixture was dialyzed extensively against 50 mM Tris-HCl, pH 7.6, at 4 °C in the preparation of the reduced (rBSA) and the reduced carboxymethylated derivatives of BSA (rcmBSA). The reduced carboxamidomethylated derivative (rcaBSA) was dialzyed at 4 °C vs. water adjusted to pH 7.6 with 1.0 M Tris base. Carboxyl groups were modified with a water-soluble carbodiimide [1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide] in the presence of ethanolamine, ethylenediamine, or taurine as the nucleophile (Hoare & Koshland, 1967). Final protein concentrations were measured by the procedure of Lowry et al. (1951).

BSA was iodinated by using either the chloramine-T method or the lactoperoxidase technique as described previously (Wilkinson & Audhya, 1981), and ubiquitin was iodinated by using the chlormaine-T method (Ciechanover et al., 1980). Radiolabeled derivatives of BSA were prepared from ^{125}I-BSA (10^4–10^5 cpm/μg) by using the same protocol described above appropriately scaled to the amount of BSA being derivatized.

Protein Degradation Assays. The ability of each derivative to act as a substrate for ATP–ubiquitin-dependent proteolysis at rate-limiting levels of ATP was assessed by using the following assay: A final volume of 0.06 mL contained 6 μg of ubiquitin, 100–150 μg of fraction II proteins, radiolabeled substrates at final concentrations ranging from 2 to 200 μg/mL, 0.3 unit creatine phosphokinase, 50 mM Tris-HCl, pH 7.6, 1 mM magnesium chloride, 0.4 mM ATP, 0.4 mM DTT, and 2 mM phosphocreatine. Assays under saturating ATP concentrations were the same except that the final concentrations of magnesium chloride, ATP, and phosphocreatine were 5-fold higher. After a 2-h incubation at 37 °C, the assay was stopped and analyzed for acid-soluble products as described before (Wilkinson & Audhya, 1981). Acid-soluble radioactivity in control incubations which lacked ubiquitin or contained 0.05 mM 2-deoxy-D-glucose and 2 units of hexokinase instead of ATP and creatine phosphokinase was also measured. Both controls gave similar values for nonspecific proteolysis (less than 1% hydrolysis per hour). The results were expressed as micrograms of substrate converted to acid-soluble material per 2 h per milligram of fraction II protein. It should be noted that there was a 20% difference in the specific activity of the fraction II proteins from different preparations. For this reason, all kinetic data for each set of determinations (high and low ATP levels) were obtained from a single preparation. The same pattern and magnitude of kinetic values were observed in all preparations.

Degradation as a function of ubiquitin concentration was measured for the rcmBSA and rcaBSA derivatives as described above exept that all assays contained 130 μg/mL substrate and ubiquitin at final concentrations ranging from 0 to 62.5 μg/mL.

Electrophoretic Methods. Formation of covalent conjugates was measured by a modification of the protein degradation assays described above. Labeled ubiquitin (1 μg, 1.56×10^5 cpm/μg) was incubated with the unlabeled substrates (rcmBSA or rcaBSA) at concentrations ranging from 0 to 300 μg/mL. This assay was stopped at 30 min, and the mixture was subjected to SDS–polyacrylamide gel electrophoresis and autoradiography as described previously (Ciechanover et al., 1980).

Protein Conformation Studies. Secondary structures of the BSA derivatives were examined by using circular dichorism spectroscopy (Jasco, Model J-20). The proteins were in 50 mM Tris–HCl, pH 7.6, at a concentration of 0.4 mg/mL. All

[1] Abbreviations: BSA, bovine serum albumin; rBSA, reduced BSA; rcaBSA, reduced carboxamidomethylated BSA; rcmBSA, reduced carboxymethylated BSA; DTT, DL-dithiothreitol; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; SDS, sodium dodecyl sulfate; kDa, kilodalton(s).

spectra were taken at 25 °C. The path length was 0.1 cm, and the wavelength range scanned was 200–265 nm. The calculation of secondary structure was done by using the method of Greenfield & Fasman (1969). Differential corrected emission spectra were measured for each derivative as well as for native BSA by using a Perkin-Elmer MPF 44N spectrophotofluorometer in the pH range 3–7 (using sodium acetate buffers at a constant ionic strength of 0.15 M). The exciting wavelength used was 275 nm, and the emission was monitored between 290 and 400 nm. Gel permeation chromatography was performed on the native and derivatized BSA with Sepharcryl S-300 Superfine in a 1 cm × 60 cm column. The column was eluted with 50 mM Tris-HCl, pH 7.6, at a flow rate of 25 mL cm^{-1} h^{-1}. The column was calibrated with ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (140 kDa), catalase (250 kDa), xanthine oxidase (300 kDa), and ferritin (800 kDa). The void and included volumes were determined by using blue dextran and DTT, respectively.

Other Methods. High-performance liquid chromatography of the BSA derivatives was performed by using a 5-μm C-8 reverse-phase column (IBM Instruments). Solvent A was 100 mM sodium perchlorate and 1 mM sodium phosphate, pH 7.0, and solvent B was acetonitrile. Elution was with a linear gradient with a flow rate of 1.0 mL/min. Double diffusion of BSA and BSA derivatives vs. anti-BSA antibody (developed in rabbit) was performed by Ouchterlony's method (Ouchterlony, 1958). The well volume was 5 μL, and the distance between the wells was 1 cm.

Amino acid analysis were done by Dr. Ray Shapira, Department of Biochemistry, Emory University School of Medicine.

Results

Several derivatives of BSA have been synthesized and tested for their ability to be substrates for the ubiquitin-dependent protein degradation system. Three of these were characterized in detail: reduced BSA (rBSA), reduced carboxymethylated BSA (rcmBSA), and reduced carboxamidomethyled BSA (rcaBSA). Native BSA is a poor substrate in the ubiquitin-dependent system (Wilkinson & Audhya, 1981). As shown by the kinetic data below, the poor substrate (native BSA) can be converted by chemical modification into two proteins which are better substrates (rcmBSA and rcaBSA) and one which is not (rBSA). Thus, in order to elucidate the parameters involved in substrate selection by the ubiquitin-dependent system, the chemical and physical properties of each derivative were examined.

Chemical and Physical Characterization of the Substrates. Since the kinetic data were collected with protein which had been radioiodinated, and the chemical and physical data with protein which had not, it was necessary to show that these two different procedures produced kinetically equivalent protein derivatives. The radioiodinated rcmBSA and rcaBSA are kinetically identical with their unlabeled analogues as demonstrated by mixing experiments (data not shown). Identical kinetic constants are obtained when the radiolabeled rcmBSA and rcaBSA are used as substrates or when the labeled substrates are diluted with the unlabeled derivatives. Thus, the iodination conditions used do not change the kinetic behavior of the proteins, and the radiolabeled proteins are valid kinetic tracers in the kinetic measurements.

Carboxymethylation and carboxamidomethylation take place via nucleophilic substitution on the iodoacetate or iodoacetamide by the free sulfhydryl, lysyl, histinyl, or methionyl side chains on the reduced BSA (Goren et al., 1968). At the pH at which the reaction was carried out (pH 7.6), substitution is primarily specific for sulfhydryl groups. Native BSA contains 1 cysteine and 17 cystine residues (Peters & Hawn, 1967). Amino acid analysis indicated that 25 cysteine residues had been modified in the rcmBSA and 21 were modified in the rcaBSA (Table I). No other modified amino acids were detected. The cysteine residues of rBSA were recovered as half-cystine and cysteic acid. Since no attempt was made to prevent reoxidation of the residues during dialysis, it is probable that some intermolecular disulfides have formed with this derivative.

Table I: Physical Properties of BSA and BSA Derivatives

property	BSA	rBSA	rcaBSA	rcmBSA
% helix[a]	15	10	10	10
% sheet[a]	65	20	20	20
aggregated[b]	no	yes	no	no
p*I*	4.9[c]	ca. 5[d]	ca. 5[d]	ca. 4.4[d]
substitution[e] (mol/mol of protein)	0	0	21	25
hydrophobicity		highest	intermediate	lowest

[a] Estimated to nearest 5% (Greenfield & Fasman, 1969). [b] Measured by gel filtration; see text. [c] Teale (1960). [d] Estimated. [e] Determined as *S*-(carboxymethyl)cysteine.

Because the ubiquitin-dependent system has been implicated in the degradation of proteins with grossly denatured structures (Etlinger & Goldberg, 1977; Chin et al., 1982; Ciechanover et al., 1980; Goldberg & Boches, 1982; Hershko et al., 1982), it was important that the secondary structures of the derivatives be examined. The circular dichroism of BSA and the BSA derivatives be examined. The circular dichroism of BSA and the BSA derivatives was measured in 50 mM Tris-HCl, pH 7.6. Table I gives a summary of the estimated percentages of α-helix, β-structure, and random coil in these proteins. It can be seen that native BSA possesses approximately 80% organized secondary structure; however, the derivatives show a large decrease in β-sheet content, with little change in the amount of helix. Ouchterlony double-diffusion plates revealed that there was precipitation of the native BSA by anti-BSA antibody; however, there was no detectable cross-reactivity with any of the BSA derivatives, indicating that antigenic determinants are disrupted when the native protein is reduced.

Half-lives of soluble liver proteins have been correlated to their susceptibility to acid denaturation (Goldberg & St. John, 1976; Bond, 1976), with the most labile showing faster degradation rates. Since the circular dichroism spectra were indicative of little optically active secondary structure, fluorescence spectroscopy was used to study the aromatic environment of BSA and BSA derivatives in the pH range 3–7. The exciting wavelength used (275 nm) excites both tyrosine and tryptophan residues. Tyrosine and tryptophan fluorescence was measured at the emission wavelengths of 305 and 335 nm for the respective residues. Figure 1 gives a comparison of the ratios of tyrosine to tryptophan fluorescence of each of the proteins as a function of pH. All of the BSA derivatives showed a generally higher tyrosine to tryptophan fluorescence ratio comapred to the native BSA. This was the result of both increased tyrosine and quenched tryptophan fluorescence in the derivatives. Examination of Figure 1 reveals that the aromatic environments of the BSA derivatives are differentially altered in the range pH 3–5.5. However, between pH 5.5 and pH 7, all of the derivatives exhibit very similar tyrosine to tryptophan fluorescence ratios.

It has been previously reported that in reticulocytes, nearly all abnormal proteins which were degraded were found in large aggregates prior to their rapid hydrolysis (Goldberg et al.,

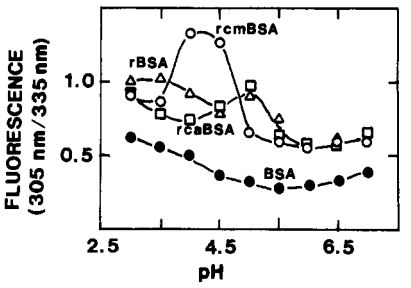

FIGURE 1: Acid denaturation of BSA and BSA derivatives monitored by fluorescence. The ratio of tyrosine (305 nm) and tryptophan (335 nm) fluorescence was determined at the indicated pH values. Details are given in the text. Values are given for BSA (closed circles), rBSA (open triangles), rcaBSA (open squares), and rcmBSA (open circles).

1978). This aggregation was presumed to arise from the exposure of hydrophobic residues of the abnormal proteins. To look at the possibility that BSA molecules become aggregated in their modified state, gel filtration chromatography was performed on the native BSA and BSA derivatives (Table I). The results suggest that native BSA, rcaBSA, and rcmBSA all exist in monomeric form with a Stokes radius consistent with an approximate globular shape. In contrast, rBSA eluted in the void volume, indicating that rBSA molecules are either present as a random coil or associated in the form of high molecular weight aggregates. The latter is most probable, since no reductant was present after dialysis of the protein.

It was observed that rcmBSA exhibits an earlier elution time upon reverse-phase chromatography than the other derivatives (data not shown). This would be consistent with the expectation that introduction of charged carboxyl groups to the protein structure would decrease its hydrophobicity. Both rBSA and rcaBSA showed similar, slightly longer retention times. Thus, it appears that the reduced derivative would be in an aggregated state, since unfolding to the random coil would be expected to expose considerably more hydrophobicity than exposed in the globular structures assumed by the alkylated derivative. This suggests that the order of hydrophobicity of these proteins is rcmBSA < rcaBSA = BSA < rBSA (Table I).

Effects of Chemical Modification on Degradation Rates. (*A*) *Effects of Iodination Conditions.* When BSA is radiolabeled by using the relatively harsh oxidizing conditions of the chloramine-T method (Ciechanover, 1980), it shows an enhanced ability to be degraded compared to the BSA iodinated by using the lactoperoxidase technique (Wilkinson & Audhya, 1981). Figure 2 compares the extent of breakdown observed when chemically or enzymatically iodinated BSA is used as substrate in the ubiquitin-dependent proteolytic system. Both preparations are iodinated to similar extents, i.e., approximately 10^6 cpm/mg. These procedures are both fairly specific for tyrosine residues in protein. The dashed lines show that about 2% of the total radioactivity is soluble in trichloroacetic acid in the absence of fraction II enzymes. Upon addition of fraction II enzymes (open symbols), an increase in soluble radioactivity is observed. This has been attributed to the "nonspecific" proteases in fraction II. Finally, upon addition of ubiquitin and fraction II enzymes (closed symbols), a further increment of degradation is observed. This increment corresponds to the "ubiquitin-dependent" rate. The ubiquitin-dependent rate for the chemically iodinated BSA is only about twice the rate attributable to nonspecific proteolysis. Furthermore, saturation behavior is not observed, and the measured rates of proteolysis vary widely with different preparations of iodinated BSA. When BSA is iodinated enzymatically, it is hydrolyzed to a lesser extent by both the nonspecific and ubiquitin-dependent systems.

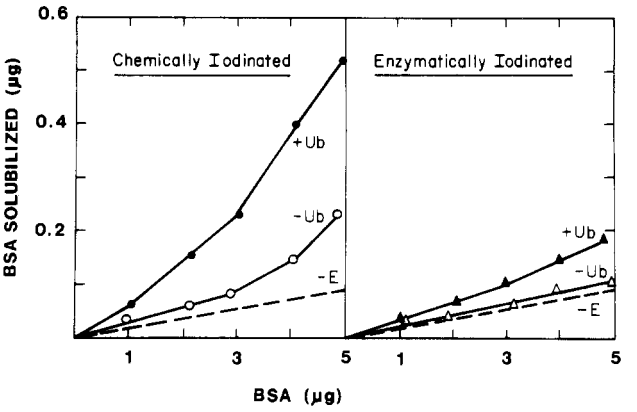

FIGURE 2: Concentration dependence of the degradation of ^{125}I-BSA labeled by the chloramine-T method (circles) and the lactoperoxidase method (triangles). The amount of BSA degraded in a 2-h incubation is plotted vs. the total BSA in the assay. Conditions are given in the text. The dashed line represents results obtained in the absence of uqiquitin and fraction II prtoeins. The open symbols were obtained in the presence of fraction II proteins but in the absence of ubiquitin and the closed symbols in the presence of the complete system.

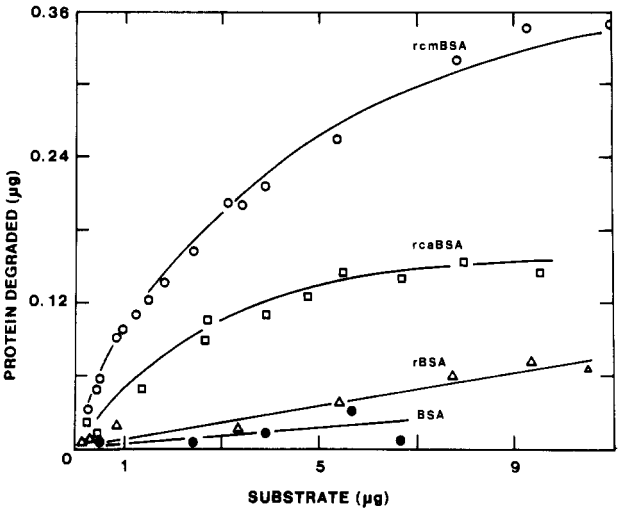

FIGURE 3: Concentration dependence of the degradation of BSA and BSA derivatives. The amount of protein degraded is plotted vs. the total amount in the assay under conditions of limiting ATP concentration. Details are given in the text. The proteins were BSA (closed circles), rBSA (open triangles), rcaBSA (open squares), and rcmBSA (open circles).

(*B*) *Effects of Reduction and Alkylation.* Figure 3 shows the amount of substrate converted to acid-soluble material during a 2-h incubation as a function of the total amount of substrate present. It can be seen that both rcmBSA and rcaBSA show simple saturation kinetics while the native BSA and rBSA do not show saturation behavior in the range tested. From the initial slopes of such plots, it can be estimated in the range tested. From the initial slopes of such plots, it can be estimated the apparent second-order rate constant for degradation of these proteins is increased by a factor of about 100 upon alkylation of the reduced BSA.

The products of these reactions are similar in all cases studied. That is, no intermediates of significant size are observed during the degradation of ^{125}I-proteins when examined on SDS gel electrophoresis. Thus, the kinetic event being observed corresponds to the net degradation of BSA and its derivatives to small peptides or free amino acids which are soluble in trichloroacetic acid. Additionally, longer incubations lead to the degradation of virtually all of the labeled substrate

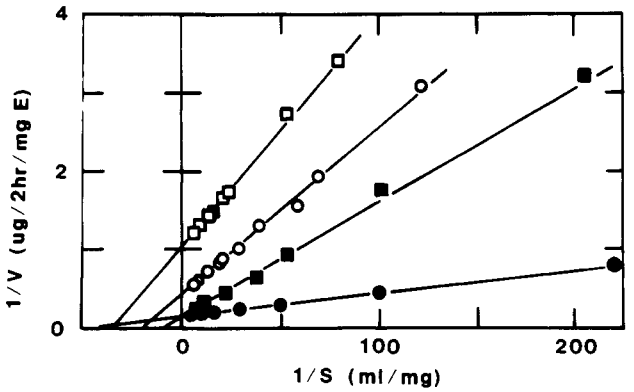

FIGURE 4: Double-reciprocal plots of the rate of degradation of BSA derivatives at high and low ATP concentrations. Rates of degradation of rcaBSA (squares) and rcmBSA (circles) were determined at 2.0 mM ATP (closed symbols) and at 0.4 mM ATP (open symbols) as described in the text.

present, indicating that there is not a subpopulation of labeled moleucles which are preferentially degraded by the system. Both of the above arguments suggest that these are kinetically homogeneous substrates undergoing comparable reactions.

(*C*) *Effects of Altering the Net Charge.* The above results would suggest that the charge on the protein may be a significant contributor to the selectivity of degradation. If that is true, it should be possible to change the net charge on the protein by other chemical reactions and demonstrate the importance of charge. Therefore, three other BSA derivatives were synthesized and tested as substrates (data not shown). Native BSA was reacted with 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide and either ethanolamine, ethylenediamine, or taurine (Hoare & Koshland, 1967). These modifications will replace the negative charge of the carboxyl groups with a zero (ethanolamine), positive (ethylenediamine), or a negative (taurine) charge. All of these derivatives are degraded at significant rates by fraction II in the absence of ubiquitin; however, none of these BSA derivatives are degraded by the ubiquitin-dependent pathway. Thus, it is not primarily charge which targets a protein for ubiquitin-dependent degradation.

Kinetic Studies on Alkylated BSA Derivatives. It has been observed that the cellular ATP concentration must be dropped by about 85–90% before all intracellular proteolysis ceases (Goldberg & St. John, 1976). It is also known that the specificity of protein degradation changes markedly during stress such as starvation (Goldberg & St. John, 1976; Dice et al., 1978). Thus, it seemed important to examine the substrate selectivity under sufficient and limiting ATP concentrations. Because the preparation of enzymes (fraction II) used contains ATPase activity and an ATP regeneration system is added to the assay, it is difficult to specify the exact concentration of ATP during the assay. Therefore, protein degradation assays will be referred to as containing ATP in "high" (2.0 mM) or "low" (0.4 mM) concentrations.

Figure 4 gives the double-reciprocal plots of the degradation rates of rcmBSA and rcaBSA derivatives at two different concentrations of ATP. Both of these derivatives show simple saturable kinetic behavior. Since we observe simple saturation behavior for these substrates, we can calculate apparent kinetic constants describing this behavior. These apparent kinetic constants for the various derivatives are given in Table II.

Assays were done to determine the dependence of rcmBSA and rcaBSA degradation rate on ubiquitin concentration. At low ATP concentrations, the apparent K_m for ubiquitin is identical for both rcmBSA and rcaBSA and is equal to 9.0 μg/mL (Table II). At high ATP concentrations, an interesting characteristic of the ubiquitin dependence of the rcmBSA degradation rate is the fact that the double-reciprocal plot is concave upward (Figure 5A). This result could be explained if the conjugation reaction has a strong preference for rcmBSA molecules already conjugated to ubiquitin. This affinity for preexisting conjugates in the subsequent addition of ubiquitin ligands to protein substrates has been observed, even under conditions of excess substrate (Hershko et al., 1980). The corresponding plot for degradation of rcaBSA (Figure 5B) does not show this curvature. The apparent K_m for ubiquitin from the linear portion of the curve for rcmBSA is 5.4 μg/mL and that for rcaBSA is 4.5 μg/mL. The similarlity of these apparent kinetic constants suggests that the degradation of both proteins requires ubiquitin and shows the same concentration dependence.

Table II: Summary of Kinetic Constants for BSA Derivatives[a]

constant	low [ATP]		high [ATP]	
	rcaBSA	rcmBSA	rcaBSA	rcmBSA
V_{max} [μg (2 h)$^{-1}$ (mg of E)$^{-1}$]	1.0	2.4	6.7	6.5
K_m (μg/mL)	29	50	83	18
K_i (μg/mL)	370	80	40	25
$(V_{max}/K_m) \times 10^{-3}$	35	48	80	361
K_i/K_m	12.8	1.7	0.5	1.4
K_m(ubiquitin) (μg/mL)	9.0	9.0	4.5	5.4

[a] All kinetic constants are apparent constants under the conditions specified. Details are given in the text.

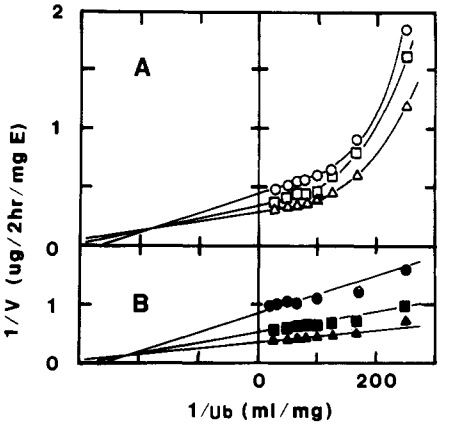

FIGURE 5: Ubiquitin dependence of the degradation rate of BSA derivatives at high ATP concentration. Details are given in the text. (A) Degradation rate of rcmBSA was determined at 6.3 μg/mL (circles), 12.3 μg/mL (squares), and 24 μg/mL (triangles) in the presence of the indicated concentrations of ubiquitin. (B) Degradation rate of rcaBSA was determined at 12.4 μg/mL (circles), 24.2 μg/mL (squares), and 39.2 μg/mL (triangles) in the presence of the indicated concentrations of ubiquitin.

The alternate substrate inhibition patterns were determined with each protein at two ATP concentrations. In these experiments, a radiolabeled substrate and another unlabeled substrate were present in the assays simultaneously. Figure 6 shows the family of lines obtained from these studies of the inhibition of rcmBSA degradation by rcaBSA at low ATP concentration. The inhibition patterns under all conditions were found to be competitive. The fact that each protein is a competitive inhibition of the other suggests that they are degraded by the same system. At high ATP concentrations, the inhibition constants and Michaelis constants were similar for each protein (K_i/K_m of 0.5 for rcaBSA and 1.4 for rcmBSA). At low ATP concentrations, however, the ratio of the inhibition constant to the Michaelis constant is very different from unity for rcaBSA (1.7 for rcmBSA and 12.7 for

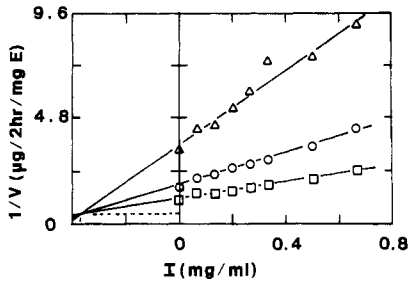

FIGURE 6: Alternate substrate inhibition pattern at low ATP concentration. The rate of degradation of rcmBSA at 7.2 μg/mL (triangles), 16 μg/mL (circles), and 23 μg/mL (squares) was determined in the presence of the indicated amounts of rcaBSA. Details are given in the text. The dashed horizontal line is drawn at the expected value for V_{max} determined from the previous experiments.

rcaBSA). These results suggest that substrates or substrate-derived polypeptides interact with the enzymes of the system at no less than two different sites (see Discussion).

Nature of the Rate-Limiting Step(s). To examine the nature of the rate-limiting step(s) for each of the ATP concentrations, the level of steady-state conjugates between ubiquitin and the proteins was measured by using SDS–polyacyrlamide gel electrophoresis as described under Experimental Procedures. At low concentrations of ATP, the system does not form measurable amounts of steady-state conjugates as measured by ^{125}I-ubiquitin incorporation into high molecular weight forms. Thus, it appears as though the rate-limiting step(s) occur before or upon conjugate formation under these conditions. Since ATP is required for conjugate formation, this observation is consistent with what one might expect to see at low ATP concentrations.

Measurement of the steady-state levels of conjugates at high ATP concentrations demonstrated that significant conjugate formation occurs. Thus, the rate-limiting step under conditions of high ATP concentration would appear to be either further addition of ubiquitin molecules to existing conjugates or proteolysis.

The nature of the rate-limiting step(s) with BSA derivatives was examined by using ^{125}I-ubiquitin. In the absence of added substrates, approximately half of the ubiquitin present is conjugated to endogenous proteins (Figure 7, curve 1). In the presence of saturating levels of rcmBSA, a number of new bands are observed (Figure 7, curve 2). These correspond to a molecular weight which must be fragments of the BSA derivative. There is little evidence for intact albumin–ubiquitin conjugates in the steady state. Thus, it would appear that a later proteolytic step is rate limiting under these conditions. When rcaBSA is used as the substrate, the pattern of new bands occurs at a higher molecular weight range. Most of these new bands occur near or above the parent protein. Thus, it would appear that with this protein, the rate-limiting step is conjugation of additional ubiquitin molecules or subsequent early proteolysis steps.

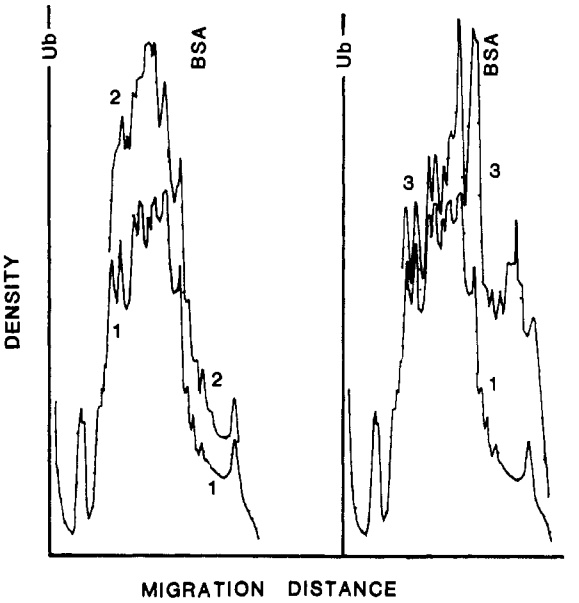

FIGURE 7: Steady-state conjugates of ubiquitin in the presence of alkylated BSA derivatives. Shown are the densitometer traces of the radioautogram from SDS–polyacrylamide gel electrophoresis of the following: (1) steady-state conjugates between ^{125}I-ubiquitin and endogenous proteins of fraction II; (2) same as (1) except in the presence of 0.2 mg/mL rcmBSA; (3) same as (1) except in the presence of 0.2 mg/mL rcaBSA. The positions of ubiquitin (Ub) and BSA are indicated at the top of the panels. Details are given in the text.

Discussion

Selectivity of Proteolysis. Very little is understood about what features of protein structure are recognized by the intracellular protease systems. It is clear, however, that there is marked selectivity to the turnover of intracellular proteins. Thus, the elucidation of the features of protein structure which are recognized by the cellular enzymes responsible for protein breakdown is important. In this study of the ubiquitin-dependent system, we have synthesized and characterized several derivatives of BSA in order to investigate the effect of protein structure on the rate of ubiquitin-dependent degradation by this system. Substrates which show simple saturable kinetic behavior have been described. These substrates exhibit relatively low K_m values and significantly higher rates of degradation than previously used substrates, enabling their use for assay of the intact system in crude extracts. The availability of these substrates will assist in the detection of the system in other cell types and extracts. Also, since these substrates show interpretable kinetic behavior and competitive alternate substrate inhibition patterns, they are useful in screening for other substrates. Putative substrates can be detected by assaying fractionated cellular proteins for competitive inhibitors of the degradation of alkylated bovine serum albumin. Further characterizations and kinetic studies on proteins which are rapidly degraded by this system and demonstrate interpretable kinetics may further define the general features of protein structure which are recognized by these enzymes. These types of studies will be important in narrowing the field of possible in vivo substrates, thereby eliminating a "trial and error" type search for the physiological substrates of the ubiquitin-dependent pathway of protein degradation.

Obviously, BSA is not a physiological substrate for the ubiquitin-dependent system. The rationale for using it in this study is that it is converted to a good substrate by oxidative damage (Wilkinson & Audhya, 1981). This may well be related to the observation that oxidatively damaged proteins are degraded rapidly in erythrocytes (Goldberg & Boches, 1982). This suggests that if proteins could be converted to good substrates by more defined chemistry, we could use the parent proteins and the derivatives as a model system in which to evaluate the general characteristics of protein which are recognized by the system.

Evaluation of the selectivity of this pathway is a complex task, since there are proably six or seven different proteins required for the overall reaction. Only one of these, the enzyme which adenylylates ubiquitin, has been purified to homogeneity (Ciechanover et a., 1982; Hershko et al., 1983).

Thus, it is not possible at this time to examine the specificity of each enzyme separately. It is possible, however, to begin characterizing the substrate selectivity of the crude reticulocyte system. This approach allows preliminary screening of substrates which may be useful in further studies with either whole cells or purified enzymes. Any approach taken to define the selectivity of this system requires careful kinetic characterization in order to evaluate the susceptibility of various proteins to degradation. The usefulness of such an approach is demonstrated by the fact that good substrates have been produced and have allowed the preliminary description of selectivity which follows.

Correlation of Physical Properties and Degradation Rates. First of all, denaturation and aggregation of the protein do not appear to be sufficient to make BSA a good substrate for the system. Evidence for this is given by the fact that the rBSA, which is denatured and aggregated (Table I), does not show significant degradation in this system (Figure 3). From the gel permeation and reverse-phase chromatography data summarized in Table I, it can be infered that the rBSA molecules exist as high molecular aggregates (molecular mass > 1.5 MDa, from Sephacryl S-300 fractionation range). This tends to obscure the effect of denauration on degradation rate. However, the reduced BSA is both denatured and extensively associated, suggesting much hydrophobic character. Therefore, neither of these properties per se endows the protein with the characteristics of a substrate which is easily degraded by this system. This is to be contrasted with the findings that aggregated proteins are preferred substrates for energy-dependent proteolysis in the intact reticulocyte (Goldberg et al., 1978).

It appears that isoelectric point is possibly a factor affecting rates of degradation in the ubiquitin-dependent system. The rcmBSA, which shows a greatly enhanced rate of breakdown over BSA, has a lower isoelectric point than the value of 4.9 observed for native BSA (Teale, 1960). However, rcmBSA's acidic isoelectric point cannot fully account for the observed differences in degradative rates. The isoelectric points of rcaBSA and BSA are similar since the net charge on the native protein is not altered significantly by the chemical modification steps. Yet rcmBSA is degraded more readily than both native and reduced BSA (Figure 3). Furthermore, the derivatives obtained from BSA which was reacted with carbodiimide (whose isoelectric points were the same or more basic than that of native BSA) did not show any significant ubiquitin-dependent breakdown. Thus, the isoelectric point cannot be the sole determinant of substrate selectivity. This pattern of selectivity is different than that observed for soluble liver protein (Dice & Goldberg, 1975; Dice et al., 1978, 1979).

There also was no apparent correlation between the acid lability of the proteins (Figure 1) and their ability to be substrates for the system. Lability has been defined by measuring the pH-dependent conformational changes around the aromatic residues using fluorescence. It is reasonable to equate this with at least local denaturation, since the exposure of aromatic residues to an aqueous environment is energetically unfavorable and expected to perturb the fluorescent properties of the aromatic residues. Acid denaturability has previously been suggested to correlate to in vivo half-lives (Goldberg & St. John, 1976; Bond, 1976).

The only property of the proteins studied which may correlate with degradation rate is hydrophobicity (Table I). In the cell, enhanced degradation of hydrophobic proteins has been implicated (Dean, 1975; Segal et al., 1976; Bohley & Reimann, 1977). The selectivity observed with the ubiquitin-dependent system seems to counter this, with the more hydrophilic proteins serving as better substrates.

This study suggests that the selectivity of the ubiquitin-dependent system depends on features of protein structure which are more subtle than the gross characteristics we have examined. It should also be recognized that there are at least two enzymes (or enzyme systems) that contribute to observed selectivity in the overall degradation of proteins by this system. These include the system which conjugates ubiquitin to the target protein and the system which is responsible for the proteolysis of that conjugate. These two systems must show different selectivity for the two protein substrates characterized here, resulting in different kinetic constants.

ATP Dependence of Selectivity. At low ATP concentrations and saturating substrate concentrations, the maximal rate of proteolysis is 2.4-fold greater for the rcmBSA derivative than for the rcaBSA derivative. Under these conditions, no detectable levels of ubiquitin–substrate conjugates accumulate. This suggests that activation or conjugation of ubiquitin is rate limiting. This is in accord with our expectations that the rate of formation of the activated ubiquitin will be slow at low concentrations of ATP. Thus, V_{max} would be expected to contain rate constants for the binding of activated ubiquitin to the conjugating protein as well as the catalytic step(s) and the release of conjugates from the activating enzyme. These results indicate either that there is a higher steady-state concentration of rcmBSA conjugates or that they are better substrates for the protease(s) than conjugates of rcaBSA.

At high ATP concentration, the maximal rate of degradation is similar for both alkylated substrates. Since conjugates of ubiquitin and proteins accumulate under these conditions, the proteolysis steps must be at least partially rate limiting. Thus, at saturation with all substrates, conjugates of rcmBSA and rcaBSA are equally good substrates for the protease(s). This argues that the kinetic differences we see reflect differences in the conjugation of ubiquitin to these substrates.

It has been suggested that proteolysis is rate limiting in the degradation of proteins by the ubiquitin-dependent system in reticulocyte lysates, and by unspecified systems in *Escherichia coli* and HeLa cells (Hough & Rechsteiner, 1984). The data supporting such a conclusion were collected at ATP concentrations similar to our high ATP concentrations. It has further been suggested that there is an ATP dependence to the proteolysis of ubiquitin–lysozyme conjugates with half-maximal stimulation observed at 0.03 mM ATP (Hershko et al., 1984). Thus, both concentrations of ATP utilized in this study would have been sufficient to saturate any ATP-dependent proteolysis step. These arguments and the data of Figure 7 argue that the ATP dependence observed in these studies is due to conjugation and not proteolysis.

The similar apparent V/K values for both alkylated substrates suggest that, at low substrate and ATP concentrations, the net rate of ubiquitin conjugation is not markedly different for rcmBSA or rcaBSA (Table II). This apparent second-order rate constant should contain terms for all steps up to and including the first irreversible step, conjugation of the first ubiquitin to the substrates. This rate likely reflects the rate of activation of ubiquitin and is similar for all substrates. The apparent K_i values from alternate substrate inhibition studies should approximate the binding constant for the substrate protein to the conjugating system. These results sugest that rcmBSA binds to the conjugating enzyme(s) more tightly than rcaBSA and forms conjugates at a slightly faster rate.

The data accumulated at saturating levels of ATP (Table II) indicate a 4-fold difference in the apparent V/K values for the alkylated substrates. Thus, although net proteolysis at

saturating substrate concentrations occurs at the same rate for both of the derivatives, rcmBSA is the preferred substrate for this system at low substrate concentrations. At saturating ubiquitin, ATP, and substrate protein, the maximal velocity appears to be limited by the proteolysis of conjugates. The K_m values for substrates (or the K_i values from inhibition studies which are similar) will then begin to reflect the binding affinity of the proteases for the conjugates of substrates and ubiquitin. This suggests that the conjugate(s) of rcaBSA bind more weakly to the protease(s) than do the conjugate(s) of rcmBSA.

It appears that both substrates are being degraded by the same system in these assays since both substrates are competitive alternate substrate inhibitors of the degradation of the other. There is good agreement between the K_i values and the K_m values except for rcaBSA at low ATP concentration ($K_i/K_m = 12.8$). This deviation could indicate that substrate or substrate-derived polypeptides can interact with more than one enzyme species. This would be consistent with the postulated mechanism where substrate protein would bind to the activating enzyme, but the conjugates formed would also have to bind to the proteases. One notes that the K_i/K_m ratio at high ATP concentration is approximately unity for both substrates. This is consistent with the substrate dependence being mainly due to saturation of the protease(s) with substrate–ubiquitin conjugates. Thus, the conjugates of ubiquitin and rcaBSA are more loosely bound to the protease(s) than are the corresponding conjugates of rcmBSA.

The difference in selectivity between the two substrates could arise simply from differences in the degree of conjugation of ubiquitin to the substrate protein molecules. In fact, the curved double-reciprocal plots obtained with rcmBSA and varied ubiquitin concentration (Figure 5A) suggest that the reciprocal velocity is a function of the reciprocal ubiquitin concentration squared. This would be consistent with more than one ubiquitin being conjugated to the rcmBSA. With rcaBSA as the substrate, however, the lack of curvature in the analogous plot (Figure 5B) suggests only first-order dependence. Thus, it is possible that the different apparent K_m values reflect the number of ubiquitin molecules conjugated to the target protein. There is experimental evidence (Chin et al., 1982) which indicates that the rate of globin degradation is proportional to the extent of ubiquitin conjugation to denatured globin. The ubiquitin conjugation follows protein denaturation, and the rate of degradation prallels the intracellular concentration of conjugates in the steady state. Thus, while there is no quantiative data on this matter, it is possible that multiple conjugation to ubiquitin results in a lowered K_m for conjugate degradation.

The conclusions about the different kinetic behavior of conjugates of the two alkylated substrates are directly verified by the results shown in Figure 7. With added rcaBSA, steady-state conjugates are of large molecular weight. This suggests that such conjugates are either bound loosely by the proteases or degraded slowly. With added rcmBSA, however, the conjugates are of lower molecular weight, suggesting that a later proteolytic step is rate limiting. Such results clearly demonstrate that the selectivity of the individual steps of the system is important in determining the overall selectivity of ubiqutin-dependent proteolysis.

Implications for in Vivo Selectivity. It is instructive to consider how the characteristics of the selectivity of this system relate to our expectations about the selectivity of controlled intracellular proteolysis. First of all, the selectivity of ubiquitin-dependent system is accomplished mainly through variation of K_m values for different substrates. The K_m values for ubiquitin are approximately independent of substrate and 10–20-fold lower than the level of ubiquitin in most cells (Goldstein et al., 1975). This implies that the system would normally be saturated with ubiquitin. Controlled metabolites are usually present at or near their K_m values for the enzymes metabolizing them. Thus, an enzyme system which metabolized several substrates (proteins) would be expected to have different K_m values for each substrate, and these K_m values should approximate the in vivo concentrations of that substrate.

A second very interesting aspect of selectivity is the change in selectivity observed when the ATP concentration is varied (Table II). The levels of ATP in the cell are important in the regulation of protein turnover and control of the selection of substrates for degradation (Goldberg & Dice, 1974; Goldberg & St. John, 1976; Hershko & Ciechanover, 1982). Therefore, it is relevant that with the ubiquitin-dependent system a change in relative specificity is observed as the ATP concentration is lowered. This suggests that the ubiquitin-dependent system is responsive to the intracellular concentration of ATP and that control over the selection of substrates is exerted such that different proteins (or classes of proteins) may be preferentially hydrolyzed in vivo, depending upon the levels of high-energy compounds within the cell.

In light of these results, the generally recognized role of the ubiquitin-dependent protein degradation system needs to be reevaluated. These results demonstrate that denaturation and aggregation alone are not sufficient to endow proteins with the properties which make them good substrates for the system. The lack of correlation between the in vivo and in vitro selectivity would suggest that the ubiquitin-dependent system cannot be responsible for the bulk of intracellular protein degradation. Since the enzymes of this system can kinetically discriminate between proteins on the basis of some chemical and/or physical feature of protein structure, it is possible that the number of substrates for this system is limited.

Registry No. ATP, 56-65-5; ubiquitin, 60267-61-0; proteinase, 9001-92-7.

REFERENCES

Bohley, P., & Riemann, S. (1977) *Acta Biol. Med. Ger. 36*, 1823–1827.

Bohley, P., Langer, J., Ansorge, S., Miehe, M., Miehe, C., & Hanson, H. (1972) *Acta Biol. Med. Ger. 28*, 321–328.

Bond, J. F. (1976) *Biochim. Biophys. Acta 451*, 238–249.

Chin, D. T., Kuehl, L., & Rechsteiner, M. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 5857–5861.

Ciechanover, A., Hod, Y., & Hershko, A. (1978) *Biochem. Biophys. Res. Commun. 81*, 1100–1105.

Ciechanover, A., Elias, S., Heller, H., Ferber, S., & Hershko, A. (1980a) *J. Biol. Chem. 255*, 7525–7528.

Ciechanover, A., Heller, H., Elias, S., Haas, A. L., & Hershko, A. (1980b) *Proc. Natl. Acad. Sci. U.S.A. 77*, 1365–1368.

Ciechanover, A., Heller, H., Katz-Etzion, R., & Hershko, A. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 761–765.

Ciechanover, A., Elias, S., Heller, H., & Hershko, A. (1982) *J. Biol. Chem. 257*, 2537–2542.

Dean, R. T. (1975a) *Eur. J. Biochem. 58*, 9–14.

Dean, R. T. (1975b) *Biochem. Biophys. Res. Commun. 67*, 604–609.

Dehlinger, P. J., & Schimke, R. T. (1972) *J. Biol. Chem. 246*, 2574–2583.

Dice, J. F., & Goldberg, A. L. (1975a) *Arch. Biochem. Biophys. 170*, 213–219.

Dice, J. F., & Goldberg, A. L. (1975b) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3893–3897.

Dice, J. F., Dehlinger, P. J., & Schimke, R. T. (1973) *J. Biol. Chem. 248*, 4220–4228.

Dice, J. F., Walker, C. D., Byrne, B., & Cardiel, A. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 2093–2097.

Dice, J. F., Hess, E. J., & Goldberg, A. L. (1979) *Biochem. J. 178*, 305–312.

Duncan, W. E., Offerman, M. K., & Bond, J. S. (1980) *Arch. Biochem. Biophys. 199*, 331–341.

Etlinger, J. D., & Goldberg, A. L. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 54–58.

Glass, R. D., & Doyle, D. (1972) *J. Biol. Chem. 247*, 5234–52426.

Goldberg, A. L. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 2640–2644.

Goldberg, A. L., & Dice, J. F. (1974) *Annu. Rev. Biochem. 43*, 835–869.

Goldberg, A. L., & St. John, A. C. (1976) *Annu. Rev. Biochem. 45*, 747–803.

Goldberg, A. L., & Boches, F. S. (1982) *Science (Washington, D.C.) 215*, 1107–1108.

Goldberg, A. L., Kowit, J., Etlinger, J., & Klemes, Y. (1978) in *Protein Turnover and Lysosomal Function* (Segal, H. L., & Doyle, D. J., Eds.) pp 171–196, Academic Press, New York.

Goldstein, G., Sheid, M., Hammerling, U., Boyse, E. A., Schlesinger, D. H., & Niall, H. D. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 11–15.

Goren, H. J., Glick, D. M., & Barnard, E. A. (1968) *Arch. Biochem. Biophys. 126*, 607–623.

Greenfield, N., & Fasman, g. (1969) *Biochemistry 8*, 4108–4116.

Haas, A. L., & Rose, I. A. (1982) *J. Biol. Chem. 257*, 10329–10337.

Haas, A. L., Warms, J. V. B., Hershko, A., & Rose, I. A. (1982) *J. Biol. Chem. 257*, 2543–2548.

Hershko, A., & Ciechanover, A. (1982) *Annu. Rev. Biochem. 51*, 335–364.

Hershko, A., Ciechanover, A., & Rose, I. A. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 3107–3110.

Hershko, A., Ciechanover, A., Heller, H., Haas, A. L., & Rose, I. A. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 1783–1786.

Hershko, A., Eytan, E., Ciechanover, A., & Haas, A. L. (1982) *J. Biol. Chem. 257*, 13964–13970.

Hershko, A., Heller, H., Elias, S., & Ciechanover, A. (1983) *J. Biol. Chem. 258*, 8206–8214.

Hershko, A., Leshinsky, E., Ganoth, D., & Heller, H. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 1619–1623.

Hoare, D. G., & Koshland, D. E., Jr. (1967) *J. Biol. Chem. 242*, 2447–2453.

Hough, R., & Rechsteiner, M. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 90–94.

Knowles, S. E., & Ballard, F. J. (1976) *Biochem. J. 156*, 609–612.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Mortimore, G. E., Ward, W. F., & Schworer, C. M. (1978) in *Protein Turnover and Lysosomal Function* (Segal, H. L., & Doyle, D. J., Eds.) pp 67–87, Academic Press, New York.

Neff, N. T., DeMartino, G. N., & Goldberg, A. L. (1979) *J. Cell. Physiol. 101*, 439–458.

Ouchterlony, O. (1958) *Prog. Allergy 5*, 1–78.

Peters, T., Jr., & Hawn, C. (1967) *J. Biol. Chem. 242*, 1566–1573.

Saus, J., Timoneda, J., Hernandez-Yago, J., & Crisolia, S. (1982) *FEBS Lett. 143*, 225–227.

Segal, H. L., Winkler, J. R., & Miyagi, M. P. (1974) *J. Biol. Chem. 249*, 6364–6365.

Segal, H. L., Rothstein, D. M., & Winkler, J. R. (1976) *Biochem. Biophys. Res. Commun. 73*, 79–84.

Teale, F. W. J. (1960) *Biochem. J. 76*, 381–388.

Wilkinson, K. D., & Audhya, T. K. (1981) *J. Biol. Chem. 256*, 9235–9241.

Wilkinson, K. D., & Evans, A. C., Jr. (1983) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 42*, 1813.

Wilkinson, K. D., Urban, M. K., & Haas, A. L. (1980) *J. Biol. Chem. 255*, 7529–7532.