Structure and Function of Ubiquitin: Evidence for Differential Interactions of Arginine-74 with the Activating Enzyme and the Proteases of ATP-Dependent Proteolysis†

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ABSTRACT: Ubiquitin was modified with the anionic, arginine-specific reagent 4-(oxoacetyl)phenoxyacetic acid in order to study the relationship between structure and function of the molecule. Four different derivatives (A, B, C, and D) were purified from the reaction mixture by anion-exchange high-performance liquid chromatography and subjected to tryptic peptide mapping to determine the location of the modification(s). These derivatives were stable throughout the procedures required for purification, tryptic hydrolysis, and peptide mapping. Derivative A was modified at arginine-42, derivative B at arginine-72, derivative C at arginines-42 and -72, and derivative D at arginine-74. Modification of ubiquitin with ^{14}C-labeled 4-(oxoacetyl)phenoxyacetic acid indicated that the reagent formed a stable, 1:1 complex with arginine residues of the protein. Native ubiquitin and each of the four derivatives were tested for their ability to stimulate ^{32}P exchange between ATP and pyrophosphate, a reaction catalyzed by enzyme 1 of the ubiquitin-dependent proteolytic pathway. A and C were capable of promoting this exchange at a rate only 15% that of native ubiquitin, B stimulated the exchange to 25%, and D stimulated exchange to 60% of the native level. None of the derivatives was capable of promoting a significant level of ubiquitin-dependent proteolysis. D was capable of forming conjugates with exogenous and endogenous proteins to an extent very similar to that of native ubiquitin, suggesting that its inability to stimulate ubiquitin-dependent proteolysis was due to a defect in a step beyond that of conjugate formation. These results indicate that in this system, the integrity of arginines-42, -72, and -74 is essential for full function of ubiquitin and suggest that the ubiquitin activating enzyme (E1) and the protease(s) of the system recognize different regions or conformations of ubiquitin.

Ubiquitin is the most highly conserved protein known. Its sequence is identical from species as diverse as *Xenopus laevis*, *Ceratitis capitata* (fruit flies), and humans (Dworkin-Rastl et al., 1984; Gavilanes et al., 1982; Schlesinger et al., 1975; Wilkinson & Audhya, 1981). Furthermore, there are only three amino acid changes between the yeast and human proteins, or between the oat and human proteins (Wilkinson et al., 1986; Vierstra et al., 1985, 1986). To account for this extraordinary sequence conservation, a number of factors need to be considered. A highly critical and specific role for the protein would be expected to enhance its sequence conservation. Also, a protein which interacted with a number of different enzymes or proteins, most likely at different domains or sites, would tend to retain its sequence homogeneity.

It appears that both factors contribute to the observed sequence homology of ubiquitin from diverse sources. The activation of ubiquitin for conjugation to cellular proteins was shown to be essential for normal cell growth and development by Varshavsky and his co-workers (Finley et al., 1984; Ciechanover et al., 1984). A temperature-sensitive mutant (ts-85, developed from a spontaneous mouse mammary carcinoma) carries a defect in the coding region for the first enzyme (E1) of the pathway which leads to ubiquitin conjugation to target proteins. Such cells are capable of normal ubiquitin–protein conjugation at the permissive temperature, but the cells become cell cycle arrested and are unable to form ubiquitin–protein conjugates or to degrade short-lived and structurally abnormal proteins at the nonpermissive temperature. Thus, ubiquitin plays at least one critical role which is essential for normal cell functioning, that as a cofactor in ATP-dependent protein degradation.

In addition to the degradation of proteins, ubiquitin plays other roles which are very likely critical. Ubiquitin was found ligated to histones 2A and 2B within the nucleus (Goldknopf & Busch, 1977; West & Bonner, 1980). The ubiquitin conjugation to these histones is believed to regulate chromatin structure during transcription (Levinger & Varshavsky, 1982; Busch & Goldknopf, 1981). Recently, it has been reported (Siegelman et al., 1986; St. John et al., 1986) that ubiquitin, ligated to a glycoprotein, is the cell surface molecule associated with lymphocyte homing. The platelet-derived growth factor receptor (Yarden et al., 1986) and a component of sodium-dependent choline uptake in synaptosomes (Meyer et al., 1986) are also ubiquitinated. Furthermore, ubiquitin has been suggested to act as an immunostimulating peptide (Goldstein et al., 1975) and has been implicated in the heat-shock response (Finley et al., 1984; Levinger & Varshavsky, 1982; Bond & Schlesinger, 1985).

It is therefore apparent that ubiquitin interacts with a number of different macromolecules in carrying out its many cellular functions. It is likely that these different macromolecules interact with different conformations or regions of ubiquitin. This could apply selective pressure in a number of areas, helping to account for the high level of sequence homology observed. A convenient model system for investigating these ubiquitin–macromolecule interactions is the ubiquitin-dependent protein degradation system. It is known that ubiquitin associates with at least three different enzymes or classes of enzymes in functioning as a cofactor in protein degradation (Haas et al., 1983; Hershko et al., 1983; Pickart & Rose, 1985a). E1 activates ubiquitin in a process requiring ATP. The activated ubiquitin is then transferred to one of

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a class of acyl-carrier proteins (E2), and E3 transfers the ubiquitin from E2 to the target protein, where an amide linkage is formed between the carboxyl terminus of ubiquitin and an ϵ-amino group of the target protein. It is likely that the covalent attachment of ubiquitin may act as a signal in activating specific proteases to degrade the target protein, and this would represent one or more additional ubiquitin–enzyme interactions. Furthermore, an enzyme capable of cleaving small leaving groups from ubiquitin's carboxyl terminus has been described (Pickart & Rose, 1985b), and additional enzymes with this activity have been detected (Mayer, 1986).

To investigate the many roles played by ubiquitin, it will be helpful to have ubiquitin derivatives which are capable of participating in some, but not all, of the native ubiquitin–macromolecule interactions. With these probes, we can begin to define the parts of the molecule responsible for the different interactions and to study the mechanisms involved. The common feature of the chemistry involved in these functions is ligation of the carboxyl terminus of ubiquitin to a variety of cellular proteins. In this paper, we have examined derivatives of arginine, since three of the four arginine residues are located in the carboxyl-terminal region. We prepared a series of four stable arginine derivatives of ubiquitin, and after separation and purification, these derivatives were tested for their ability to stimulate ATP–PP_i exchange (catalyzed by E1 of the protein degradation system), their ability to form ubiquitin–protein conjugates, and their ability to participate in the entire ATP, ubiquitin-dependent protein degradation system. The results demonstrate that the activating enzyme and the proteases of the system interact differently in the region of Arg-74 of ubiquitin.

Materials and Methods

Materials. Ubiquitin was isolated from bovine erythrocytes as previously described (Haas & Wilkinson, 1985). 4-(Oxoacetyl)phenoxyacetic acid (OAPA)[1] was synthesized as described previously (Duerksen & Wilkinson, 1987). Iodo[2-^{14}C]acetamide was obtained from ICN Radiochemicals, and [^{32}P]pyrophosphate was obtained from Amersham. Fraction II was obtained from reticulocytes according to the method of Ciechanover et al. (1978). The affinity-purified antibody to ubiquitin was a generous gift from Dr. Arthur L. Haas. All other materials were purchased from commercial sources.

Modification of Ubiquitin. Modification reaction mixtures consisted of 1.0 mg/mL ubiquitin, 100 mM potassium phosphate, pH 8.0, and 1.0 mM OAPA. The reaction was allowed to proceed for 5 h at 37 °C followed by dialysis against 2 mM sodium phosphate (solvent A) for 60 h (three changes) to remove unreacted reagent. After concentration to 10 mg/mL protein (Amicon, YM 5 filter), the derivatized proteins (100-μL aliquots) were purified by HPLC, using an AX-300 Aquapore column (Brownlee) and a phosphate gradient. Sodium phosphate (0.5 M, pH 7.5) was diluted to 2 mM (solvent A) and 10 mM (solvent B). The column was equilibrated in 2 mM sodium phosphate (0% B) and maintained at that concentration for 2.8 min after injection. The percentage of buffer B was then linearly increased to 100% by 21 min and held at that composition for 5 min. The flow rate was 1.0 mL/min, and the absorbance was monitored at 220 nm. Fractions containing protein were collected, lyophilized, resuspended in water, and dialyzed 16 h against 50 mM ammonium bicarbonate at 4 °C. After centrifugation to remove any undissolved material, protein concentrations were determined (Lowry et al., 1951), correcting for the presence of ammonium bicarbonate buffer. The samples were then lyophilized in preparation for tryptic hydrolysis.

Tryptic Hydrolysis, Peptide Mapping, and Amino Acid Analysis. Both native ubiquitin and derivatized ubiquitin were digested by using trypsin and the peptide products analyzed by reverse-phase HPLC and amino acid analysis (Cox et al., 1986a).

Peptide maps of the native and OAPA-derivatized ubiquitin were compared, and peaks that differed between the two in migration position were collected, allowed to stand overnight to evaporate the acetonitrile, lyophilized, hydrolyzed, and subjected to amino acid analysis.

Synthesis of [^{14}C]OAPA. [^{14}C]OAPA was synthesized by using a modification of the previously described procedure (Duerksen & Wilkinson, 1987). *p*-Hydroxyacetophenone (244 mg) was dissolved in 2.5 mL of water containing 1 equiv of sodium hydroxide. Unlabeled iodoacetamide (304 mg) and 50 μCi of iodo[2-^{14}C]acetamide (approximately 0.7 mg) were added to the solution and allowed to react at 40 °C for 4 h. After the solution was cooled overnight, the crystals formed were harvested and washed with 0.5 mL of water. The crystals were dissolved in 5.6 mL of 6 N HCl, and the solution was refluxed 1 h. After overnight cooling, the crystals were washed with 0.5 mL of water. Water (5.4 mL) and selenium dioxide (0.27 g) were added, and the sample was refluxed 5 h. After centrifugation to remove selenium, the supernatant was transferred to a clean vial. Ether (three aliquots of 2 mL each) was used to extract starting material and byproducts. The remaining aqueous layer was concentrated by incubation in a desiccator in the presence of phosphorus pentoxide for 3 days. The crystals formed were removed by vacuum filtration. Forty-two milligrams of crystals with a specific activity of 2.9×10^{10} dpm/mol was obtained.

Determination of Reaction Stoichiometry. To determine the stoichiometry of the reaction between OAPA and ubiquitin, radiolabeled OAPA (1 mM) and ubiquitin (1 mg/mL) were incubated as described above. After dialysis and concentration, 100-μL aliquots were applied to an Aquapore AX-300 HPLC column and the products eluted with a phosphate gradient. Fractions (0.4 mL) were collected throughout each gradient. Fractions from four applications were pooled, producing combined fractions of 1.6 mL each. Protein in a portion of each combined fraction was precipitated by the addition of TCA to 5%. After centrifugation, the supernatant was removed and the protein resuspended in water. Protein concentration was then determined by the method of Lowry et al. (1951). The remainder of each combined fraction was used to determine incorporation of radioactivity by liquid scintillation counting.

ATP–PP_i Exchange Assay. The method used was similar to that described by Haas et al. (1982). Incubations (50 μL) contained 1 mM ATP, 1 mM AMP, 10 mM magnesium chloride, 0.1 mM sodium pyrophosphate, 10 mM sodium fluoride, 0.1 mM dithiothreitol, 50 mM Tris-HCl, pH 7.2, ubiquitin activating enzyme [purified as described (Ciechanover et al., 1982)], the indicated amount of native or modified ubiquitin, and approximately 1.5×10^4 cpm of [^{32}P]pyrophosphate. Reaction times were chosen such that only the initial reaction rate was measured. Reactions were quenched with 5% trichloroacetic acid, and the ATP was absorbed by adding 50 μL of 10% acid-treated charcoal (suspended in 2% trichloroacetic acid and 50 mM pyrophosphate). After 10 min,

[1] Abbreviations: OAPA, 4-(oxoacetyl)phenoxyacetic acid; HPLC, high-performance liquid chromatography; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; PAGE, polyacrylamide gel electrophoresis.

the charcoal was separated from the supernatant by filtration and was washed with 2 mL of 2% trichloroacetic acid, and the bound radioactivity was determined by measuring Cerenkov radiation. In separate control experiments, the nucleotides were extracted from the charcoal with methanol and counted directly. Both methods gave similar results.

Determination of Conjugate Formation. Native or OAPA-modified ubiquitin (derivative D) was incubated with fraction II in the presence or absence of ATP. Reactions which included ATP consisted of 10 μL of buffer A (600 mM Tris-HCl, pH 7.6, 30 mM magnesium chloride, 12 mM ATP, 18 mM dithiothreitol, and 60 mM phosphocreatine), 2 μL of creatine phosphokinase (150 units/mL), 10 μL of fraction II, and 1 or 5 μg of native or modified ubiquitin in a 60-μL reaction mix. Reactions in the absence of ATP consisted of 10 μL of buffer B (600 mM Tris-HCl, pH 7.6, 30 mM magnesium chloride, 18 mM dithiothreitol, and 50 mM glucose), 2 μL of hexokinase (5800 units/mL), 10 μL of fraction II, and 5 μg of native or modified ubiquitin in a 60-μL reaction mix. Controls included a zero-time point (SDS and β-mercaptoethanol added before fraction II) as well as reactions run in the presence and absence of ATP, but without any native or modified ubiquitin. Reactions were allowed to proceed for 1.5 h at 37 °C and then were stored at −20 °C.

SDS–polyacrylamide gel electrophoresis, followed by immunoblotting, was used to analyze for conjugate formation. Five microliter samples of each incubation were subjected to SDS–polyacrylamide gel electrophoresis. The electrophoretic transfer and immunochemical staining for ubiquitin and ubiquitin conjugates were done as described by Haas and Bright (1985), with the exception that in the antibody binding step, 1.5 μg/mL antibody was present and that step was allowed to proceed for 20 h.

To demonstrate that conjugates are formed between derivative D and the substrate protein, reduced carboxymethylated bovine serum albumin, incubations in the presence of ATP and either native ubiquitin or derivative D were performed as described above with the addition of radioactively labeled reduced carboxymethylated bovine serum albumin (2 μg/mL). Aliquots were removed at various times (1 min, 15 min, 30 min, and 1 h), quenched by the addition of SDS and β-mercaptoethanol, and subjected to SDS–polyacrylamide gel electrophoresis. The substrate protein and corresponding conjugates were detected by autoradiography.

Assays for Ubiquitin-Dependent Proteolysis. Each of the ubiquitin–OAPA derivatives was assayed for its ability to stimulate ATP-dependent proteolysis in the presence of fraction II. Incubation mixtures (containing ATP) were as described above, with the addition of ^{125}I-reduced carboxymethylated bovine serum albumin as the substrate (Evans & Wilkinson, 1985) and varying amounts of native or modified ubiquitin.

Other Methods. Protein concentrations were determined by the absorbance at 280 nm (using an extinction coefficient for a 1% solution of ubiquitin of 1.6) and/or by the method of Lowry et al. (1951). Amino acid analyses were done on a Jeol JHC 6AH analyzer after 48-h hydrolysis in 6 M HCl at 110 °C.

RESULTS

Reaction of OAPA with Ubiquitin. When ubiquitin is reacted with OAPA, a number of ubiquitin derivatives are formed which elute later than native ubiquitin on anion-exchange HPLC. Figure 1 shows the typical distribution of these products after a 5-h incubation followed by a 60-h dialysis, as described under Materials and Methods. The peak labeled

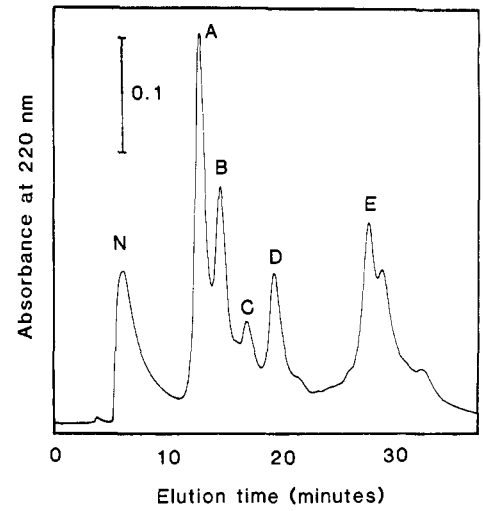

FIGURE 1: HPLC separation of ubiquitin–OAPA derivatives. Ubiquitin (1 mg/mL) was reacted with 1.0 mM OAPA in 100 mM potassium phosphate, pH 8.0, at 37 °C for 5 h. After dialysis against 2 mM sodium phosphate, pH 8.0, for 60 h and concentration to 10 mg/mL protein (Amicon, YM 5), 500 μg was applied to an anion-exchange HPLC column (Brownlee, AX-300) and eluted as described under Materials and Methods. Absorbance was monitored at 220 nm.

Table I: Structural Characteristics of Ubiquitin Derivatives

derivative	rel amount (%)[a]	residue(s) modified[b]	mol of OAPA/mol of Ub[c]
N	17.3		0
A	21.4	42	1.2
B	14.2	72	0.9
C	6.1	42, 72	1.8
D	10.6	74	1.3
E	30.4	nd[d]	nd

[a] The relative amounts were estimated by peak areas from HPLC, monitored at 220 nm (Figure 1). [b] The residues modified were determined by peptide mapping (Figure 2). [c] The moles of OAPA per mole of ubiquitin were determined by [^{14}C]OAPA incorporation into ubiquitin, as described under Materials and Methods. [d] These values were not determined.

N is unreacted ubiquitin, peaks A, B, C, and D are stably modified forms of ubiquitin, and peak E appears to be due to further reversible association of the reagent, probably via lysine residues. If the mixture is dialyzed for longer periods of time, or if lower concentrations of OAPA are used in the reaction, the amount of peak E relative to the other forms is decreased. Additionally, if peak E is purified by HPLC separation, allowed to stand 24–48 h at room temperature, and rechromatographed, a significant fraction of the material elutes at the position of native or stably modified ubiquitin (mostly as A). If the reaction is allowed to proceed for longer periods of time, or if higher concentrations of ubiquitin and OAPA are used, the remaining native ubiquitin will react. However, the stably modified forms also undergo further reaction to form derivatives which do not elute from the HPLC column under these conditions. The rate of these subsequent reactions, as well as that of the initial reactions, increases with pH and with temperature.

Comparison of the absorbances of the various derivatives at 220 nm and at 280 nm suggests that C contains approximately twice as many OAPA molecules per ubiquitin as do derivatives A, B, and D. Integration of the peak areas from the 220-nm trace (Table I) indicates the general distribution of the various species present; however, the added absorbance at 220 nm contributed by OAPA is expected to lead to an

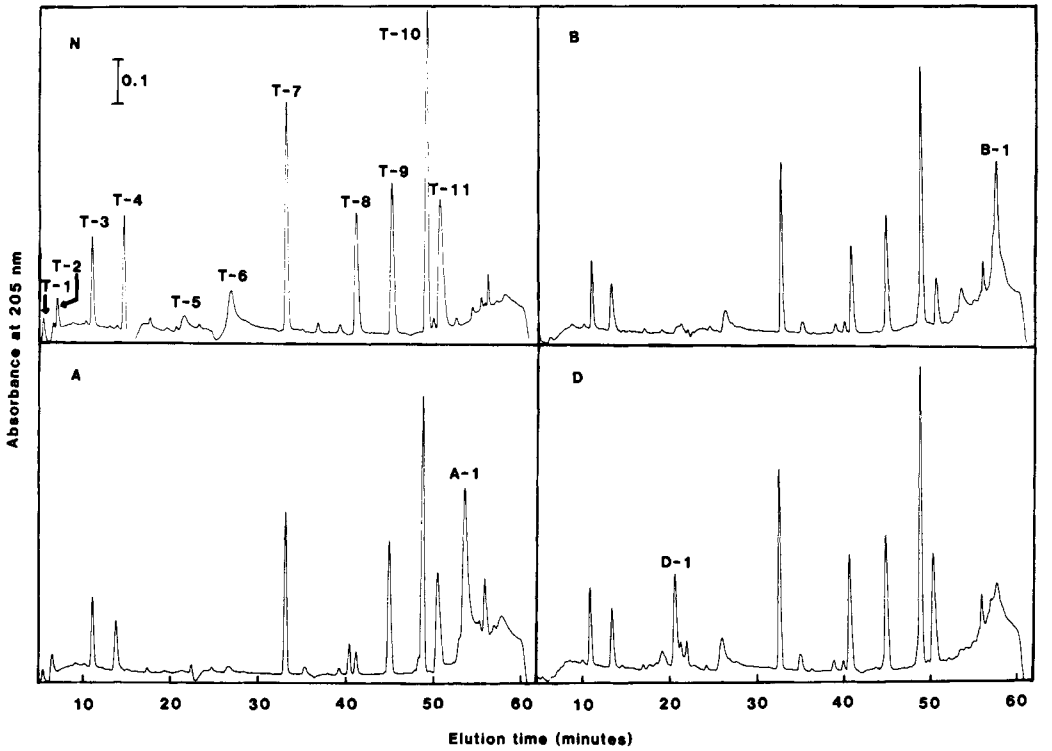

FIGURE 2: Tryptic peptide maps of native and OAPA-modified ubiquitin. OAPA-modified ubiquitin was prepared, isolated, and digested with trypsin as described under Materials and Methods. Panel N shows the peptide map of the native protein, panel A the peptide map of derivative A, panel B the peptide map of derivative B, and panel D the peptide map of derivative D. 80 μg of protein was applied in each case. The notations are those used previously (Cox et al., 1986b) and/or are explained in the text.

overestimation of the amount of derivatives containing OAPA. This is because peaks of modified proteins will contain absorbance contributions from both OAPA and the protein, while the peak of native ubiquitin is reflective only of absorbance contributions from the protein.

Characterization of the Derivatives. Modification of an arginine residue of ubiquitin should block trypsin reactivity at that particular arginine. The two flanking peptides normally formed by tryptic digestion would be missing, and a new, larger peptide would appear. Ubiquitin contains four arginines, located at positions 42, 54, 72, and 74 (Schlesinger et al., 1975). Any or all of these residues might be accessible to OAPA. To determine which arginines are susceptible, ubiquitin was incubated with OAPA as described. After separation by HPLC, derivatives A, B, C, and D were collected, lyophilized, and subjected to tryptic hydrolysis and amino acid analysis. The OAPA/arginyl residue adducts were found to be stable over the time period and conditions required for separation by HPLC, dialysis, lyophilization, and tryptic hydrolysis.

Figure 2 shows the peptide maps of native ubiquitin and derivatives A, B, and D. The migration positions of most of the peptides appear identical in native and modified ubiquitin, with a few exceptions (as noted). These identical retention times indicate that the reagent is selective. It does not form stable adducts with any of the lysyl residues, and it also displays a limited selectivity toward the arginine residues.

The top left panel in Figure 2 is a tryptic map of native ubiquitin with the peptides labeled T-1 through T-11 as described earlier. Under these conditions, trypsin does not cleave the peptide bonds at Lys-33 or at Arg-74 (Cox et al., 1986a). Derivative A (bottom left panel) exhibits a peptide map very similar to that of native ubiquitin. In A, however, peptides T-6 (amino acids 30–42) and T-8 (amino acids 43–48) are missing, and a new peptide migrating at 53 min (A-1) has appeared. Amino acid analysis confirms that this is the composite peptide consisting of amino acids 30–48. The residual amounts of T-6 and T-8 can be accounted for by contamination of the isolated A by B. We concluded that derivative A is ubiquitin modified by OAPA at a single residue, namely, Arg-42.

The top right panel shows the peptide map of derivative B. It differs from that of the native protein in not containing peptide T-2 (amino acids 73–76) and having a drastically reduced amount of peptide T-11 (amino acids 64–72). Additionally, a new peptide migrating at 57 min (B-1) has appeared. Amino acid analysis confirms that peptide B-1 is the composite peptide containing amino acids 64–76. The remaining amount of peptide T-11 can be accounted for by contamination of B by a small amount of A. We conclude that derivative B is ubiquitin modified by OAPA at Arg-72.

Derivative C appears to be doubly modified ubiquitin on the basis of its 280- and 220-nm absorbance ratio. The peptide map (not shown) and stoichiometry (determined below) support this conclusion, demonstrating that the modifications occur at Arginines-42 and -72.

The bottom right panel shows the peptide map of derivative D. Comparison with the native map shows a loss of peptide T-2 (amino acids 73–76) and the appearance of a new peptide migrating slightly after 20 min (D-1). Amino acid analysis of peptide D-1 confirms that this is the carboxyl-terminal tetrapeptide Leu-Arg-Gly-Gly, amino acids 73–76. This indicates that derivative D arises from OAPA modification only at Arg-74.

The modification of arginines-42, -72, and -74 was apparently reversed under the acid hydrolysis conditions preceding amino acid analysis, as the expected amount of arginine was eluted at the position of the unmodified amino acid. We saw no evidence that Arg-54 was modified under these conditions; the peptide ending with this residue (T-4) and the following peptide (T-7) appear unaffected in all the derivatives we examined.

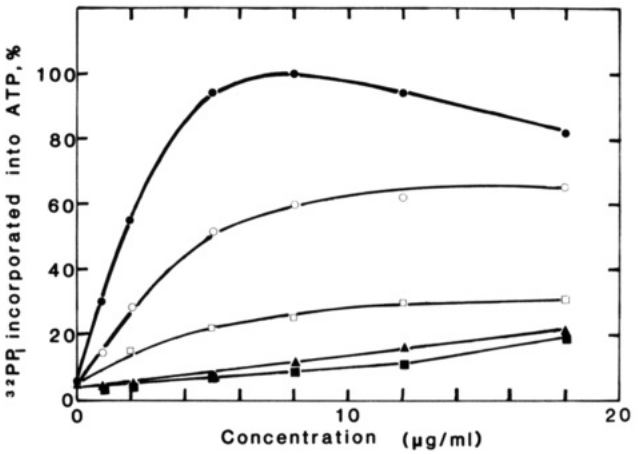

FIGURE 3: Ability of native and modified ubiquitin to stimulate E1-catalyzed ATP–PP_i exchange. Various concentrations of native ubiquitin (●) as well as derivative A (▲), B (□), C (■), or D (○) were incubated with ATP, [^{32}P]pyrophosphate, and E1 as described in the text, and the exchange of ^{32}P between ATP and pyrophosphate was measured. Results are reported as the percentage of control (the maximal incorporation observed with the native protein under these conditions) incorporation observed.

To determine the stoichiometry of the reaction, ^{14}C-labeled OAPA was reacted with ubiquitin. After separation of the derivatives by HPLC, fractions were analyzed for both protein content and incorporation of radioactivity. The results are presented in Table I. The stoichiometry observed corresponds to one molecule of OAPA per arginine modified. It is known that the 2:1 adduct observed previously (Duerksen & Wilkinson, 1987) rearranges to a secondary product. These data demonstrate that the secondary product is a 1:1 adduct, probably as a result of a Canizarro rearrangement.[2] In confirmation of the data obtained from the peptide maps and 280-nm:220-nm absorbance ratio, derivative C contains twice as many OAPA groups as do derivatives A, B, and D.

Activity of Derivatives. Derivatives A, B, C, and D were tested in three different assays to determine their ability to participate in the ubiquitin and ATP-dependent proteolysis system. The ATP–PP_i exchange reaction has been frequently used to study the ability of the ubiquitin activating enzyme (E1) to catalyze the ubiquitin-dependent exchange of radioactivity between pyrophosphate and adenosine trisphosphate. Native ubiquitin, as well as derivatives A, B, C, and D, was incubated with E1, [^{32}P]pyrophosphate, and buffer, and the exchange of radioactivity into ATP was monitored. The results are shown in Figure 3. Derivatives A (modified at Arg-42) and C (modified at Arg-42 and Arg-72) had little activity (<15%) in this assay, and B (modified at Arg-72) had approximately 25% activity. However, D (modified at Arg-74) demonstrated 60% of the native activity. Since arginines-42, -72, and -74 are all near each other on the surface of the ubiquitin molecule (Vijay-Kumar et al., 1985), and since E1 catalyzes the adenylation of ubiquitin at the carboxyl terminal (Gly-76), only two residues away from the modification, this result was quite unexpected.

To determine whether derivative D was capable of forming ubiquitin–protein conjugates, native ubiquitin as well as derivative D was incubated with fraction II in the presence and absence of ATP. After reaction, aliquots were subjected to SDS–polyacrylamide gel electrophoresis, electrophoretically transferred to nitrocellulose paper, and immunochemically

[2] P. J. Duerksen-Hughes and K. D. Wilkinson, unpublished experiments.

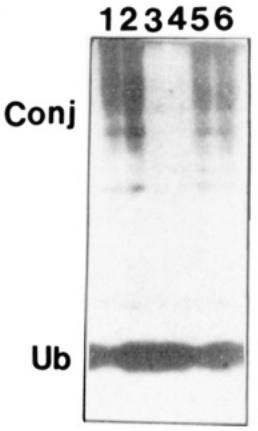

FIGURE 4: Ability of native ubiquitin and derivative D to form steady-state levels of conjugates. Reaction mixtures containing fraction II and native ubiquitin (lanes 1–3) or derivative D (lanes 4–6) were incubated in the presence of ATP (lanes 1, 2, 5, and 6) or in its absence (lanes 3 and 4). Lanes 1 and 5 each contained 1 μg of native or derivatized ubiquitin while lanes 2, 3, 4, and 6 each contained 5 μg of native or derivatized ubiquitin. Samples were electrophoresed (SDS–PAGE) and blotted onto nitrocellulose paper, and ubiquitin-containing bands were detected by using antibodies directed against SDS-denatured ubiquitin as described (Haas & Bright, 1985).

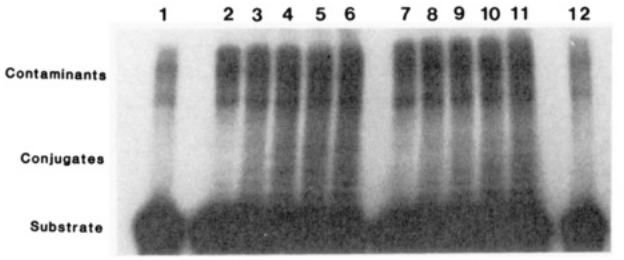

FIGURE 5: Formation of conjugates of exogenous substrate protein. Radiolabeled reduced carboxymethylated bovine serum albumin was incubated with either native ubiquitin (lanes 2–6) or derivative D (lanes 7–11) as described under Materials and Methods. At various times, aliquots were removed and subjected to SDS–polyacrylamide gel electrophoresis. Lanes 2 and 7 are samples removed at 1 min; lanes 3 and 8 are samples removed at 15 min; lanes 4 and 9, 30 min; lanes 5 and 10, 1 h; and lanes 6 and 11, 2 h. Lanes 1 and 12 show the substrate before incubation.

stained for ubiquitin and ubiquitin conjugates. Figure 4 shows that derivative D forms a variety of steady-state conjugates very similar in size and amounts to those formed with the native protein. In separate experiments, it was shown that conjugates were not formed in the absence of either ATP or ubiquitin and that no detectable conjugates were present in the preparation of fraction II used. This indicates that the conjugates observed in Figure 4 arose from de novo synthesis. It should be noted from Figure 4 that the antibody cross-reacts to a similar extent with free D and with native protein.

To verify that derivative D forms conjugates with the exogenous substrate, reduced carboxymethylated bovine serum albumin, as well as with endogenous proteins, native ubiquitin and derivative D were incubated with radioactively labeled reduced carboxymethylated bovine serum albumin. After reaction and separation by SDS–polyacrylamide gel electrophoresis, the gel was subjected to autoradiography. The results are shown in Figure 5. With ubiquitin or derivative D, a "ladder" of higher molecular weight albumin conjugates is appparent. Further, the distribution of these conjugates, and the rate at which they accumulate, is similar for both ubiquitin and derivative D. These results demonstrate that derivative D is active in all of the steps up to, and including, conjugate formation with both endogenous proteins and exogenous substrates.

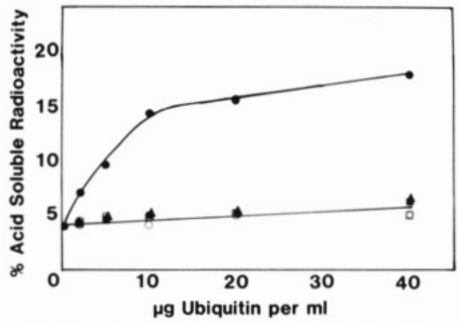

FIGURE 6: Ability of native and derivatized ubiquitin to stimulate proteolysis of reduced–carboxymethylated bovine serum albumin. Incubations containing various amounts of native ubiquitin (●), derivative A (○), derivative B (□), derivative C (■), or derivative D (▲), fraction II, and ^{125}I-labeled substrate were quenched with trichloroacetic acid, and the percentage of acid-soluble radioactivity was determined, as described under Materials and Methods.

Finally, native ubiquitin and also derivatives A–D were tested for their ability to stimulate ATP-dependent proteolysis of the exogenous substrate reduced carboxymethylated bovine serum albumin. None of the derivatives was capable of stimulating detectable levels of ATP-dependent proteolysis (Figure 6). Thus, D is defective in some step beyond that of conjugate formation.

DISCUSSION

A recently described arginine modification reagent, 4-(oxoacetyl)phenoxyacetic acid (OAPA) (Duerksen & Wilkinson, 1987), was used to modify ubiquitin. The stable derivatives thus formed were purified by HPLC, characterized by peptide mapping, and tested for their ability to participate in ubiquitin, ATP-dependent protein degradation. The results indicate that modification at any one of arginines-42, -72, or -74 is sufficient to prevent the protein from stimulating ATP-dependent proteolysis; however, one of the derivatives, modified at arginine-74, is capable of reacting with enzyme 1 of the system and of forming ubiquitin–protein conjugates.

Structure of Ubiquitin and the Arginine Derivatives. The 1:1 stoichiometry observed between OAPA molecules and arginine residues is consistent with earlier reports of phenylglyoxal/protein stoichiometries (Borders & Riordan, 1975; Philips et al., 1978, 1979) but contrasts with other observations of a 2:1 adduct (Takahashi, 1968; Tancini et al., 1985). It is clear that glyoxal reagents are capable of forming more than one type of adduct, possibly rearranging from one form to another. This would explain why different results are obtained, depending on the reagent, the protein or arginine derivative, and the reaction conditions.

The protein modification data suggest a number of important points. The stability of the derivatives and the negative charge of OAPA, combined with the influence of nonionic interactions between the protein and the solid support, made it possible to purify the variously modified forms of the protein by HPLC. Thus, the different derivatives could be purified and rigorously characterized.

A marked stability characterized these adducts during the purification and analytical steps. Reversal of the binding has been observed in some cases of arginine modification, especially when reagents such as 1,2-butanedione were used in the absence of borate, although modifications with phenylglyoxal are generally agreed to be more stable. The reaction with OAPA was reversed, however, during the hydrolysis preceding amino acid analysis of the peptides.

On the basis of the relative amounts of the singly modified derivatives (derivatives A, B, and D) noted earlier, the apparent

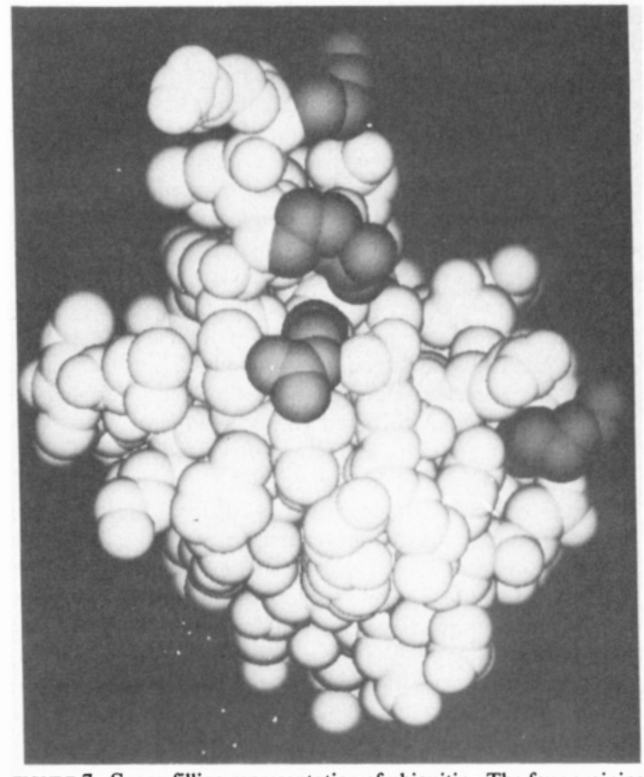

FIGURE 7: Space-filling representation of ubiquitin. The four arginine residues are shaded. From top to bottom: Arg-74, Arg-72, Arg-42, and Arg-54.

relative reactivities and/or accessibilities of the four arginines in ubiquitin with respect to this reagent can be ranked as Arg-42 > Arg-72 > Arg-74 ≫ Arg-54. It is interesting to note that the three-dimensional structure of ubiquitin shows that Arg-42, Arg-72, and Arg-74 are all located in a cluster at one end of the ubiquitin molecule with Arg-54 being somewhat remote from this area (Vijay-Kumar et al., 1985). Figure 7 shows a space-filling representation of the ubiquitin molecule, with the arginine residues highlighted in grey. Analysis of the environment of Arg-54 of ubiquitin indicates that this arginine is near two acidic residues, Asp-58 and Glu-51. This negatively charged environment probably reduces the reactivity of Arg-54 with the anionic reagent.

Activities of the Arginine Derivatives. The ability of the arginine derivatives to participate in various stages of ATP-dependent proteolysis has been examined. We have divided the reactions of proteolysis into three easily assayed steps; ATP–PP_i exchange catalyzed by E1; conjugation of ubiquitin to proteins catalyzed by E1, E2, and E3: and overall proteolysis catalyzed by the complete system.

The first step of proteolysis is the activation of the carboxyl terminus of ubiquitin by adenylation and subsequent intermolecular thiol ester formation (Haas et al., 1982). The function of this enzyme, E1, can be assayed by measuring the ubiquitin-dependent ATP–PP_i exchange reaction. In the presence of excess AMP, there is no net conversion to the E1–ubiquitin thiol ester. All four derivatives show a reduced ability to support this exchange reaction, although derivative D is 60% as active as native ubiquitin. These results suggest that the structure about the carboxyl terminus is recognized by the activating enzyme, an expected result since the adenylation occurs at the carboxyl-terminal residue. A closer examination of the available data, however, suggests that the region about residues 42 and 72 is particularly important. These two residues are clustered near and on the same face of the molecule as the protruding carboxyl terminus (Figure

7). The introduction of a bulky, charged group upon modification of these arginines apparently interferes with the required E1–ubiquitin interactions necessary for catalysis. In contrast, arginine-74 is oriented opposite to the protruding carboxyl terminus. The fact that the derivative of this residue has 60% activity in this assay suggests either that E1 does not interact strongly with this region of the molecule or that there is a sufficiently large substrate binding pocket to accommodate excess bulk in this region of the ubiquitin molecule.

After ubiquitin is activated by E1, two additional proteins, E2 and E3, participate in transferring the molecule to the target protein via the intermediacy of intermolecular thiol esters. Because of the significant level of ATP–PP_i exchange activity expressed by derivative D, we have examined the ability of native ubiquitin and derivative D to form steady-state conjugates. The ability of D to form a comparable level of steady-state conjugates from endogenous proteins (Figure 4) indicates that the modified protein is capable of participating in the remainder of the steps leading to conjugate formation. Furthermore, derivative D forms conjugates of reduced carboxymethylated bovine serum albumin at a rate very similar to those formed by the native protein (Figure 5). Thus, the modification of Arg-74 has little effect on the function of the protein with the enzymes of conjugation (E1, E2, and E3). The fact that essentially native levels of conjugates of both endogenous and exogenous proteins are formed with derivative D confirms that this modified protein can interact well with the activating enzyme, in support of the data reported above in the pyrophosphate exchange assays.

The final activity which we have examined is the stimulation of overall protein degradation and requires the function of E1, E2, E3, and the protease(s). None of the arginine derivatives is capable of supporting overall protein degradation. In particular, even though derivative D is fully functional in forming conjugates, these conjugates are not substrates for the proteolytic events. This suggests that the region about Arg-74 is vital for the function of some steps subsequent to conjugation. There are at least three different enzyme activities which could affect the net rate of proteolysis of these conjugates.

First, ubiquitin carboxyl-terminal hydrolytic activity may be necessary to regenerate free ubiquitin for subsequent catalytic cycles (Pickart & Rose, 1985b; Hershko & Rose, 1987). If the derivatization of Arg-74 interferes with this activity, it might explain our results. Two lines of evidence suggest this is not the case. First, the steady-state level of conjugates formed is similar with both derivative D and native ubiquitin (Figures 4 and 5). If the defect were at the level of ubiquitin carboxyl-terminal hydrolase, we would expect to see low molecular weight conjugates accumulate, and this is clearly not the case. Second, we have found that under the conditions of our assays, complete inhibition of UCH activity with ubiquitin aldehyde has no effect on the rate of proteolysis.[3] This appears to be due to the fact that ubiquitin is present in vast excess over that needed to account for the amount of proteolysis that occurs (Hershko & Rose, 1987).

The second possibility is that conjugates of derivative D are better substrates for the isopeptidase activity (Kanda et al., 1986) which deconjugates ubiquitin before proteolysis can occur. If that were the case, we would again expect to see a different steady-state level or pattern of conjugates accumulated with derivative D. It appears likely that the isopeptidase and the protease(s) act on different subpopulations of conjugates. Consistent with this, inhibition of isopeptidase activity with ubiquitin aldehyde increases the levels of steady-state conjugates without affecting the rate of proteolysis (Hershko & Rose, 1987). We have also observed that the inclusion of ubiquitin aldehyde (6 μg/mL) in experiments like those shown in Figure 5 has no effect on the rate of formation, or steady-state levels of ubiquitin–albumin conjugates (data not shown). This demonstrates that isopeptidase activity is not responsible for the decreased activity with derivative D.

The simplest explanation of our results is that modification of Arg-74 interferes with the ability of conjugates to be bound to or acted upon by the proteases of the system. Thus, the effect of this modification is opposite of the effects of iodination which increase the interaction of conjugates with the proteases of the system (Cox et al., 1986b).

Conclusions. These and earlier results (Cox et al., 1986b; Cox, 1986) begin to describe the structure and conformations of ubiquitin which react with enzymes of the protein degradation system. It is apparent that many regions of the molecule interact differently with the various enzymes of the system. In this study, we have shown that the region around Arg-42 and Arg-72 interacts with the activating enzyme, and that around Arg-74 interacts with the proteases. In an earlier study (Cox et al., 1986b), we showed that the modification of His-69 by iodination did not interfere with activation or conjugation of ubiquitin but destabilized the protein and led to an increased activity of the proteases on conjugates of this derivative. Alterations of residues 19, 24, and 28 as in yeast ubiquitin (Wilkinson et al., 1986), or residues 19, 24, and 57 as in oat ubiquitin (Vierstra et al., 1985, 1986), have no effect on its functioning in the proteolytic system. Finally, a series of Tyr-58 derivatives have revealed that the interactions of the protein with E1 are sensitive to this portion of the molecule.[4]

The knowledge gained from these studies is important for a number of reasons. These studies begin to explain the molecular interactions which participate in the specific degradation of intracellular proteins. Selective pressure on some portions of the ubiquitin molecule can be explained by these interactions. The lack of effect observed on alteration of some residues suggests that other functions of ubiquitin are responsible for selective pressure in these regions. A detailed map of these interactions could be exploited in designing specific inhibitors of these processes, tools which would be invaluable in defining the roles of ubiquitin and in possible pharmacological intervention. These studies also establish the possibility of using the much more powerful and flexible approach of site-directed mutagenesis. The fact that we can alter the structure and function of ubiquitin, without totally abrogating its activities, suggests that genetic approaches will be fruitful as probes of ubiquitin structure and function.

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