Site-Directed Mutagenesis of Ubiquitin. Differential Roles for Arginine in the Interaction with Ubiquitin-Activating Enzyme†

Timothy J. Burch and Arthur L. Haas*

Department of Biochemistry, The Medical College of Wisconsin, Milwaukee, Wisconsin 53226

Received January 27, 1994; Revised Manuscript Received April 5, 1994®

ABSTRACT: The strict evolutionary conservation of ubiquitin suggests an essential role for each residue in the folding, stability, or function of the protein but precludes identification of such contributions through interspecies comparison of ubiquitin sequences. However, site-directed mutagenesis potentially allows assignment of specific function(s) for each residue. The four arginines present on ubiquitin at positions 42, 54, 72, and 74 were independently mutated to leucine and their effects on the interaction of the resulting polypeptides with ubiquitin-activating enzyme (E1) were characterized. All of the mutants except UbR54L exhibited altered kinetics for E1-catalyzed ATP:PP_i exchange compared to wild-type ubiquitin. In addition, the UbR72L mutant altered the mechanism of E1 from strictly order addition of substrates to random addition with respect to ATP and ubiquitin. Values for the intrinsic K_d of ubiquitin binding were determined by coupling the net forward reaction of E1 to the $E2_{32K}$-catalyzed conjugation of histone H2B. Only R54 and R72 residues participate in the initial binding of free ubiquitin, resulting in a 6- or 58-fold increase in K_d for UbR54L or UbR72L, respectively, compared to wild type. More significant effects of the UbR42L and UbR72L mutants were observed for binding of their respective ubiquitin adenylate intermediates within the E1 active site. Wild-type ubiquitin adenylate binds to E1 with an estimated $K_d \leq 8 \times 10^{-12}$ M while intermediates formed with UbR42L or UbR72L each bind with *ca.* 10^3-fold lower affinity, representing a destabilization of ≥7 kcal/mol. In contrast, neither R54 or R74 significantly contribute to ubiquitin adenylate binding since their mutations did not exhibit the same behavior. These results indicate that three of the arginines contribute to the interaction between ubiquitin and E1 but do not quantitatively account for polypeptide binding, indicating roles for other residues. Moreover, the differential contributions of these residues to binding of either free ubiquitin or adenylate and the relative magnitudes of the effects are consistent with a significant reorientation of the active site during the catalytic cycle of E1.

All eukaryotes contain the highly conserved 8600-Da polypeptide ubiquitin and an enzyme pathway responsible for its covalent ligation to other intracellular protein targets [reviewed most recently by Hershko and Ciechanover (1992)]. This post-translational modification serves in a number of cellular functions including transcriptional regulation, cell cycle control, DNA repair, the stress response, receptor modification, and intracellular protein degradation (Hershko & Ciechanover, 1992). The ultimate fate for a subpopulation of these conjugates is degradation by the 26S multicatalytic protease complex (Hershko & Ciechanover, 1992). The system of enzymes catalyzing conjugation of ubiquitin to target proteins is initiated by the ATP-coupled activation of the carboxyl terminus of ubiquitin by E1,[1] the ubiquitin-activating enzyme [discussed in Haas et al. (1988)]. The resulting E1–ubiquitin thiol ester is then transferred in an exchange reaction to a conserved cysteine residue present on any of a family of low molecular weight carrier enzymes, collectively termed E2. The aminolytic cleavage of the E2–ubiquitin thiol ester is coupled to formation of an isopeptide bond between the carboxyl terminal glycine of ubiquitin and ε-amino groups present on the target protein in a step that may require participation of a third component, ubiquitin:protein isopeptide ligase (E3).

While much is known about the diverse roles of ubiquitin, very little is known about the exact mechanism(s) by which ubiquitination targets proteins for degradation or the precise interactions between the polypeptide and the enzymes responsible for its ligation. We have initiated a study to answer these questions using site-directed mutagenesis of ubiquitin. Ubiquitin is ideally suited for mutagenesis studies since the protein is relatively small, its physical properties and folding kinetics are known in some detail (Briggs & Roder, 1992; Woolfson et al., 1993), and a high-resolution crystal structure is available (Vijay-Kumar et al., 1987). In addition, the enzymes responsible for ubiquitin conjugation have been purified to apparent homogeneity, allowing chemically-defined mechanistic studies.

Our experimental approach has been to start from the carboxyl terminus and advance over the surface of ubiquitin selectively mutating single residues. We have initially concentrated on charged side chains for which mutation to relatively isosteric apolar amino acids obviates potential coulombic or hydrogen-bonding interactions. In this paper we present the first report of these studies, characterization of interactions between the four arginines of ubiquitin and E1. The E1-catalyzed step was chosen for these initial studies because it is the best characterized. In addition, our results can be compared to earlier chemical modification studies by Wilkinson and co-workers showing that R42, R72, and R74 contribute to the degradative targeting by ubiquitin and that chemical modification at R42 or R72 is more effective than

† This work was supported by United States Public Health Service Grant GM34009 (to A.L.H.).

* Author to whom correspondence should be addressed.

® Abstract published in *Advance ACS Abstracts,* May 15, 1994.

[1] Abbreviations: BSA, bovine serum albumin; DTT, dithiothreitol; E1, ubiquitin-activating enzyme; E2, ubiquitin carrier protein (subscript denotes relative molecular weight by SDS–PAGE); E3, ubiquitin:protein isopeptide ligase; EDTA, ethylenediaminetetraacetic acid; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PPase, yeast inorganic pyrophosphatase; SDS, sodium dodecyl sulfate; Ub, ubiquitin; UCH, ubiquitin carboxyl-terminal hydrolase.

0006-2960/94/0433-7300$04.50/0

at R74 in inhibiting E1-catalyzed ATP:PP_i exchange (Duerksen-Hughes et al., 1987). However, the chemical modification employed previously substituted a large anionic group for the positively charged imido moiety of arginine. Consequently, possible secondary steric effects make an unambiguous interpretation of the data difficult.

The mechanism of ubiquitin activation by E1 is known in detail, the three principal steps of which are represented below (Haas & Rose, 1982):

$$E1 + Ub + ATP{\cdot}Mg \rightleftharpoons E1^{\cdot AMP\text{–}Ub} + PP_i \quad (1)$$

$$E1^{\cdot AMP\text{–}Ub} \rightleftharpoons E1_{s\text{–}Ub} + AMP \quad (2)$$

$$E1_{s\text{–}Ub} + Ub + ATP{\cdot}Mg \rightleftharpoons E1_{s\text{–}Ub}^{\cdot AMP\text{–}Ub} + PP_i \quad (3)$$

In step 1, a tightly-bound mixed anhydride between ubiquitin and AMP is formed between the carboxyl terminus of ubiquitin and the α-phosphate of AMP. The ubiquitin adenylate is transferred in step 2 to a reactive cysteine on E1 to form an enzyme-bound ubiquitin thiol ester and free AMP. In step 3 formation of a second ubiquitin adenylate yields the final ternary complex. There are three salient features of this mechanism: (1) both E1-catalyzed ATP:PP_i and ATP:AMP exchange reactions occur only in the presence of ubiquitin (Haas et al., 1982; Haas & Rose, 1982); (2) the sequence of substrate addition and product release is strictly ordered with ATP binding before ubiquitin and PP_i dissociating before AMP (Haas & Rose, 1982); and (3) the enzyme-bound ubiquitin adenylate does not measurably dissociate so that a 1:1 stoichiometry exists, in the absence of PP_i, between enzyme and intermediate in the final ternary complex of step 3. The present results show differential effects for binding of ubiquitin or ubiquitin adenylate to E1 following mutation of single arginine residues, suggesting substantial reorientation of the active site during the catalytic cycle of the enzyme. In addition, a single mutation at R72 is unique in changing the normal ordering of substrate addition.

EXPERIMENTAL PROCEDURES

Materials. Bovine ubiquitin and HPLC purified yeast inorganic pyrophosphatase were obtained from Sigma Chemical Co. Ubiquitin was radioiodinated by the chloramine T procedure as previously described (Haas et al., 1982). Carrier-free Na[^{125}I], [2,8-^{3}H]ATP, and [^{32}P]PP_i were purchased from NEN-Dupont. Fresh rabbit reticulocytes were prepared by phenylhydrazine induction (Haas & Rose, 1981) and used to prepare E1 and $E2_{32K}$ (Haas & Bright, 1988). Histone H2B was provided by Dr. Vaughn Jackson (Medical College of Wisconsin). The recombinant L3 isozyme of ubiquitin carboxy terminal hydrolase (UCH-L3) was the generous gift of Dr. Keith D. Wilkinson (Emory University Medical School).

Generation of Ubiquitin Mutants. The synthetic human ubiquitin gene contained in the plasmid pNMhUb (Ecker et al., 1987) was used as the template to generate all mutants. Mutagenesis to yield UbR42L, UbR72L, and UbR74L was based on the in vitro method of Eckstein (Amersham). For mutation to yield UbR54L, the full-circle PCR method of Hemsley and co-workers (Hemsley et al., 1989) was employed using pPLhUb, a shortened construct of pNMhUb generated by restricting the latter with AatII and EcoRI. The AatII and EcoRI fragment containing the Ub gene and promoter sequences was subcloned into the complementary sites of pUC18, retaining ampicillin resistance selection and the origin of replication. The resulting pPLhUb plasmid (3500 bp) was substantially smaller than pNMhUb (5200 bp), allowing for more efficient amplification in the full-circle PCR method.

Purification of the mutant proteins was modified from that described by Haas and Wilkinson (1985). Briefly, 10 L of *E. coli* strain AR58 were grown at 30 °C in a 20-L fermenter (Vitris). When the cells reached an A_{600} of 1.0, ubiquitin induction was initiated by ramping the temperature to 42 °C (1 °C/min). Induction continued for an additional 1.5 h. The cells were harvested by centrifugation, resuspended to 30% (w/v) in 25 mM NH_4–acetate buffer (pH 7.0) and then lysed by two passes through a French pressure cell (SLM-AMINCO) at 15,000 psi. The original heat and TCA precipitation steps (Haas & Wilkinson, 1985) were omitted to avoid possible irreversible denaturation of the mutant proteins which would complicate interpretation of later data. Instead, the cell lysate was centrifuged 90 min at 100000*g* to remove insoluble debris. The resulting supernatant was batch absorbed onto 300 mL of a 50% (v/v) slurry of DEAE-52 (Whatman) equilibrated in the lysis buffer. The unbound fraction from DEAE-52 was separated by mild vacuum filtration through 3MM filter paper (Whatman). The DEAE-52 bed was washed with two volumes of lysis buffer and pooled with the unabsorbed fraction. The sample was then adjusted to pH 4.5 with glacial acetic acid. Precipitated protein was removed by centrifugation at 10000*g* for 10 min. The supernatant was applied to a 2.5- × 10-cm CM-52 column (Whatman) equilibrated in 25 mM NH_4–acetate (pH 4.5). The column was washed with two bed volumes of equilibrating buffer and then eluted with 50 mM NH_4–acetate, pH 5.5 (Haas & Wilkinson, 1985). Ubiquitin-containing fractions were dialyzed overnight against 4 L of distilled water at 4 °C using 3.5-kDa exclusion tubing. After dialysis, the pH was adjusted to 4.5 with acetic acid. The sample was applied to a HR 10/10 Mono-S FPLC column (Pharmacia) equilibrated in 25 mM NH_4–acetate buffer (pH 4.5). Ubiquitin eluted at 0.2 M NaCl in a linear gradient (2.7 mM/min). If necessary, trace contaminants were removed by subsequent passage through a HR 5/10 Mono-Q FPLC column (Pharmacia) equilibrated in 20 mM ethanolamine (pH 9.5) or gel filtration on an HR 10/30 Superose-12 column equilibrated in 50 mM Tris-Cl (pH 7.5), 50 mM NaCl. Absolute ubiquitin concentrations for the homogeneous preparations were calculated spectrophotometrically from the empirically determined extinction coefficient of 0.16 $(mg/mL)^{-1}$ at 280 nm (Haas & Wilkinson, 1985). Typical yields of pure protein ranged from 1 to 15 mg/L of culture.

ATP:PP_i Exchange Assay. The assay was performed as previously described (Haas et al., 1982). Incubations of 50 μL final volume contained 50 mM Tris-Cl (pH 7.5), 1 mM ATP, 10 mM $MgCl_2$, 1 mM DTT, 0.1 mM [^{32}P]PP_i (20–40 cpm/pmol), 0.25 pmol of E1, and the indicated concentrations of ubiquitin or ubiquitin mutant. The reactions were quenched after 20 min at 37 °C by the addition of 500 μL of 5% (w/v) TCA containing 4 mM carrier PP_i. The ^{32}P-labeled ATP was isolated by the addition of 300 μL of 10% (w/v) slurry of activated charcoal in 2% TCA. The charcoal was separated by centrifugation, and the pellet was washed with 3 × 800 μL of 2% TCA. Radioactivity incorporated into ATP was determined by Cerenkov counting. Data represent duplicate determinations which routinely agreed within 5% of the mean value. The blank in the absence of E1 generally represented less than 0.1% of total radioactivity.

Ubiquitin [^{3}H]Adenylate Assays. The production of the ubiquitin [^{3}H]adenylate intermediate was as previously described (Haas et al., 1982). Wild-type or mutant ubiquitin

was incubated for the indicated times at 37 °C in a final volume of 50 μL containing 50 mM Tris-Cl (pH 7.5), 0.76 μM [^{3}H]ATP (20,000 cpm/pmol), 10 mM $MgCl_2$, 1 mM DTT, 1 IU PPase, 1 mg/mL carrier BSA, and 1 pmol E1. The blank in the absence of E1 generally represented less than 0.2% of total radioactivity. The reactions were quenched by the addition of 200 μL of 20% TCA. The reactions were kept on ice for 10 min and then centrifuged for 15 min at 15 000 rpm to pellet the precipitated protein. The protein pellet was rinsed twice with 1 mL of 2% TCA and then dissolved in 200 μL of 0.2 M triethanolamine–HCl (pH 8.0). The dissolved pellet was quantitatively transferred to a vial containing 5 mL of Econo-Safe (RPI) for liquid scintillation counting.

The production of free ubiquitin [^{3}H]adenylate was carried out for each of the mutants in a similar manner as described above, except the reactions contained 11.5 pmol of E1 and saturating concentrations of ubiquitin or mutant (Haas et al., 1983). The reactions were quenched as above with 200 μL of 20% TCA and rinsed twice with 2% TCA. The protein pellets were dissolved in 200 μL of 0.1 M Tris-Cl (pH 7.5) and then used as the source of free labeled intermediate.

Ubiquitin–H2B Conjugation Assays. The ability of wild-type or mutant ubiquitin to support covalent conjugation to histone H2B was tested as described (Haas et al., 1988). Briefly, the indicated concentrations of ^{125}I-labeled wild-type or mutant ubiquitin were incubated for 30 min at 37 °C in a 50 μL volume containing 50 mM Tris-Cl (pH 7.5), 2 mM ATP, 10 mM $MgCl_2$, 1 mM DTT, 1 IU PPase, 15 μM H2B, 20 nM E1, and 30 nM $E2_{32K}$. Reactions were quenched with 50 μL of SDS sample buffer containing 3% (v/v) 2-mercaptoethanol and then boiled for 5 min. Samples were resolved by 10% SDS–PAGE after which the gel was dried and visualized by autoradiography using Kodak X-Omat film. The band corresponding to monoubiquitinated H2B was excised from the dried gel and the amount of radioactivity was determined by γ-counting. Data were corrected for radioactivity present in control lanes derived from incubations performed in the absence of E1 and E2.

RESULTS

Differential Effects of Arginine Mutants on the Rates of ATP:PP$_i$ Exchange. Mutants were initially tested for their ability to support the ATP:PP$_i$ exchange reaction catalyzed by ubiquitin-activating enzyme. Initial rates were determined in the absence of AMP in order to measure step 3 in which ^{32}PP$_i$ exchanges through the ubiquitin adenylate intermediate of the ternary complex (Haas et al., 1982; Haas & Rose, 1982). The concentration dependence on the rates of exchange for wild-type and mutant ubiquitins is shown in Figure 1. Wild-type ubiquitin exhibits a marked substrate inhibition above 4 μM that is characteristic of the ordered addition of reactants and the submicromolar K_d for binding of free ubiquitin to the thiol ester form of the E1·ATP complex (Haas & Rose, 1982). All four arginine mutants support ATP:PP$_i$ exchange;[2] however, they differ in their concentration dependence from that of the wild-type polypeptide. The UbR42L, UbR54L, and UbR74L mutants resemble wild type in exhibiting substrate inhibition for the exchange reaction, indicating an ordered mechanism in which ATP is the leading and ubiquitin the trailing substrate (Haas & Rose, 1982). The results of Figure 1 agree with the earlier studies of

[2] The four mutants also show stoichiometric formation of their respective E1 thiol esters and, thus, presumably also support ATP:AMP exchange (not shown).

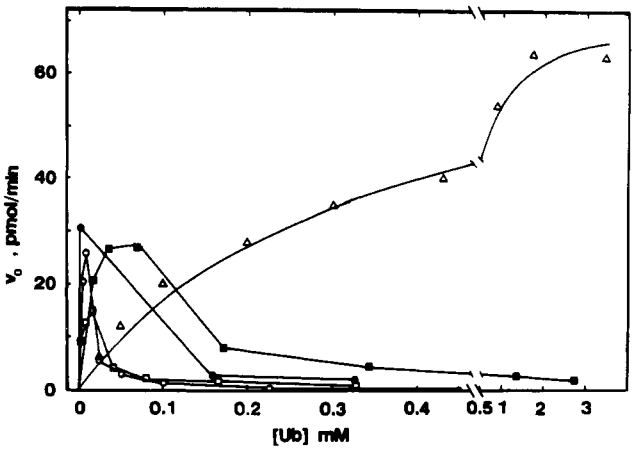

FIGURE 1: Ubiquitin concentration dependence for E1-catalyzed ATP:PP$_i$ exchange. The ubiquitin dependence on ATP:PP$_i$ exchange was determined as described in the Experimental Procedures. The initial velocity of exchange was measured at the indicated concentrations of wild-type (●), UbR42L (■), UbR54L (○), UbR72L (△), or UbR74L (□) ubiquitins. The curve through the data for UbR72L represents a theoretical fit based on the kinetic constants listed in Table 1.

Table 1: Kinetics of ATP:PP$_i$ Exchange[a]

	$K_{1/2}$ (μM)	k_{cat} (s^{-1})	$v_{limiting}$ (pmol/min)
w.t.	0.78 ± 0.24	2.7 ± 0.21	0.89 (2.1%)
R42L	18.3 ± 1.8	4.1 ± 0.17	2.3 (3.7%)
R54L	1.9 ± 0.17	2.8 ± 0.09	0.53 (1.2%)
R72L	317 ± 32	4.0 ± 0.04	65 (100%)
R74L	17.3 ± 1.5	5.2 ± 0.35	0.19 (0.25%)

[a] Values of $K_{1/2}$ and k_{cat} (± SE) were determined as described in the Experimental Procedures at ubiquitin concentrations below the onset of substrate inhibition. The limiting velocity for substrate inhibition ($v_{limiting}$) was determined at saturating concentrations for wild type (320 μM), UbR42L (2.74 mM), UbR54L (655 μM), and UbR74L (450 μM). Values in parentheses are the percent of the maximal velocity graphically determined from corresponding reciprocal plots at ubiquitin concentrations below the onset of substrate inhibition.

Duerksen-Hughes *et al.* (1987) using the arginine-specific reagent 4-(oxoacetyl)phenoxyacetate. In those studies, ubiquitin stoichiometrically modified at R74, R72, or R42 was capable of supporting E1-catalyzed ATP:PP$_i$ exchange, although with altered kinetics. The effect of modification at R54 was not examined since derivatives at this residue could not be obtained. Exchange kinetics with the chemically modified ubiquitins were not conducted at sufficiently high concentrations to observe substrate inhibition; however, in the V/K region the rank order of the ubiquitins in supporting ATP:PP$_i$ exchange was wild type > R74 > R72 > R42. In contrast, the results of Figure 1 within a comparable V/K region before onset of substrate inhibition indicate a rank order of wild-type > R54 > R74, R42 > R72. The inversion of order for R42 *versus* R72 between the results obtained from chemical modification or mutagenesis likely reflects effects of the bulky modifying group on the internal steps of E1.

Double reciprocal plots were linear for wild-type ubiquitin and the UbR42L, UbR54L, and UbR74L mutants at concentrations below the onset of substrate inhibition. The kinetic constants of exchange, $K_{1/2}$ and V_{max}, were determined for each of the polypeptides from these plots and are summarized in Table 1. In Table 1, V_{max} is expressed as the corresponding k_{cat} since $[E1]_o$ could be accurately determined by the stoichiometric ubiquitin [^{3}H]adenylate assay described in the Experimental Procedures (Haas et al., 1982). The $K_{1/2}$

for wild type determined here is in good agreement with the value of 1.2 μM previously reported (Haas & Rose, 1982). The UbR54L mutant exhibited a $K_{1/2}$ value approximately twice that of wild-type ubiquitin while both UbR42L and UbR74L mutants yielded values approximately 20-fold greater. Two of the mutants also exhibited k_{cat} values statistically greater than wild-type ubiquitin; however, the standard error in k_{cat} values listed in Table 1 probably underestimates the actual variation in these constants due to the restrictions imposed on the reciprocal plots by the observed substrate inhibition, as discussed previously (Haas & Rose, 1982).

The UbR72L mutant was unique in showing hyperbolic kinetics over the full range of concentrations tested (Figure 1) and yielded a value of $K_{1/2}$ that was 400-fold greater than that of wild-type ubiquitin, Table 1. The onset of substrate inhibition for the other three arginine mutants occurred at concentrations close to that of their respective $K_{1/2}$ values (Table 1); however, no substrate inhibition by UbR72L was observed at a concentration of 3.7 mM, 10-fold greater than the experimentally determined $K_{1/2}$ and 100-fold greater than the intrinsic K_d for UbR72L binding subsequently determined in a coupled kinetic assay (below). The latter observation indicates that the UbR72L mutation results in either an inverted or random order of substrate addition. The former interpretation was ruled out by the hyperbolic kinetics observed for the ATP dependence on the reaction at saturating UbR72L (not shown). In addition, the $K_{1/2}$ for ATP determined from the double reciprocal plot for UbR72L was identical within experimental error to that of wild-type ubiquitin. Therefore, the most significant effects of the UbR72L mutation are an altered order of substrate addition and a marked decrease in the apparent affinity of the ubiquitin variant.

Determination of the Intrinsic K_d for the Arginine Mutants. The values of $K_{1/2}$ determined from the ATP:PP_i exchange kinetics (Table 1) cannot be directly equated with the respective K_d values of the mutants since the $K_{1/2}$ is a complex constant composed of the internal rate and equilibrium constants for formation of the ternary E1 species (Haas & Rose, 1982). Moreover, the k_{cat} can reflect either a forward or reverse step since the system is at equilibrium by definition (Haas et al., 1982; Haas & Rose, 1982). For these reasons K_d values were determined for ^{125}I-labeled mutant and wild-type ubiquitins by measuring the rate-limiting net forward reaction of E1 in a reaction coupled to the $E2_{32K}$-catalyzed monoubiquitination of histone H2B (Haas & Bright, 1988; Haas et al., 1988; Haas et al., 1991).

Three observations indicate that this approach yields accurate estimates of the intrinsic K_d for ubiquitin: (1) the k_{cat} for $E2_{32K}$-catalyzed monoubiquitination of H2B at saturating substrates and the E1 ternary complex is significantly less than the k_{cat} for either ATP:PP_i or ATP:AMP exchange (Haas & Rose, 1982; Haas et al., 1988), allowing for equilibration of ubiquitin binding to E1 and for E1 ternary complex binding to the E2 isozyme; (2) E1 and $E2_{32K}$ concentrations were empirically set to be saturating with respect to binding of the carrier protein to the E1 ternary complex (K_d = 0.4 μM; Haas et al., 1988); and (3) the K_d of 0.39 μM for wild-type ubiquitin shown in Table 2 agrees favorably with the intrinsic K_d of 0.58 μM determined by equilibrium methods (Haas & Rose, 1982).

Comparing the data from Tables 1 and 2, there is little relationship between the $K_{1/2}$ determined by ATP:PP_i exchange and the K_d determined in the coupled assay. The UbR42L and UbR74L mutants possess $K_{1/2}$ values that are

Table 2: Effect of Mutations of the Net Forward Reaction of E1[a]

	K_d (μM)	V_{max} (pmol/min)
w.t.	0.39 ± 0.24	0.09 ± 0.01
R42L	0.55 ± 0.03	0.06 ± 0.03
R54L	2.35 ± 0.96	0.31 ± 0.05
R72L	22.8 ± 5.2	0.13 ± 0.02
R74L	0.33 ± 0.17	0.09 ± 0.01

[a] The K_d of ubiquitin was determined in a coupled assay linking the ubiquitin dependence of E1 activation to $E2_{32K}$-catalyzed H2B formation as described in the Experimental Procedures. Values of K_d represent the intrinsic dissociation constants for ubiquitin binding to E1.

20-fold greater than that of wild-type ubiquitin but K_d values which are statistically equivalent to wild type. This indicates that the UbR42L and UbR74L mutations affect steps within the E1 reaction other than that of polypeptide binding. In contrast, the other two arginine mutations affect ubiquitin binding and possibly other steps within the activation mechanism. From the K_d values of Table 2, the contribution to ubiquitin recognition follows the order R72 > R54 > R42, R74. The comparable V_{max} values among the ubiquitin variants indicates that the four arginine mutations have no measurable effect on the E3-independent, $E2_{32K}$-catalyzed conjugation step. This is because the rate-limiting step, when E1 is saturated with respect to ubiquitin, lies within the $E2_{32K}$-catalyzed ubiquitination of the H2B substrate, as shown by the linearity in V_{max} *versus* $[E2_{32K}]_o$ (not shown). Therefore, the V_{max} values reflect potential interactions between ubiquitin and $E2_{32K}$ within their resulting thiol ester adduct.

The Arginine Mutants Alter Binding of the Ubiquitin Adenylate Intermediate. Values of K_d for substrate and product binding to E1 as well as the internal equilibrium constants for E1 ternary complex formation have previously been determined by quantitating enzyme-bound ubiquitin [^{3}H]-adenylate formation in response to altered concentrations of reactants and products (Haas & Rose, 1982). Such studies are possible because of the unique nature of E1 ternary complex formation in isolation, the stoichiometric formation of ubiquitin adenylate in the absence of AMP and PP_i products, and the extremely high affinity for binding of the adenylate intermediate to the activating enzyme (Haas et al., 1982; Haas & Rose, 1982; Haas et al., 1983). When similar equilibrium studies were attempted with the four arginine mutants, the results were consistent with formation of the corresponding adenylates in excess of the amount of E1 present (not shown). This conclusion was confirmed in experiments similar to that shown in Figure 2 for the UbR72L mutant which compares the time course for formation of wild-type ubiquitin [^{3}H]-adenylate to UbR72L [^{3}H]adenylate in the presence of 1 pmol of E1.

For wild-type ubiquitin, formation of the adenylate intermediate is stoichiometric with the amount of E1 and is complete by the first time point (30 s), Figure 2. Assuming at least 95% completion at 30 s, then the first-order rate constant for formation of wild-type ubiquitin [^{3}H]adenylate within the E1 ternary complex can be estimated as ≥6 min^{-1}. In contrast, the first-order rate constant for formation of UbR72L [^{3}H]-adenylate (0.53 min^{-1}) is less than one-tenth that of wild type and reaches an end point that is 0.75 pmol in excess of the E1 present in the incubation. The latter results are consistent with dissociation of UbR72L adenylate from E1; however, the results constrain the free and bound pools to equilibrate rapidly since total intermediate does not continue to accumulate, Figure 2. The other arginine mutants were tested for their ability to form ubiquitin adenylates in excess of the stoichiometric amount of E1. Incubations were for 5 min

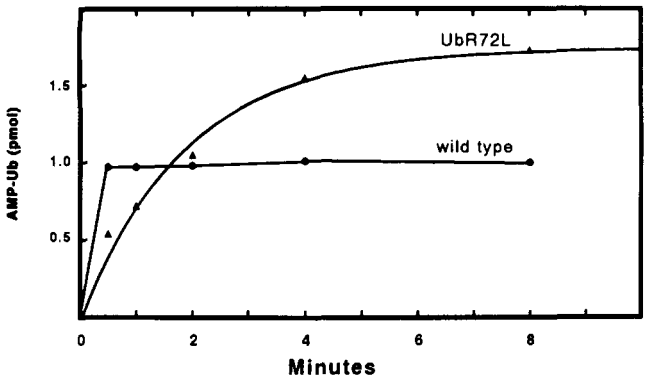

FIGURE 2: Time course of ubiquitin [^{3}H]adenylate formation. Incubations of 0.275 mL were as described in the Experimental Procedures and contained 20 nM E1, 0.76 μM [^{3}H]ATP (29 900 cpm/pmol), 5.5 IU HPLC-purified yeast inorganic pyrophosphatase, and 100 μM of either wild-type (●) or R72L ubiquitin (▲). At the indicated times 50-μL aliquots were removed and quenched into 200 μL of 20% (w/v) TCA. Ubiquitin [^{3}H]adenylate formed was calculated from the TCA precipitated radioactivity using the specific activity of the [^{3}H]ATP. The curve through the data for UbR72L represents a theoretical fit for a first-order reaction having a k_o of 0.53 min and an end point of 1.74 pmol.

Table 3: Steady-State Levels of Ubiquitin [^{3}H]Adenylate[a]

	Ub–AMP (pmol)	$\%E_{total}$
w.t.	1.02 ± 0.02	100
R42L	1.16 ± 0.06	114
R54L	1.00 ± 0.03	98
R72L	1.75 ± 0.02	175
R74L	1.13 ± 0.01	110

[a] Ubiquitin [^{3}H]adenylate assays were performed as described in the Experimental Procedures. Activating enzyme (1 pmol) was incubated for 5 min with the indicated wild-type or mutant ubiquitin, 0.76 μM [^{3}H]ATP, and 1 IU PPase in a final volume of 50 μL. The data represent assays performed in triplicate to yield the average ± SE. Ubiquitin concentrations were 50 μM for wild type or UbR42L and 100 μM for UbR72L, and UbR74L.

under conditions identical to those used in Figure 2. Parallel studies showed that the incubation time was sufficient for the putative free pool to reach equilibrium (not shown). The UbR72L mutant accumulated to 175% of the stoichiometric amount of E1, Table 3, in good agreement with the results of Figure 2. In addition, the UbR42L and UbR74L mutants also produced a small but statistically significant excess in their respective adenylates, Table 3. The R54L ubiquitin was the only mutant to consistently produce an adenylate intermediate equal to that of E1.

To test whether the excess ubiquitin adenylate represented a free pool of intermediate, its susceptibility to trapping by ubiquitin carboxyl terminal hydrolase was examined. Ubiquitin carboxyl terminal hydrolases are a class of ubiquitin-specific enzymes thought to serve a salvage role in regenerating functional ubiquitin from free small molecule adducts such as ubiquitin adenylate, ubiquitin–glutathione thiol ester, and ubiquitin–lysine amide that may form by dissociation or as side reactions (Pickart & Rose, 1985). Incubations identical to those of Table 3 were conducted in the absence or presence of 1.3 mIU of recombinant UCH-L3, the major human B cell isozyme (Wilkinson et al., 1989). In the presence of UCH-L3 no change in the amount of wild-type ubiquitin adenylate was noted (Table 4), indicating that bound intermediate is protected from hydrolytic trapping by the hydrolase. In contrast, free ubiquitin adenylate generated by TCA precipitation of the E1 ternary complex followed by neutralization and solubilization of the intermediate (Haas et al., 1983) was

Table 4: Effect of UCH on the Steady-State Level of Ubiquitin [^{3}H]Adenylate[a]

	– UCH (pmol)	+ UCH (pmol)
w.t.	0.97	0.99
R42L	1.22	1.02
R54L	0.79	0.80
R72L	1.86	0.61
R74L	1.10	1.06

[a] Ubiquitin [^{3}H]adenylate assays were performed as described in the Experimental Procedures for 5 min in a final volume of 50 μL containing 1 pmol E1, 0.76 μM [^{3}H]ATP, 1 IU PPase, and 100 μM ubiquitin. Parallel incubations were conducted in either the absence or presence of 1.3 mIU UCH-L3.

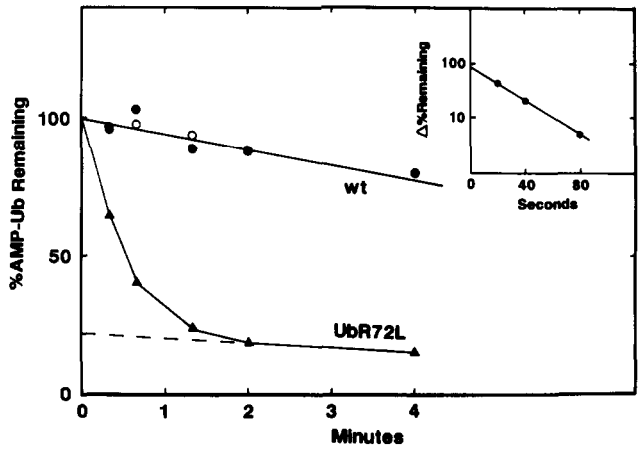

FIGURE 3: Kinetic determination of free and bound adenylate. Wild-type (●) or UbR72L (▲) ubiquitin [^{3}H]adenylate was formed in a 5-min incubation as described in the legend to Figure 2. Zero time samples of 50 μL were taken and quenched into 200 μL of 20% (w/v) TCA after which further adenylate formation was blocked by addition of EDTA to a final concentration of 10 mM. Additional aliquots were taken at the indicated times to monitor the loss of adenylate in the absence (○) or presence of 1.3 mIU of recombinant UCH-L3. Inset: first-order replot of the difference between data points for the rapid phase of UbR72L [^{3}H]adenylate loss and the extrapolated slower linear phase.

completely hydrolyzed with a $t_{1/2}$ of 30 s in the presence of 1.3 mIU of hydrolase (not shown).[3] Significant reduction in the steady-state level of ubiquitin adenylate was noted in the presence of UCH-L3 for the UbR42L and UbR72L mutants, Table 4. A small reduction in the UbR74L adenylate was also noted; however, the difference was within the reproducibility of the assay (ca. ± 2%). The UbR54L mutant consistently failed to show any loss of adenylate in the presence of UCH-L3, Table 4. In separate control experiments all of the free mutant ubiquitin adenylates were hydrolyzed by 1.3 mIU of UCH-L3 with $t_{1/2}$ ranging from 30 s for UbR74L to 59 s for UbR42L (not shown). The latter observations rule out a free pool of mutant adenylate resistant to trapping by the hydrolase.

Estimation of K_d for Mutant Ubiquitin Adenylate Binding to E1. Because the steady-state amount of UbR72L adenylate was less than that of E1 in the presence of UCH, the data of Table 4 suggest this mutant produces either a lower steady-state level of enzyme-bound intermediate than wild-type polypeptide or the bound intermediate is sensitive to UCH. To distinguish between these two possibilities the time course for trapping by UCH-L3 was examined, Figure 3. Activating enzyme (1 pmol) was loaded with either wild type or UbR72L

[3] This important control experiment is possible because ubiquitin and its corresponding adenylate are stable to TCA precipitation (Haas et al., 1983).

ubiquitin for 5 min to allow equilibration between bound and free pools (Figure 2). The incubations were then adjusted to 10 mM EDTA to block further loading, after which 1.3 mIU of UCH-L3 was added to the reactions. Aliquots were taken at the indicated times and the remaining ubiquitin [^{3}H]-adenylate determined by TCA precipitation.

The rates of loss for E1 ternary complex-bound wild-type ubiquitin adenylate were identical in the absence or presence of hydrolase and compared well with similar values arising by the combined effects of hydrolysis of the bound intermediate and transthiolation to DTT present in the incubations (Haas et al., 1982). In contrast, trapping of UbR72L adenylate exhibited biphasic kinetics, Figure 3. By extrapolating the slower limiting rate of adenylate loss to t_0 and plotting the difference between this line and the time course, it was possible to resolve the two kinetic components (inset to Figure 3). The initial rapid loss of adenylate was first order as shown by the linearity of the difference plot and corresponded to a rate constant of 0.018 ± 0.002 s^{-1} (see inset). The slower limiting rate for loss of mutant adenylate was similar to that observed with wild-type ubiquitin in either the absence or presence of UCH (Figure 3) or for mutant adenylate in the absence of added hydrolase (not shown). These observations are consistent with the slow dissociation of the mutant adenylate from E1 with a $t_{1/2}$ substantially longer than the duration of the experiment. In contrast, the first-order rate constant for rapid initial loss of mutant adenylate was proportional to the amount of UCH in the incubations (not shown), indicating that trapping rather than dissociation of a weakly bound pool of intermediate was rate limiting. This conclusion was supported by the good agreement between the rate constants determined for the first-order hydrolysis of free UbR72L adenylate by UCH-L3 determined above and that of the initial exponential loss in Figure 3.

The results of Table 4 and Figure 3 are consistent with free and bound pools of UbR72L adenylate, only the former of which is sensitive to trapping by UCH. If one extrapolates the limiting rate to zero time, then the rapidly hydrolyzed fraction (79%) must represent the free pool and the residual (21%) must correspond to the bound pool. This model represents the following dissociation equilibrium of mutant adenylate from the ternary complex

$$E_{s\text{-}Ub}^{AMP\text{-}Ub} \rightleftharpoons E_{s\text{-}Ub} + AMP\text{–}Ub_{(free)}$$

from which a K_d of 49×10^{-9} M for UbR72L adenylate can be calculated from the appropriate equilibrium expression. Wild-type ubiquitin adenylate binds to E1 with an affinity too high to experimentally determine; however, if one assumes the minimum amount of free wild-type ubiquitin adenylate that could have been detected corresponds to the measurement error of 2% for ubiquitin [^{3}H]adenylate, then the corresponding K_d for wild-type intermediate is $\leq 8 \times 10^{-12}$ M, in good agreement with the value of $\leq 4 \times 10^{-12}$ M estimated from the internal equilibrium constants (Haas & Rose, 1982).

Experiments similar to that of Figure 3 were conducted with the other arginine mutants. Both UbR74L and UbR54L failed to show biphasic kinetics but exhibited slow linear loss of intermediate identical to wild-type ubiquitin. These results are consistent with the conclusion from Table 4 that UbR74L and UbR54L do not generate a significant pool of free intermediate. However, the results with UbR42L were similar to those of Figure 3 for UbR72L (not shown). Extrapolation of the time course yielded estimates for the free and bound UbR42L adenylate pools of 59% and 41%, respectively, from which a K_d of 16×10^{-9} M was calculated.

DISCUSSION

The systematic mutagenesis of ubiquitin provides an experimentally tractable approach for mapping active site interactions of the enzymes required for ubiquitin conjugation in the absence of additional structural information for these proteins. From this rationale we have mutated each of the four arginines present on ubiquitin to leucine, a substitution that retains the overall steric profile of the polypeptide but obviates potential coulombic or hydrogen bonding interactions with the guanidinium side chain of arginine. To demonstrate the utility of this approach these mutants have been exploited to probe interactions within the first step of ubiquitin conjugation, the activation of its carboxyl terminus catalyzed by E1. The results extend previous group specific modification studies of these arginine residues (Duerksen-Hughes et al., 1987) and demonstrate that the four residues contribute differentially to interactions between E1 and bound ubiquitin or the resulting adenylate intermediate.

By coupling the E1 reaction to the $E2_{32K}$-catalyzed conjugation of H2B as a model substrate for E3-independent ligation, the net forward reaction of ubiquitin activation and the intrinsic K_d for binding of free ubiquitin could be measured (Table 2) since established equilibrium approaches (Haas & Rose, 1982) were precluded by dissociation of some mutant adenylates. The validity of this kinetic approach was confirmed by the good agreement between the K_d of 0.39 ± 0.24 μM for wild-type ubiquitin determined kinetically in this study and 0.58 μM reported from equilibrium measurements (Haas & Rose, 1982). Only two of the four arginine residues, R54 and R72, significantly contribute to binding of free ubiquitin to the thiol ester form of the E1·ATP binary complex. Arginine-72 is the most important of the four arginine residues in binding of free ubiquitin since the UbR72L mutant yields a K_d of 22.8 ± 5.2 μM, representing a 58-fold increase over that for wild-type polypeptide, Table 2. Therefore, interaction-(s) between E1 and R72 accounts for 2.4 kcal/mol of the 8.7 kcal/mol apparent binding energy for ubiquitin. The contribution of R54 to E1–ubiquitin interactions reflected in the intrinsic K_d is 1 order of magnitude less than that of R72. The K_d of 2.35 ± 0.96 μM for UbR54L represents a 6-fold increase for this dissociation constant or approximately 1.1 kcal/mol for E1 binding. The data of Table 2 indicate that neither R42 or R74 significantly participates in E1–ubiquitin interactions. The absence of a contribution by R74 to ubiquitin binding was unexpected since the carboxyl terminal LRGG sequence is disordered and completely exposed to solvent (Vijay-Kumar et al., 1987), making this residue a prime candidate for such interactions. However, it remains possible that R74 participates in orienting the carboxyl terminus of ubiquitin within the active site and that the energy of this interaction is below our limit of kinetic detection.

Early work demonstrated that the obligatory ubiquitin adenylate intermediate is tightly bound to E1 with an estimated $K_d \leq 4 \times 10^{-12}$ M such that no free pool of this species is normally observable (Haas et al., 1982; Haas & Rose, 1982). This characteristic has been exploited as a stoichiometric functional assay for active E1 (Haas et al., 1982) and a probe for experimentally determining the internal equilibrium constants and K_d for substrates and products (Haas & Rose, 1982). Analogous experiments in the present studies demonstrate that two of the four mutants form steady-state levels of ubiquitin [^{3}H]adenylate significantly in excess of the stoichiometric amount of E1 present, Table 3. A fraction of the steady-state ubiquitin [^{3}H]adenylate pool for UbR42L and UbR72L is labile to hydrolysis by the action of exogenous

tRNA synthetase					
val (S. cer.)	191	NVTGALHIG-----HA	696	VRDAQGRKMS-KSLGNVIDP	
val (N. crass.)	180	NVTGALHCG-----HA	685	IRDSEGRKMS-KSLGNVIDP	
val (human)	345	NVTGSLHLG-----HA	856	VRDAHGRKMS-KSLGNVIDP	
gln (E. coli)	34	EPNGYLHIG-----HA	261	RLNLEYTVMS-KRKLNLLVT	
met (E. coli)	13	YANGSIHLG-----HM	324	YVTVNGAKMS-KSRGTFIKA	
glu (B. stear.)	13	SPTGHLHIG-----GA	245	IVNEQRKKLS-KRDESIIQF	
tyr (B. stear.)	39	PTADSLHIG-----HL	223	VTKADGTKFG-KTESGTIWL	
Activating enzyme					
uba1 (human)	324	SRPAQLHIGFQAL-HQ	293	SQVKVPKKISFKSLVASLAE	
uba1 (mouse)	324	SRPAQLHIGFQAL-HQ	293	SQVKVPKKISFKSLPASLVE	
uba1 (S. cer.)	288	DRAAQLHLGFQAL-HQ	247	TEVKVPRKISFKSLKQQLSN	
uba1 (wheat)	322	ERPPLLHLAFQALDKK	289	TQVKPPKVIKFKPLKEAMSE	

FIGURE 4: Comparison of HIGH and KMSKS sequences. Regions containing the consensus HIGH and KMSKS sequences are compared between selected aminoacyl tRNA synthetases (top) and the four reported E1 sequences (bottom).

UCH-L3, Table 4. The labile fraction of mutant adenylate likely represents a pool of free intermediate since tightly-bound wild-type ubiquitin adenylate is totally refractory to trapping by UCH-L3, Table 4. These observations do not preclude an alternative model in which the UCH-L3-labile pool represents tightly bound but solvent-exposed adenylate susceptible to hydrolysis by the hydrolase; however, this interpretation appears unlikely since the first-order rate constant for UCH-L3 cleavage of UbR72L adenylate (inset to Figure 3) is identical to that of free intermediate present after acid denaturation of the resulting E1 ternary complex (see text).

For the two mutants, the kinetics for trapping of ubiquitin [^{3}H]adenylate by UCH-L3 displayed biphasic kinetics consistent with free and bound pools of intermediates, Figure 3. Graphical analysis of the kinetics for trapping allowed quantitation of the absolute pools of bound and free mutant adenylate for UbR42L and UbR72L, from which values of K_d could be calculated from the equilibrium expression. These calculations yield values of K_d for adenylate binding of 49×10^{-9} M for UbR72L and 16×10^{-9} M for UbR42L, representing a *ca.* 10^3-fold decrease in affinity from the estimated upper limit of 8×10^{-12} M for wild-type intermediate, respectively. In contrast, the data do not support a role for either R54 or R74 in ubiquitin adenylate binding, Table 4. These results are remarkable for two reasons. First, the data suggest a significant reorientation of the E1 active site during formation of the adenylate intermediate since there is a marked shift in relative contribution of the arginines to binding. The R54 and R72 residues contribute to binding of free polypeptide while R42 and R72 participate in binding of the resulting ubiquitin adenylate intermediate. Second, the contributions of R42 and R72 are significantly greater for adenylate binding than for the initial interaction with free polypeptide, an observation also consistent with reorganization of the active site. At present it is unclear whether this proposed conformation change represents a change in the active site of E1, a reorientation of ubiquitin within a static active site, or a combination of the two effects. However, this conclusion is consistent with data by Wilkinson and co-workers using adenosylphosphoubiquitinol, a nonhydrolyzable analog of ubiquitin adenylate that binds competitively with respect to ATP but noncompetitively with respect to free ubiquitin, indicating discretely different binding interactions for the two forms of the polypeptide (Wilkinson et al., 1990).

The kinetics of E1-catalyzed ATP:PP_i exchange have been used routinely in the past to characterize genetically and chemically altered ubiquitins (Ecker et al., 1987; Duerksen-Hughes et al., 1987). The present studies demonstrate that the exchange reaction is a relatively insensitive probe for quantitating the effects of such protein modifications in the absence of additional measurements of the intrinsic K_d for polypeptide binding, Tables 1 and 2. Therefore, the kinetics of exchange should be interpreted with care in assessing the effects of such modifications and can only be reliably used to identify gross alterations in the internal equilibria and rates of the E1 catalytic cycle. In spite of this cautionary note, exchange kinetics are valuable in defining the order of substrate binding and product release (Haas & Rose, 1982). The UbR72L mutant is unique among the arginine modifications in shifting the mechanism of E1 from a strictly ordered addition to that of random binding with respect to ATP and ubiquitin. Structural information about the E1 active site is necessary to provide a model for the observed change in binding order.

The four E1 sequences reported to date bear significant sequence homology within this class but no homology to other proteins present in protein sequence data bases (Handley et al., 1991; McGrath et al., 1991; Disteche et al., 1992; Hatfield & Vierstra, 1992). However, the E1 mechanism is analogous to that of the amino acyl tRNA synthetases which have been well characterized previously. Both classes of enzymes activate the α-carboxyl of an amino acid by formation of a tightly enzyme-bound adenylate and then transfer this intermediate to an acceptor in a second step. The cognate tRNA is the acceptor for the synthestases while the active site cysteine serves this function within the E1 active site. As a class the synthetases have little global sequence homology; however, they share limited homology within two peptide sequences located in their N-terminal region (Webster et al., 1984). The consensus tetrapeptide His-Ile-Gly-His (the "HIGH" sequence) constitutes part of the adenylate binding site, with the first histidine residue hydrogen bonding to the ribose ring oxygen and the second histidine binding to the γ-phosphate of ATP (Brick et al., 1988). A second consensus KMSKS sequence constitutes part of a mobile loop that envelopes the active site during the catalytic cycle, allowing binding of the lysine residues to the β- and γ-phosphates of ATP in the ternary complex and to pyrophosphate following adenylate formation (Brick et al., 1988; Fersht et al., 1988). We have identified homologous regions among the four existing E1 sequences, summarized in Figure 4, which suggests a similar active site topology between the two enzyme classes. The E1 sequences contain small peptide insertions within the two consensus sequences; however, these insertions may not adversely affect their roles in adenylate binding.

We speculate that the active site of E1 contains a cationic residue that participates in binding the phosphate(s) of ATP and that unfavorably interacts with R72 in the absence of ATP to prevent binding of ubiquitin as the leading substrate. This model requires interaction of ATP with the putative cation to neutralize the charge on the latter and allow subsequent binding of ubiquitin as the trailing substrate. The UbR72L

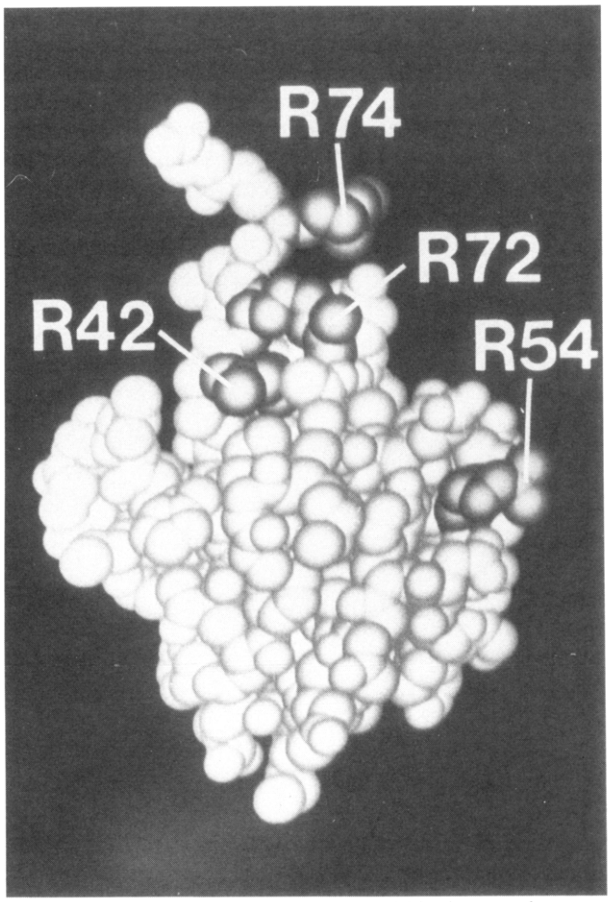

FIGURE 5: Position of the arginine residues within the crystal structure of ubiquitin. The four arginine residues examined in the present study have been highlighted on the 1.8-Å crystal structure of ubiquitin reported by Vijay-Kumar et al. (1987).

mutant would relieve the repulsive interaction between the cation and R72, allowing the substrates to bind randomly. It is unlikely that either the histidine or lysine residues within the identified consensus sequences represent this putative cationic group since these same residues do not participate in the initial binding of ATP based on active site modeling (Fersht et al., 1988). However, tyrosyl tRNA synthetase contains an additional lysine residue at position 88 that does contribute to the initial binding of ATP (Brick et al., 1988; Fersht et al., 1988). A similar lysine within the E1 active site would be correctly positioned to interact with R72 of ubiquitin. Unfortunately, K88 of the tyrosyl tRNA synthetase does not lie within a sequence conserved among this enzyme class, making it impossible to identify a candidate residue within E1. Work is currently in progress to identify potential E1 active site residues using photoactivated ATP analogs.

Figure 5 shows the refined 1.8-Å crystal structure of ubiquitin (Vijay-Kumar et al., 1987) onto which the arginine residues examined in this study have been highlighted to reveal their relative orientation within the "basic face" identified by Wilkinson (1988). The vertical dimension of the protein from the carboxyl terminal G76, located at the top of the figure, to the lower margin is 42 Å while the horizontal dimension is 30 Å. The R72 and R54 residues required for binding of ubiquitin to the E1·ATP binary complex are separated by a distance of 17 Å, providing a favorable axis of separation for these two groups to facilitate the correct orientation of the polypeptide within the active site. However, as noted above these residues account for only 3.5 kcal/mol of the 8.7 kcal/mol apparent binding energy for free ubiquitin, requiring participation of additional groups on the surface of the polypeptide. Recent work by Chen and Pickart (1990) precludes participation in E1 binding of residues present on the lower half of the molecule. In this study free K48-linked diubiquitin formed by recombinant $E2_{25K}$ was indistinguishable from wild-type monoubiquitin in competition with ^{125}I-ubiquitin for E1-ubiquitin thiol ester formation, indicating that both mono- and diubiquitin share comparable K_d for binding to E1. The crystal structure of diubiquitin reveals that the lower half of the ubiquitin molecule shown in Figure 5 would be severely occluded by the second ubiquitin domain (Cook et al., 1992). The contact surface for the two ubiquitin domains extends roughly from a line segment linking R42 and R54 to the lower margin of the molecule, although these two arginine residues remain exposed for binding interactions with E1. This packing arrangement predicts extreme steric hindrance for diubiquitin if residues on the lower half of the polypeptide were required for binding. Therefore, it is likely that the additional residues required to account for the apparent binding energy of ubiquitin lie on the acidic face of the molecule (Wilkinson, 1988) which extends from the upper surface of the ubiquitin structure in Figure 5 between R72 and R54 to the opposite side of the molecule (not shown). Studies are in progress to examine the participation of these additional residues in interactions with E1.

ACKNOWLEDGMENT

The authors wish to thank David Ecker for providing the pNMhUB ubiquitin expression clone, Keith Wilkinson for providing recombinant UCH-L3, and Joseph T. Barbieri for technical advice in generating the mutants.

REFERENCES

Brick, P., Bhat, T. N., & Blow, D. M. (1988) *J. Mol. Biol. 208*, 83–98.

Briggs, M. S., & Roder, H. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 2017–2021.

Chen, Z., & Pickart, C. M. (1990) *J. Biol. Chem. 265*, 21835–21842.

Cook, W. J., Jeffrey, L. C., Carson, M., Chen, Z., & Pickart, C. M. (1992) *J. Biol. Chem. 267*, 16467–16471.

Disteche, C. M., Zacksenhaus, E., Adler, D. A., Bressler, S. L., Keitz, B. T., & Chapman, V. M. (1992) *Mammal. Genome 3*, 156–161.

Duerksen-Hughes, P. J., Xu, X. X., & Wilkinson, K. D. (1987) *Biochemistry 26*, 6980–6987.

Ecker, D. J., Khan, M. I., Marsh, J., Butt, T. R., & Crooke, S. T. (1987) *J. Biol. Chem. 262*, 3524–3527.

Fersht, A. R., Knill-Jones, J. W., Bedouelle, H., &Winter, G (1988) *Biochemistry 27*, 1581–1587.

Haas, A. L., & Rose, I. A. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 6845–6848.

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

Haas, A. L., & Wilkinson, K. D. (1985) *Prep. Biochem. 15*, 49–60.

Haas, A. L., & Bright, P. M. (1988) *J. Biol. Chem. 263*, 13258–13267.

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

Haas, A. L., Warms, J. V., & Rose, I. A. (1983) *Biochemistry 22*, 4388–4394.

Haas, A. L., Bright, P. M., & Jackson, V. E. (1988) *J. Biol. Chem. 263*, 13268–13275.

Haas, A. L., Reback, P. B., & Chau, V. (1991) *J. Biol. Chem. 266*, 5104–5112.

Handley, P. M., Mueckler, M., Siegel, N. R., Ciechanover, A., & Schwartz, A. L. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 258–262.

Hatfield, P. M., & Vierstra, R. D. (1992) *J. Biol. Chem. 267*, 14799–14803.

Hemsley, A., Arnheim, N., Toney, M., Cortopassi, G., & Galas, D. (1989) *Nucl. Acids Res. 17*, 6545–6551.

Hershko, A., & Ciechanover, A. (1992) *Ann. Rev. Biochem. 61*, 761–807.

McGrath, J. P., Jentsch, S., & Varshavsky, A. (1991) *EMBO J. 10*, 227–236.

Pickart, C. M., & Rose, I. A. (1985) *J. Biol. Chem. 260*, 7903–7910.

Vijay-Kumar, S., Bugg, C. E., & Cook, W. J. (1987) *J. Mol. Biol. 194*, 531–544.

Webster, T., Tsai, H., Kula, M., Mackie, G. A., & Schimmel, P. (1984) *Science 226*, 1315–1317.

Wilkinson, K. D. (1988) in *Ubiquitin* (Rechsteiner, M., Ed.) pp 5–38, Plenum Press, New York.

Wilkinson, K. D., Lee, K. M., Deshpande, S., Duerksen-Hughes, P., Boss, J. M., & Pohl, J. (1989) *Science 246*, 670–673.

Wilkinson, K. D., Smith, S. E., O'Conner, L., Sternberg, E., Taggart, J. J., Burges, D.A., & Butt, T. (1990) *Biochemistry 29*, 7373–7380.

Woolfson, D. N., Cooper, A., Harding, M. M., Williams, D. H., & Evans, P. A. (1993) *J. Mol. Biol. 229*, 502–511.