Conjugation of the 15-kDa Interferon-induced Ubiquitin Homolog Is Distinct from That of Ubiquitin*

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The biological effect of type 1 interferons is proposed to arise in part from the conjugation of ubiquitin cross-reactive protein (UCRP), the ISG15 gene product, to intracellular target proteins in a process analogous to that of its sequence homolog ubiquitin, a highly conserved 8.6-kDa polypeptide whose ligation marks proteins for degradation via the 26 S proteasome. Inclusion of CoCl₂ during the purification of recombinant UCRP blocks the proteolytic inactivation of the polypeptide occurring by cleavage of the carboxyl-terminal glycine dipeptide required for activation and subsequent ligation. Intact UCRP supports a low rate of ubiquitin-activating enzyme (E1)-dependent ATP:PPᵢ exchange but fails to form a stoichiometric E1-UCRP thiol ester or undergo transfer to ubiquitin carrier protein (E2). The binding affinity of E1 for UCRP is significantly diminished relative to that of ubiquitin. These results suggest that UCRP conjugation proceeds through an enzyme pathway distinct from that of ubiquitin, at least with respect to the step of activation. This was confirmed for an in vitro conjugation assay in which ¹²⁵I-UCRP could be ligated in an ATP-dependent reaction to proteins present within an A549 human lung carcinoma cell extract and could be competitively inhibited by excess unlabeled UCRP but not ubiquitin. Other results demonstrate that ¹²⁵I-UCRP conjugation is significantly increased in cell extracts after 24 h of incubation in the presence of interferon-β, consistent with the late induction of UCRP conjugating activity. Thus, interferon-responsive cells contain a pathway for UCRP ligation that is parallel but distinct from that of ubiquitin.

The interferons exert their biological effects through induction of a subset of cellular genes whose patterns of expression define the cell- and tissue-specific responses characteristic of these cytokines. Several of these interferon-induced genes have been the subject of considerable study (reviewed in Ref. 1); however, the mechanisms by which many of these proteins contribute to the interferon response remain poorly understood. One such protein within the latter group is the 15-kDa polypeptide originally identified by Farrell et al. (2) and later characterized by Knight and co-workers (3, 4). Interferon-induced expression of the 15-kDa protein is regulated by an upstream interferon-stimulated response element typical of all early genes induced by type 1 interferons, IFN-α, and IFN-β (5). Subsequently, the sequence of the 15-kDa protein was noted to possess significant homology to a tandem diubiquitin sequence, accounting for its cross-reaction with affinity-purified anti-ubiquitin antibodies (6). The carboxyl-terminal LRLLGG sequence of ubiquitin essential for its conjugation to cellular proteins is conserved within the carboxyl terminus of the ubiquitin cross-reactive protein (UCRP), leading to the proposal that UCRP contributes to the type 1 interferon response through an analogous conjugation reaction (6). More recently, high molecular weight UCRP conjugates have been detected constitutively and within interferon-induced cultured cell lines using anti-UCRP-specific antibodies (7, 8).

Ubiquitin is one of the most highly conserved proteins found widely distributed among eucaryotes. The best studied function of ubiquitin is to target cellular proteins for degradation through a post-translational modification wherein the carboxyl terminus of ubiquitin is covalently linked via isopeptide bond to primary amines on target proteins (9). The resulting conjugates are degraded by a multienzyme ATP-dependent pathway requiring the 26 S multicatalytic protease complex (proteasome) (10). Conjugation of ubiquitin to cellular proteins proceeds through a three-step pathway, reviewed in Hersko and Ciechanover (10) and Pickart (11). The ubiquitin-activating enzyme (E1) catalyzes an ATP-coupled activation of the carboxyl-terminal glycine of ubiquitin to generate an enzyme-bound ubiquitin adenylyl intermediate and free PPᵢ (12). Transfer of activated ubiquitin to an active site cysteine of E1 releases AMP and generates a covalent E1-ubiquitin thiol ester (12). In the second step, ubiquitin is transferred by transacylation to a cysteine residue conserved among all members of a family of ubiquitin carrier proteins, E2 (13, 14). The third step involves aminolysis of these E2 thiol esters to form isopeptide bonds between the carboxyl-terminal glycine of ubiquitin and ε-amino groups of lysine residues on target proteins in both E3 (ubiquitin:protein ligase)-dependent and -independent mechanisms (15).

A persistent question has been whether the regulatory pathway of ubiquitin ligation is unique within the cell or if other similar pathways exist. Discovery of UCRP and its conjugates suggests such ligation events represent a general regulatory strategy within cells. However, a remaining unresolved question is whether ubiquitin and UCRP ligation mechanisms share a common set of enzymes or proceed through parallel but distinct pathways. In order to address the latter question, we have examined the ability of recombinant human UCRP to support the reaction catalyzed by ubiquitin-activating enzyme.

*This work was supported by United States Public Health Services Grant GM47426 (to A. L. H.), Postdoctoral Fellowship Grant GM15977 (to J. N.) and an American Heart Association (Wisconsin Affiliate) Predoctoral Fellowship Grant (to J. L. P.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: IFN, interferon; E1, ubiquitin activating enzyme; E2, ubiquitin carrier protein; E3, ubiquitin:protein isopeptide ligase; PAGE, polyacrylamide gel electrophoresis; UCRP, ubiquitin cross-reactive protein; DTT, dithiothreitol; MOPS, 4-morpholinepropane-sulfonic acid.
The results suggest that conjugation of ubiquitin and UCRP proceed through distinct enzymes for their initial activation, a conclusion supported by competition studies utilizing an in vitro UCRP ligation assay.

MATERIALS AND METHODS

Boxine ubiquitin and yeast inorganic pyrophosphatase were purchased from Sigma. In all preparations the absolute concentration of ubiquitin was determined using an empirically determined ε_{280nm} of 0.16 ml/mg cm (16). Carboxypeptidase B was purchased from Boehringer Mannheim. Recombinant ubiquitin carboxyl-terminal hydrolase (L3) was a generous gift from Dr. Keith Mannheim (Emory University School of Medicine). Human recombinant interferon bearing a C175 mutation to enhance stability (IFN-β) was supplied by Trint Biociences. Carrier-free Na^{125}I, [2,8-3H]ATP, and Na^{4}[32P]PPi, were obtained from DuPont NEN. Ubiquitin and UCRP were labeled with [32P]Pi by the chloramine-T (17) and the IODO-GEN methods (18), respectively. Precast isoelectric focusing gels having a broad pH range (3.5-9.5) were obtained from Pharmacia Biotech Inc. The ubiquitin-activating enzyme (E1) was purified by modification of the published procedure (19) from rabbit reticulocytes, rabbit liver, and human erythrocytes.

Purification of Recombinant UCRP—Isolation of mature recombinant UCRP was modified from the procedure of Loeb and Haas (7). All steps were conducted at 4°C and included 5 mM CoCl₂ in buffers to prevent chelation by the polymerase. Ten liters of Escherichia coli BL21(DE3) harboring the PETUCRP expression plasmid were grown to OD_{600}nm of 0.8, then harvested after a 90-min induction in the presence of 0.4 mM isopropyl-β-D-thiogalactopyranoside (7). The centrifuged cells were resuspended in 50 mM Tris-Cl (pH 7.5) containing 5 mM CoCl₂ and lysed by French press. All intermediate steps were identical to those published previously with the exception that the unadsorbed fraction from the DE-52 column was concentrated in a 200-ml Amicon cell fitted with a PM10 membrane then dialyzed against 20 mM MOPS-Cl buffer (pH 7.2) containing 5 mM CoCl₂. The dialyze was adjusted to 1.6 M ammonium sulfate then applied to a 2.5 × 22-cm phenyl-Superose FAST column containing 50 mM Tris-Cl (pH 7.2), containing 5 mM CoCl₂ and 1.6 M ammonium sulfate. Recombinant UCRP was eluted from the phenyl-Sepharose column as a single peak using a negative linear gradient of ammonium sulfate (5 mM/ml). Following concentration in an Amicon cell fitted with a PM10 membrane, the sample was resolved on a 2.5 × 36-cm preparative column of Superdex-75 equilibrated with 50 mM Tris-Cl (pH 7.5), 50 mM NaCl, and 5 mM CoCl₂ to remove trace high molecular weight contaminants. Fractions containing UCRP were pooled and adjusted to 1 mM DTT, then dialyzed against 50 mM Tris-Cl (pH 7.5) containing 1 mM DTT. Concentration of the apparently homogeneous recombinant UCRP (>99% by SDS-PAGE followed by Comassie Blue staining) was determined spectrophotometrically using an ε_{280nm} of 0.82 ml/mg cm (7). Addition of CoCl₂ to the buffers during purification presumably reduced the molar extinction coefficient of UCRP via the single cysteine residue (4, 19). The addition of 1 mM DTT to the final dialysis step resulted in a dark brown precipitate that was removed by centrifugation. Loss of protein in the precipitate was minimal.

Activity Assays—The ability of recombinant UCRP to substitute for ubiquitin was compared in three types of activity assays. Rates of ATP:PP, exchange were measured at 37°C in a final volume of 50 µl containing 50 mM Tris-Cl (pH 7.6), 2 mM ATP, 10 mM MgCl₂, 100 µM [32P]PPi, (200 cpm/µmol), 1 mM DTT, 1 pmol of rabbit reticulocyte ubiquitin-activating enzyme, and a range of UCRP concentrations (12). The incorporation of [32P]PPi into ATP was measured by adsorbing the resulting radioactivity to charcoal and determining the bound radioactivity on E1 was determined after resolving incubations by SDS-PAGE under nonreducing conditions, then determining [32P]-associated radioactivity in the E1 thiol ester band by γ counting (12, 20). Incubations were identical to those for measuring adenylate formation except that 2 µM unlabeled ATP was used in place of the radiolabeled nucleotide. Five pmol of rabbit liver E1 and 32 µM recombinant UCRP were used in the reaction at 37°C for 5 min. SDS-PAGE sample buffer containing 4% SDS and 5 M urea was used to quench the reaction. β-Mercaptoethanol was excluded from the sample buffer. Due to covalent aggregation of [125I]-UCRP during iodination and the low specific activity of radiolabeled polyepideotide, Western blotting was used for the detection of E1-bound UCRP thiol ester (21). Blots were immunostained with 10 µg/ml affinity-purified anti-UCRP antibody followed by 125I-protein A and visualized by autoradiography. Control reactions were included in which E1 was quenched prior to addition of UCRP. A 7% gel was used to increase transfer efficiency and resolve the difference in electrophoretic mobility between the ubiquitin and UCRP thiol esters formed with E1.

Cell Culture and Preparation of Extracts—Confluent monolayers cultures of A549 human lung carcinoma cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum. Cultures were maintained at 37°C in a humidified 5% CO₂ atmosphere. Cultures of A549 cell were induced by adding IFN-β to a final concentration of 1000 IU/ml (7). Following 24 h of induction, cells were rinsed twice with phosphate-buffered saline, then harvested by scraping directly into 50 mM Tris-Cl (pH 7.6) containing 2 mM ATP, 10 mM MgCl₂, and 1 mM DTT (100 µl-100-mm plate). The cells were then briefly sonicated, and the extracts were used without further fractionation.

RESULTS

Integrity of the UCRP Carboxyl Terminus—Previously we have been unable to demonstrate conjugation of recombinant [125I]-UCRP when added to fresh extracts of A549 cell (7, 19). In addition, recombinant UCRP failed to support E1-catalyzed ATP:PP, exchange (7, 19) in spite of the marked homology between UCRP and ubiquitin. The latter negative result suggested that either E1 was incapable of interacting with UCRP or that the recombinant protein was isolated in an inactive form. Subsequent experiments have confirmed that recombinant UCRP isolated as described previously (7) was inactive due to proteolytic cleavage of the carboxyl-terminal glycine dipeptide.

Mature forms of ubiquitin and UCRP share a common LRLR-Arg carboxyl terminus (6). Previous work has shown that the carboxyl-terminal glycine dipeptide of ubiquitin is acutely sensitive to cleavage by trypsin-like activities (22, 23). The resulting des-Gly-Gly ubiquitin retains a pI characteristic of native ubiquitin (22, 23). However, removal of the carboxyl-terminal glycine dipeptide exposes Arg-74 which is subject to cleavage by carboxypeptidase B to yield a product exhibiting a pl shift to 5.4 (22, 23). The specificity of carboxypeptidase B for lysine and arginine precludes a similar pl shift for intact ubiquitin in which Arg-74 is masked by the glycine dipeptide (22). When a similar experiment was conducted with recombinant UCRP isolated by our published protocol (22), the polypeptide exhibited a shift in pl from the value of 6.7 characteristic of UCRP to 5.4 upon incubation with carboxypeptidase B (Fig. 1, lanes 3 and 4). This indicated that the carboxyl-terminal glycine dipeptide was absent from UCRP.

The pl shift upon incubation of ubiquitin with carboxypeptidase B was used as a convenient assay to test for carboxyl-terminal glycine dipeptide processing in BL21 extracts. These experiments revealed that the inactive enzyme was a periplasmic carboxypeptidase similar in specificity to carboxypeptidase A; that is, the activity readily cleaved the glycine dipeptide from ubiquitin but failed to remove the resulting Arg-74 carboxyl terminus. Similar inactivating activity was
found in all expression strains of *E. coli* tested, except for that of AR58 used in the heat-inducible expression of recombinant ubiquitin (24). However, the AR58 expression system was pre-
duced for UCRP since the polypeptide fails to fold into a soluble, native conformation at the elevated temperature (42 °C) required for induction (19). Various inhibitors were unsuccess-
fully screened for their ability to block ubiquitin car-
boxyl-terminal inactivation by BL21 extracts. In contrast, 5 mM
EDTA stimulated the rate of ubiquitin inactivation, suggesting that endogenous divalent metal(s) present in the lysates acted as natural inhibitors of the activity. Among the metal salts subsequently tested, CoCl₂ was the most effective in inhibiting ubiquitin inactivation; therefore, 5 mM CoCl₂ was included in all buffers used in the isolation of recombinant UCRP, since this concentration quantitatively blocks ubiquitin and UCRP glycine dipeptide excision.

Recombinant UCRP purified in the presence of CoCl₂ failed to show a pI shift upon incubation with carboxypeptidase B (Fig. 1, lanes 1 and 2), suggesting the presence of an intact native carboxyl terminus. However, the pI of UCRP shifted from 6.7 to 5.4 following successive incubation with car-
boxypeptidase A, to remove the glycine dipeptide, followed by carboxypeptidase B at 37 °C for 15 min, then resolved by isoelectric focusing gel electrophoresis.

Subsequent rate studies using UCRP preparations having variable amounts of intact carboxyl terminus yielded comparable values of *k₅₀* but different values for *Kᵥₒ* that were inversely proportional to the fraction of total UCRP possessing an intact carboxyl terminus, as judged by relative Coomassie staining intensity in the pl shift assay following incubation with carboxypeptidase B (not shown). The latter observations are consistent with the inability of des-Gly-Gly UCRP to support the E1-catalyzed reaction. The dependence of ubiquitin concentra-
tion on E1-catalyzed ATP:PP exchange exhibits pronounced substrate inhibition at high concentrations, characteristic of the obligatory ordered addition of substrates for which ATP binds prior to ubiquitin (12). However, no substrate inhibition was observed at the highest concentration of UCRP tested (50 μM), presumably because this concentration remains below the zone of inhibition for the polypeptide. Insolubility of UCRP at higher concentrations prevented testing for substrate inhibi-
tion by UCRP.

### Stoichiometry of E1-bound UCRP Adenylate Formation—The ATP:PP₁ exchange reaction proceeds through a tightly E1-bound (Kᵥₒ ≤ 10⁻¹⁴ M) (12, 24) ubiquitin adenylate interme-
diate that is stoichiometric with the activating enzyme in the

| Ubiquitin  | UCRP |
|------------|------|
| *k₅₀* (s⁻¹) | 9.6  |
| *Kᵥₒ* (μM)  | 1.2  |

*Values taken from Haas and Rose (12).*
tic acid precipitation could represent a similar equilibrium between bound and free forms since this method is otherwise unable to distinguish between the pools, as has been discussed previously (24). The amount of UCRP \(^{3}H\)adenylate formed was unaffected by incubation in the presence of 0.4 IU/ml recombinant ubiquitin carboxyl-terminal hydrolase (Table II), precluding the existence of a free pool of intermediate. Separate control studies demonstrated that the UCRP \(^{3}H\)adenylate was completely cleaved by the hydrolase if the intermediate was first dissociated from E1 by trichloroacetic acid precipitation and then resolubilized in the presence of 0.2 M triethanolamine-Cl (pH 8.0), not shown. These results indicate that non-conservative sequence differences between ubiquitin and the carboxyl-terminal ubiquitin-like domain of UCRP affect the equilibrium formation of UCRP adenylate but not the tight binding characteristic of this intermediate.

Stoichiometry of E1-UCRP Thiol Ester Formation—In the catalytic cycle of ubiquitin activation, tightly E1 bound ubiquitin adenylate reacts with an active site cysteine to yield a covalently bound E1-ubiquitin thiol ester with release of AMP (12, 20). Thiol ester formation between E1 and \(^{125}\)I-ubiquitin is typically quantitated following resolution by nonreducing SDS-PAGE (12, 20). Qualitatively, formation of the corresponding thiol ester between UCRP and E1 was observed after nonreducing SDS-PAGE and Western blotting by immunostaining the blots with anti-UCRP antibody followed by detection with \(^{125}\)I-protein A and autoradiography (Fig. 2). A parallel reaction using \(^{125}\)I-ubiquitin was carried out and resolved by SDS-PAGE as described previously (20) (Fig. 2). The E1 thiol ester formed to UCRP is of slightly higher molecular weight when resolved by 7% SDS-PAGE than that formed with \(^{125}\)I-ubiquitin, the difference approximately equaling that between UCRP and ubiquitin (Fig. 2). In parallel incubations the E1 thiol esters formed to \(^{125}\)I-ubiquitin and UCRP were labile to brief boiling in the presence of \(\beta\)-mercaptoethanol (not shown). This control experiment demonstrates that the linkage between E1 and UCRP is a thiol ester rather than an isopeptide bond (20).

Western blotting and detection with anti-UCRP antibody proved a facile means of routinely detecting E1-UCRP thiol ester formation; however, the method was inadequate for determining the stoichiometry of this step. Conversely, the low specific activity of \(^{125}\)I-UCRP and covalent aggregation during radiolabeling made direct detection of \(^{125}\)I-UCRP thiol esters difficult except at elevated levels of E1. Using 10 pmol of E1 in the standard thiol ester incubation (see "Materials and Methods") we were able to detect \(^{125}\)I-UCRP thiol ester formation with E1 at a level corresponding to 5% of that formed with \(^{125}\)I-ubiquitin (not shown) following a 15-min incubation. In these experiments, the specific activity of \(^{125}\)I-UCRP was determined by estimating the amount of radiolabeled 15-kDa band by Coomassie staining relative to that of a series of known amounts of unlabeled UCRP determined spectrophotometrically using the empirically determined extinction coefficient at 280 nm (see "Materials and Methods"). The amount of \(^{125}\)I-UCRP thiol ester formation did not increase on prolonged incubation, indicating that the 20-fold lower stoichiometry for \(^{125}\)I-UCRP formation was an equilibrium rather than a kinetic effect.

**Table II**

| UCH | UCRP \(^{3}H\)adenylate (pmol) ± S.D.
|-----|----------------------|
| -   | 0.112 ± 0.008        |
| +   | 0.113 ± 0.004        |

**Figure 2.** Thiol ester formation between E1 and UCRP. Thiol ester formed between UCRP (Panel A) or \(^{125}\)I-ubiquitin (Panel B) and 5 or 1 pmol of rabbit liver E1, respectively, was detected as described under "Materials and Methods." Lane 1 in each panel represent a control in which the E1 was quenched prior to the reaction.
with affinity-purified E1 isolated from rabbit liver and human erythrocytes. Values for the $K_d$ of ATP, ubiquitin, and PP, binding show good agreement between rabbit liver and human erythrocyte E1 and with the values previously reported for rabbit reticulocyte enzyme (12). These results with enzyme isolated from both a different rabbit tissue and species/tissue confirm earlier preliminary conclusions that ubiquitin activation is saturated with respect to intracellular ATP concentrations normally encountered (12). The results of Table III also indicate that ubiquitin activation is saturating with respect to polypeptide (12), based on quantitative estimates of free ubiquitin pools in a number of cultured cell lines and tissues (21, 26). In addition, the equilibrium constants for formation of enzyme-bound ubiquitin adenylate of 0.2 and 0.13 for liver and erythrocyte E1 enzymes, respectively, agree very well with the value of 0.16 reported for the rabbit reticulocyte form (12).

The results of Table III also demonstrate that the amino acid substitutions present in UCRP have significant effects on the interaction of the polypeptide with ubiquitin-activating enzyme. The $K_d$ for binding of UCRP is 80-fold higher than that of ubiquitin for liver E1 and 19-fold higher for erythrocyte enzyme. In addition, the equilibrium constant for formation of enzyme-bound ubiquitin adenylate is approximately 20-fold lower for both forms of E1, accounting in part for the diminished stoichiometry for equilibrium formation of this intermediate (Table II). The increase in $K_d$ for UCRP binding and the decrease in equilibrium constant for formation of the adenylate intermediate is consistent with earlier proposals that tight binding of the polypeptide is functionally coupled to this catalytic step compared to predicted formation constants based on model organic reactions (12). The significant decrease in $K_d$ for ATP and PP, binding in the presence of UCRP observed for both enzymes is puzzling since such an effect cannot be accounted for directly by the presently accepted mechanism for E1 (12). This effect on binding of cosubstrate and product suggests that binding of UCRP, and presumably ubiquitin, is also coupled to those of ATP and PP.

The significantly diminished affinity of ubiquitin-activating enzyme for UCRP suggests that the enzyme does not normally function within the conjugation pathway of the ubiquitin homolog. This conclusion is supported by the argument that free ubiquitin, normally present at 5-10 $\mu$M within cells (21, 26), would effectively competitively block a kinetically significant rate of UCRP activation, since free concentrations of the latter are constitutively present at 0.1 $\mu$M and accumulate to only about 1 $\mu$M after 24 h induction with saturating concentrations of type 1 interferons (7). Therefore, it is likely that cells contain a separate enzyme for the activation of UCRP.

In Vitro Conjugation of UCRP to Cellular Proteins—Previous reports by Loeb and Haas (7, 8) have indirectly demonstrated the presence of UCRP conjugates within interferon-responsive cultured cells. The subsequent inability to show in vitro conjugation of radiodinated recombinant UCRP in cell culture extracts is accounted for by the proteolytic inactivation of the polypeptide during isolation. The autoradiogram of Fig. 3 demonstrates that intact $^{125}$I-UCRP isolated in the presence of CoCl$_2$ shows measurable rates of in vitro conjugation when added to extracts obtained from uninduced and interferon-$\beta$ treated human A549 lung carcinoma cells.

For the incubations of Fig. 3, $^{125}$I-UCRP was added to a final concentration 50-fold greater than that of endogeneous UCRP present in the extracts to obviate effects of isotope dilution. Radioiodinated UCRP exhibits a low but measurable rate of conjugation to cellular proteins present in extracts obtained from uninduced cells (−IFN) after 90 min of incubation. The rate of $^{125}$I-UCRP conjugation is significantly greater in extracts from parallel A549 cultures induced for 24 h in the presence of 1000 U/ml interferon-$\beta$ (+IFN). Enhanced in vitro conjugation of $^{125}$I-UCRP agrees with the previous results demonstrating the late induction of UCRP conjugating activity by IFN-$\beta$ (7, 19) Conjugation of $^{125}$I-UCRP in both extracts is absolutely dependent on the presence of ATP and a creatine phosphate/creatine phosphate ATP-regenerating system (not shown). That the conjugation of $^{125}$I-UCRP is specific for the homolog is demonstrated by the marked inhibition by isotope dilution when an excess of unlabeled UCRP (+UCRP) but not ubiquitin (+Ub) is included in parallel incubations.

Table III

| Step$^a$ | Liver E1 | Human E1 |
|---------|----------|----------|
| $E_{\text{ATP}} + U$ → $E_{\text{ATP}} + U + ATP$ | 13 $\mu$M | 2 $\mu$M |
| $E_{\text{ATP}} + U$ → $E_{\text{ATP}} + U + PP$ | 0.3 $\mu$M | 24 $\mu$M |
| $E_{\text{ATP}} + U + PP$ | 0.9 $\mu$M | 14 $\mu$M |
| $E_{\text{ATP}} + U + PP + P_P$ | 0.3 $\mu$M | 11 $\mu$M |

$^a$U represents ubiquitin or UCRP in the reaction.
latter observations suggest that conjugation of UCRP and ubiquitin proceed through distinct ligation pathways, consistent with the earlier conclusion that UCRP activation is unlikely to require ubiquitin-activating enzyme.

The significant number of additional low molecular weight radiolabeled bands obvious at 0 min and persisting after 90 min of incubation is not due to contaminating proteins present in the recombinant UCRP preparation since the polypeptide was >99% pure (see "Materials and Methods") but, rather, results from covalent aggregation of the radioiodinated polypeptide during the labeling reaction. Preliminary studies indicate that this aggregation results in part from disulfide dimerization of UCRP through the single cysteine residue present in the sequence under the nonreducing conditions required for radioiodination.

**DISCUSSION**

Identification of ubiquitin conjugation as a required step in targeting proteins for ATP-dependent degradation by the 26 S proteasome poses the question of whether this novel post-translational modification is unique or represents the first translational modification is unique or represents the first novel post-targeting proteins for ATP-dependent degradation by the 26 S proteasome. The present results indicate that the UCRP and ubiquitin ligation pathways are distinct, at least with respect to activation of their respective carboxyl termini. This conclusion is supported by the inability of ubiquitin to compete with 125I-UCRP during in vitro conjugation under conditions for which unlabeled UCRP effectively blocks addition of the radiolabeled polypeptide (Fig. 3). Although intact recombinant UCRP supports a modest rate of E1-dependent activation (Table I), the affinity for UCRP is considerably lower than that for ubiquitin (Table III). Because of this difference in affinity between the two proteins, it is unreasonable to assume that UCRP could compete with ubiquitin for activation by E1, particularly since intracellular constitutive concentrations of UCRP within uninduced cells is approximately 50–100-fold lower than that of ubiquitin and only accumulate to 10% of the free pool of ubiquitin following interferon induction (7). Differences in affinity and concentration thus necessitate a distinct enzyme for UCRP activation.

Recent structure-function studies exploiting site-directed mutagenesis of ubiquitin provide some insight into the sequence differences that may in part account for the inability of UCRP efficiently to support the E1-catalyzed reaction. Position Arg-72 of ubiquitin, retained as Arg-152 in UCRP (7), represents a major contribution in the initial binding of polypeptide to E1 and is absolutely required for defining the ordered addition of substrates (24). However, the additional significant binding contribution of the ubiquitin Arg-54 site (24) is lost through mutation at the corresponding Leu-134 of UCRP (7). This sequence difference probably accounts in part for the decreased binding of UCRP to E1, although the magnitude of the effect is greater than predicted from this sequence change alone (24), indicating that other nonconserved sites or steric factors associated with the increased size of UCRP must also contribute to diminished binding. For ubiquitin, the single His-68 residue is essential for function since mutation at this site blocks the ability of the polypeptide to support ATP-dependent degradation (28). We have recently found that ubiquitin H68N duplicates this effect and results from an impaired rate of conjugation resulting from a kinetic defect in formation of the E1-ubiquitin thiol ester. It is likely that a similar effect in UCRP, for which His-68 is replaced by Phe-148 (7), accounts for the observed low steady state level of enzyme-bound E1-UCRP thiol ester (Fig. 2); however, additional steric effects cannot be ruled out.

In the course of these studies we compared the ability of ubiquitin and UCRP to support the E1-catalyzed reaction using enzymes isolated from two different sources (Table III). A similar analysis had only been previously reported for E1 isolated from rabbit reticulocytes (12). The new data support and extend the previous conclusions that E1 is saturating with respect to both ATP and free ubiquitin within cells (12). Therefore, the rate-limiting step for ubiquitin ligation cannot normally reside in the activation step and changes in steady state levels of ubiquitin conjugates within cells cannot be directly explained by a substrate dependence on ubiquitin concentration, as has been discussed previously (26).

The present results argue that UCRP ligation represents a parallel but distinct pathway to that of ubiquitin conjugation. In spite of the sequence similarities between the two polypeptides, they most likely utilize different activating enzymes. Interestingly, several recent cDNA sequences have recently been reported that are similar to those of the ubiquitin-activating enzyme (29–31). Whether these sequences represent the putative UCRP-dependent E1 is uncertain at present. Also uncertain is whether other steps in the ubiquitin and UCRP ligation mechanisms share common components. Nonetheless, the ubiquitin and UCRP conjugation pathways represent an unparalleled opportunity for comparative biochemical studies of related processes.

Acknowledgment—We are grateful to Dr. Keith Wilkinson for the generous gift of L3 ubiquitin carboxyl-terminal hydrolase.

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J. Biol. Chem. 1996, 271:324-330.
doi: 10.1074/jbc.271.1.324

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