Multiple Forms of Ubiquitin–Protein Ligase. Binding of Activated Ubiquitin to Protein Substrates†

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Received August 23, 1985; Revised Manuscript Received January 3, 1986

ABSTRACT: Enzyme activities that catalyzed the covalent attachment of ubiquitin to protein substrates (ubiquitin–protein ligase, UbL) were purified from the extracts of human red blood cells. These activities required the presence of ubiquitin-activating enzyme and ATP for activity. Four fractions (UbL A, B_1, B_2, and C) were resolved and showed different specificities toward added substrates [carboxymethylated bovine serum albumin (CM-BSA), G-actin, lysozyme, and α-lactalbumin]. The enzyme fractions gave different products with a given substrate. UbL A and UbL B_1 were exclusively active with CM-BSA and α-lactalbumin, respectively. UbL B_2 was most active toward CM-BSA with substantial activities to G-actin and α-lactalbumin and with no activity to lysozyme. UbL C showed significant activities with all four substrates, having a highest activity toward CM-BSA. There were many endogenous proteins present in the erythrocyte extract which were efficient substrates for ubiquitin conjugation. In particular, a pair of substrates were identified from eythrocyte extracts which were far more efficient substrates than the denatured proteins exogenously added.

Ub[1] is a small, heat-stable protein that is universally present in eukaryotic cells (Goldstein et al., 1975). It is found either in a free form or covalently attached to many cellular proteins. Two biological functions have been identified. It is an essential component of ATP-dependent protein degradation in the reticulocytes (Ciechanover et al., 1978) and is involved in the covalent modification of certain histones (Goldknopf et al., 1975; West & Bonner, 1980). In the reticulocyte system, a three-enzyme sequence was proposed for the covalent attachment of Ub to protein substrates (Hershko et al., 1983).

$$2ATP + 2Ub + E1 \overset{Mg^{2+}}{\rightleftharpoons} E1\overset{AMP-Ub}{\underset{S-Ub}{}} + 2PP_i + AMP$$

$$E1\text{–}S\text{–}Ub + E2 \rightleftharpoons E2\text{–}S\text{–}Ub + E1$$

$$E2\text{–}S\text{–}Ub + RNH_2 \overset{E3}{\rightleftharpoons} E2 + Ub\text{–}NHR$$

The Ub-activating enzyme (E1) which activates Ub to a thiol ester has been isolated and extensively studied (Ciechanover et al., 1982). The activated Ub is then transferred to thiol groups on carrier proteins (E2s) (Pickart & Rose, 1985). Ub–protein ligase (E3) catalyzes the transfer of Ub from one or more E2–thiol esters to amino groups of protein substrates to form alkaline-stable peptide linkages with the carboxyl terminus of Ub (Hersko et al., 1983). The system has several notable similarities to the multienzyme system that catalyzes the synthesis of the circular decapeptide gramicidin S (Gevers et al., 1969), where two thiol ester intermediates are involved in the synthesis of each peptide bond.

Ub–protein ligase (E3) from human red cells can be separated into several fractions each of which requires the Ub-activating enzyme for activity. The fractions appear to have different specificities toward protein substrates. The different enzyme fractions give different products with a given substrate.

Materials and Methods

Ub was prepared from outdated human blood (Ciechanover et al., 1980a,b). It was iodinated with $Na^{125}I$ by the chloramine T method (Ciechanover et al., 1980a,b). DEAE-Sepharose and "activated" CH-Sepharose were obtained from Pharmacia. DEAE-cellulose (DE-52) was from Whatman. Hydroxylapatite was prepared by the Tiselius procedure (Levin, 1962). Bovine serum albumin, α-lactalbumin, and lysozyme were from Sigma Chemical Co. Bovine serum albumin was carboxymethylated by the procedure of Crestfield et al. (1963). G-Actin was prepared from rabbit muscle acetone powder (Spudich & Watt, 1971). It was partially denatured by dialysis against 2 mM EDTA which inhibited its assembly to F-actin.

Purification of the Ub-Activating Enzyme. Extracts of human erythrocytes from freshly drawn blood was absorbed to DEAE-cellulose and eluted with high salt to obtain "fraction II" (Ciechanover et al., 1978). The Ub-activating enzyme was then purified by chromatography on DEAE-Sepharose and hydroxylapatite (see below). The final purification step, covalent affinity chromatography on Ub–Sepharose, was by previously published methods (Ciechanover et al., 1982). The Ub-activating enzyme after two initial steps was completely bound to Ub–Sepharose. The affinity resin contained 10–20 mg of Ub/g of dried activated CH-Sepharose. The activating enzyme was eluted from the column with 1 mM AMP and 0.2 mM PP_i at pH 7.5. It was concentrated by precipitation with 75% saturated ammonium sulfate, dialyzed against 20 mM Tris-HCl, pH 7.5, 0.5 mM $MgCl_2$, and 0.5 mM DTT (buffer A), and stored at −80 °C.

Purification of Ub–Protein Ligase. Fraction II (300 mL) from 4 pt of human blood was dialyzed against 20 mM potassium phosphate buffer, pH 6.5, 0.5 mM $MgCl_2$, and 0.5

† Supported by NIH Grants AM 23847, GM 24667, AG 03949, HL 16387, and HL 34171 and by grants from the Muscular Dystrophy Association, New York, and American Heart Associations. C.F.M. is an Established Investigator of the American Heart Association. V.B.H. is a recipient of the Ann Weinberg Cystic Fibrosis Research Scholarship Award and is an Established Fellow of the New York Heart Association. The data in this paper are from a thesis to be submitted in partial fulfillment of the requirements of the degree of Doctor of Philosophy in the Sue Golding Graduate Division of Medical Sciences, Albert Einstein College of Medicine, Yeshiva University.

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[1] Abbreviations: CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; CM-BSA, carboxymethylated bovine serum albumin; Ub, ubiquitin; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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mM DTT (buffer B) containing 20 mM KCl and absorbed to a DEAE-Sepharose CL-6B column (4 × 50 cm) equilibrated with the same buffer. The column was eluted with a 4-L gradient to 500 mM KCl. Three peaks of Ub–protein ligase activity were eluted to KCl concentrations of approximately 100 (UbL A), 180 (UbL B), and 250 mM (UbL C) (see Figure 1). The three peaks of activity were concentrated by precipitation with 75% saturated ammonium sulfate and dialyzed against buffer A. Fractions UbL A and UbL C were not purified further. Fraction UbL B, which contained approximately 60% of the Ub–protein ligase activity and the Ub-activating enzyme, was absorbed to a hydroxylapatite column (2 × 30 cm) equilibrated with 10 mM potassium phosphate, pH 7.0, 0.5 mM $MgCl_2$, and 0.5 mM DTT. The column was eluted in steps of 100 mL each of increasing potassium phosphate concentration (50, 100, 150, 200, and 250 mM). The Ub-activating enzyme eluted in the 50 mM buffer along with approximately one-tenth of the Ub-protein ligase activity (fraction UbL B_1). Most of the Ub–protein ligase activity eluted in the 200 mM buffer (UbL B_2). Both fractions were concentrated with ammonium sulfate and dialyzed against buffer A. The first fraction (UbL B_1) was further purified by Ub–Sepharose covalent affinity chromatography as described for the Ub-activating enzyme. The Ub–protein ligase activity absorbed to the column in the presence of Ub-activating enzyme and MgATP. Most of the activity eluted from the column after the activating enzyme with 2 mM DTT at pH 9.

Assays of Ub-Activating Enzyme and Protein–Ligase Activities. The Ub-activating enzyme was assayed by the Ub-dependent ATP $\rightleftharpoons$ [^{32}P]PP_i activity as described by Ciechanover et al. (1981). The incubation was at pH 7.6 for 20 min at 37 °C. A unit of enzyme activity was defined as 1 μmol of [^{32}P]PP_i exchanged into ATP per minute.

The Ub–protein ligase was assayed on Sephadex G-75, which separated Ub–protein conjugates from free Ub. The reaction mixture contained, in 100 μL, 10 μmol of Tris-HCl, pH 9, 0.2 μmol of ATP, 0.5 μmol of $MgCl_2$, 0.05 μmol of DTT, 0.5 IU of inorganic pyrophosphatase, 0.5 × 10^{-3} unit of Ub-activating enzyme, 10–30 pmol of ^{125}I-labeled Ub (approximately 10^5 cpm), and where indicated, 100 μg of added protein acceptor. After 15 min at 37 °C, 10 μL of 5 N NaOH was added to stop the reaction, and the incubation was continued for 10 min to hydrolyze the Ub–thiol ester(s). The pH was brought to 8 with 100 μL of 2 M Tris-HCl, and the reaction mixture was passed into a Sephadex G-75 column (0.7 × 15 cm) equilibrated with 20 mM Tris-HCl, pH 7.5, 1 mg/mL bovine serum albumin, and 0.2% sodium azide. Fractions of 0.3 mL were collected and counted. Three peaks of ^{125}I label were observed. The radioactive counts in the void volume were taken as the amount of alkaline-stable ^{125}I-labeled Ub–protein conjugates formed. The second peak was unreacted Ub, and the third peak was free ^{125}I. A unit of enzyme activity was defined as 1 pmol of Ub–protein conjugate formed per minute at 37 °C.

Thiol Ester Formation. Thiol ester formation was performed under the same conditions as for conjugation except that the reaction was stopped with NaOH or formic acid to a final concentration of 0.4 N. Thiol ester formation was taken as the amount of alkaline labile ^{125}I-ubiquitin-bound species eluting with the void volume on Bio-gel P-60 columns.

SDS–Polyacrylamide Gel Electrophoresis. SDS–polyacrylamide gel electrophoresis was carried out essentially according to the method of Robbi and Lazarow (1978). Autoradiography was performed with Kodak XAR 5 SB film and Cronex High Plus or Quanta III intensifying screens. The proteins used for molecular weight standards were bovine serum albumin (M_r 68 000), pyruvate kinase (M_r 57 000), glyceraldehyde-3-P dehydrogenase (M_r 36 500), carbonic anhydrase (M_r 30 000), soybean trypsin inhibitor (M_r 21 000), and α-lactalbumin (M_r 14 000). Densitometry measurements were made by using an EC apparatus densitometer equipped with a strip chart recorder. The peaks were cut out and weighed.

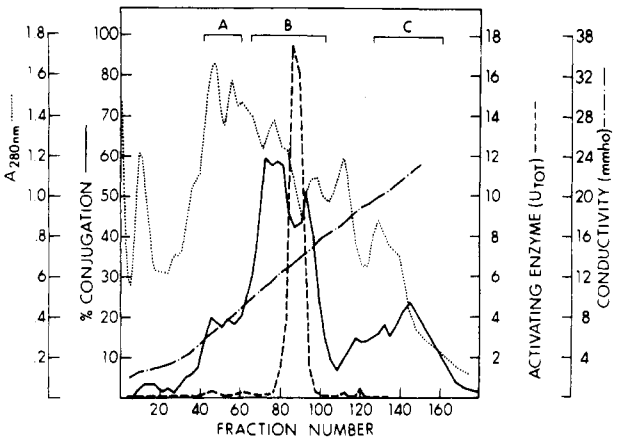

FIGURE 1: DEAE-Sepharose profile of Ub–protein ligase activity. Fresh human red blood cell fraction II was chromatographed on DEAE-Sepharose CL-6B equilibrated with buffer B containing 20 mM KCl. Ub–protein ligase and Ub-activating enzyme were eluted with a linear gradient from 20–500 mM KCl and assayed as described under Materials and Methods.

RESULTS

Purification of Ub–Protein Ligase. Comparable activities of Ub–protein ligase were found in the extracts of rabbit reticulocytes, human red blood cells, and rabbit muscle, even though an ATP-dependent proteolysis has not successfully been demonstrated in the extracts of the two latter tissues. The better recovery of Ub–protein ligase after extensive purification was obtained from human red blood cells, for which the reason was not clear. Therefore, Ub–protein ligase was purified from fraction II of fresh human red cells prepared as described under Materials and Methods.

Three peaks of activity were separated when fraction II was chromatographed on DEAE-Sepharose (Figure 1). The middle peak appeared to contain several components that were only partially resolved from each other. The peak activities were pooled as indicated and labeled UbL A, UbL B, and UbL C. The Ub-activating enzyme eluted as a single, sharp peak along with UbL B (dotted line in Figure 1).

The fraction of ligase activity (UbL B) that coeluted with the activating enzyme was then chromatographed on hydroxylapatite and eluted with a stepwise concentration of phosphate buffer (Figure 2). A small fraction of the ligase activity was eluted with the Ub-activating enzyme in 50 mM phosphate buffer, while most of the activity eluted with 200 mM phosphate buffer. The hydroxylapatite fractions were labeled UbL B_1 and UbL B_2. After these steps, four Ub–protein ligase fractions were obtained of which UbL A, UbL B_2, and UbL C were free of Ub-activating enzyme activity.

In order to determine if thiol ester forms (E1, E2) were associated with the Ub–protein ligase fractions, Ub thiol ester formation was measured. Little, if any additional thiol ester formed in the presence of the Ub protein ligase fractions over the thiol ester formed in the presence of the Ub-activating enzyme fraction. Ubiquitin conjugation did not occur in the absence of Ub-activating enzyme or if the thiol binding sites

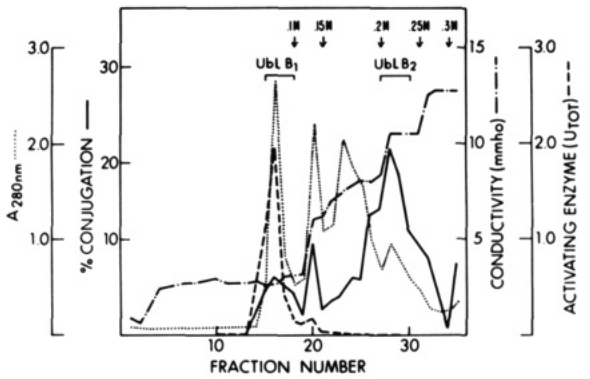

FIGURE 2: Hydroxylapatite chromatography of UbL B. UbL B following DEAE-Sepharose chromatography (Figure 1) was chromatographed on hydroxylapatite equilibrated with 10 mM potassium phosphate buffer, pH 7.0, 0.5 mM DTT, and 0.5 mM $MgCl_2$. Ub–protein ligase activity was eluted with a step gradient of 50, 100, 150, 200, 250, and 300 mM potassium phosphate, pH 7.0, 0.5 mM DTT, and 0.5 mM $MgCl_2$.

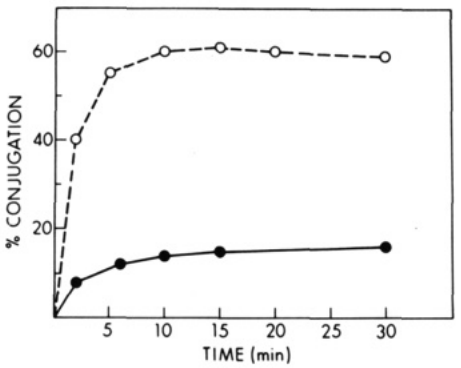

FIGURE 3: Time course of Ub–protein ligase activity. The activity was measured with 6 (●) and 24 μg (○) of UbL B_2 as described under Materials and Methods.

Table I: Specific Activities of UB–Protein Ligase Fractions[a]

	total protein (mg)	total activity (unit)	specific activity (unit/mg)	% reaction with CM-BSA
UbL A	180	1520	9	32
UbL B_2	19	670	37	47
UbL C	160	3020	20	55
UbL B_1	61	305	6	0[b]

[a] Ub–protein ligase activity was measured under the conditions described under Materials and Methods using 75 μg of UbL A, 25 μg of UbL B_1, 24 μg of UbL B_2, 67 μg of UbL C, and 0.24 μg of Ub-activating enzyme except the reaction was incubated for 2 or 5 min. The picomoles of Ub conjugated per minute were averaged for the two time points. The percent of CM-BSA-dependent activity was determined from densitometry measurements of Ub–protein ligase in the presence or absence of CM-BSA (Figures 4 and 5). [b] No net increase in ligase activity was observed.

in the Ub-activating enzyme fraction were inactivated with 5 mM iodoacetamide. Ubiquitin conjugation catalyzed by either 50 mM iodoacetamide or iodoacetic acid treated or untreated Ub protein ligase fractions were identical. In our experimental system the thiol ester forms were associated with the Ub-activating enzyme fraction.

Time Course of Ub Conjugation. Each of the Ub–protein ligase fractions contained protein contaminants that were substrates for Ub conjugation. Figure 3 shows the time course of Ub conjugation observed with UbL B_2. The reaction rapidly reached an end point which increased when more enzyme was added. With CM-BSA present at a concentration of 1 mg/mL, the reaction was biphasic because of the rapid reaction

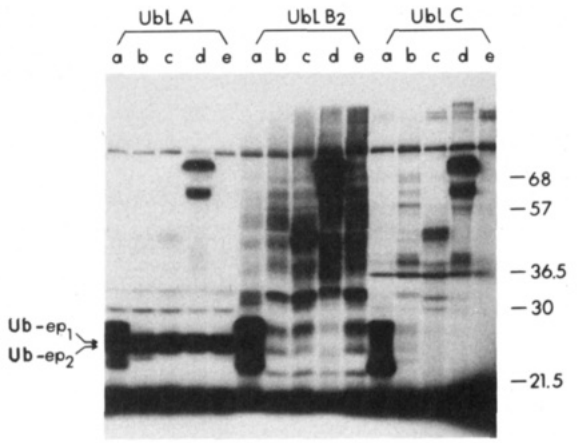

FIGURE 4: Substrate specificity of Ub–protein ligase by SDS–PAGE. UbL A (75 μg), UbL B_2 (24 μg), and UbL C (67 μg) activities were assayed in a reaction mixture containing 100 mM Tris-HCl, pH 9.0, 5 mM $MgCl_2$, 2 mM ATP, 0.5 mM DTT, 0.5 IU of pyrophosphatase, 30 pmol (2×10^5 cpm) of ^{125}I-Ub, 0.5 milliunit (0.15 μg) of Ub-activating enzyme, and 50 μg of α-lactalbumin (lane a), lysozyme (lane b), G-actin (lane c), CM-BSA (lane d), or no substrates (lane e). The 10% SDS gel electrophoresed as described under Materials and Methods, and the autoradiogram was exposed for 5 h. Ub–ep_1 and Ub–ep_2: Ub conjugates with endogenous protein acceptors 1 and 2.

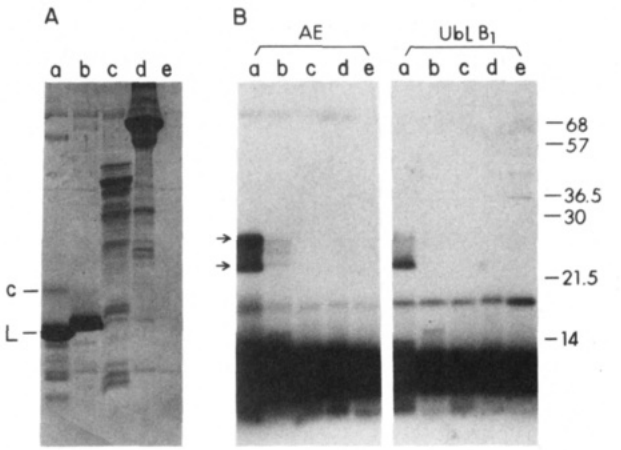

FIGURE 5: Substrate specificity of the Ub–protein ligase contaminating the Ub-activating enzyme by SDS–PAGE. (Panel A) SDS–PAGE (15%) of the reaction mixtures with AE in panel B stained with Coomassie Blue. (Panel B) The rabbit muscle Ub–protein ligase that copurifies with the Ub-activating enzyme through Ub–Sepharose affinity chromatography (AE, 0.35 μg) and the red blood cell Ub–protein ligase that copurifies with the Ub-activating enzyme through hydroxylapatite (UbT B_1, 6.3 μg) were assayed under identical conditions as described for Figure 4 except Ub-activating enzyme was deleted for the reaction with UbL B_1. The reaction mixture contained 50 μg of the following substrates: α-lactalbumin (lane a), lysozyme (lane b), G-actin (lane c), CM-BSA (lane d), or no substrates (lane e). The autoradiogram is exposed for 10 h.

with the endogenous protein substrates and the slower reaction with CM-BSA (data not shown). Examination of the Ub conjugates by SDS–PAGE confirmed that the rate and extent of reaction with endogenous proteins was not affected by the presence of CM-BSA (see the following section).

The specific activities of the four Ub–protein ligase fractions are summarized in Table I. The activity was determined on 2- and 5-min time points, before the plateau of Ub conjugation had been reached. The fractions were stimulated to varying degrees by CM-BSA. One fraction, UbL B_1, had no apparent activity toward CM-BSA.

Specificities toward Protein Substrates. To demonstrate if the four ligase fractions represent different forms of Ub–protein ligase, their activities toward four protein acceptors

Table II: Summary of UB–Protein Ligase Activity with Substrates[a]

substrate	relative activity				
	UbL A	UbL B_1	UbL B_2	UbL C	Ub AE
CM-BSA	66	0*	100	100	+
G-actin	19	0*	30	40	+
lysozyme	+	0*	0*	40	+
α-lactalbumin	+	100	20*	73	30
ep_1 and ep_2	335*	–	–	–	–

[a] Ub–protein ligase activity toward several substrates is summarized from densitometry measurements made on the SDS–PAGE data described in Figures 4 and 5 except that UbL B_2 and the reaction of UbL A with ep_1 and ep_2 were measured from a 1-h exposure (asterisk). The numbers indicate relative Ub conjugation with various substrates subtracted for the contribution by endogenous substrates and, where applicable (UbL A, UbL B_2, and UbL C), for the contribution by Ub-activating enzyme toward each substrate. Ub AE, Ub–protein ligase activity present in the muscle Ub-activating enzyme.

(α-lactalbumin, lysozyme, G-actin, and CM-BSA) were compared. Each substrate was added at a concentration of 1 mg/mL.

As shown in Figures 4 and 5 and quantitated in Table II, the fractions varied in their activities toward the various substrates. One fraction, UbL C, was almost equally active toward all four substrates. Another, UbL B_1, was only active toward α-lactalbumin. A third fraction, UbL B_2, had substantial activity toward three of the substrates, but it was inactive toward lysozyme. Thus, the fractions appeared to contain different Ub–protein ligases with varying substrate specificities. Since the enzymes were only partially purified, it is possible that some, particularly UbL C and UbL B_2, were mixtures of other activities with different substrate specificities.

The ligase activity of the Ub-activating enzyme alone is included in Figure 5. The Ub-activating enzyme fraction exhibited approximately 3% conjugation with CM-BSA. Its activity was substrated from the activities of UbL A, UbL B_2, and UbL C to obtain the net ligase activity in Table II. Its specificity was similar to that of UbL B_1, from which it was obtained by Ub–Sepharose affinity chromatography, although from a different enzyme preparation.

When commercial α-lactalbumin was used as a substrate, two ^{125}I-Ub-labeled products of M_r 22 500 and 25 800 resulted. The former corresponded to the molecular weight expected for a monovalent Ub conjugate, but the latter was half way in between a monovalent and a bivalent Ub conjugate. Most likely, the conjugate of M_r 25 800 is due to a contaminant in the commercial preparation of α-lactalbumin, because some of our α-lactalbumin preparations did not display this band when used as substrate for UbL B_2.

There appeared to be some contaminants in the CM-BSA preparation. However, these were shown to be artifacts from the carboxymethylation step, representing smaller pieces of BSA.

In the case of one substrate, lysozyme, many higher molecular weight Ub conjugates were formed. With UbL C, in particular, ^{125}I-Ub conjugates were formed corresponding in relative molecular weights to conjugates containing one to six Ub molecules per lysozyme molecule (Figure 4). However, there were other Ub conjugates that did not have the correct molecular weights, and they may represent conjugates with impurity or degradative products.

Endogenous Substrates. As pointed out in the previous section and in Figure 3, the time course of Ub conjugation rapidly approached a plateau in the absence of added substrates because of the reaction with acceptor(s) present in the ligase fractions. SDS–PAGE examination of several time points showed that the ^{125}I-Ub conjugates with endogenous

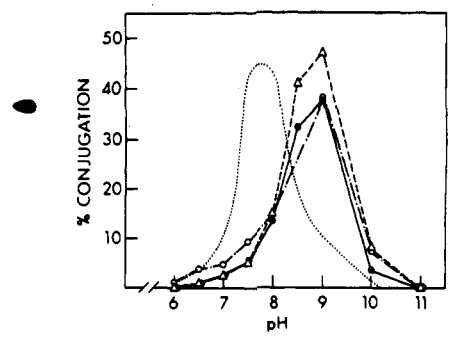

FIGURE 6: pH profile of Ub–protein ligase activity. Ub–protein ligase activity was measured essentially as described under Materials and Methods except the following buffers were used to establish the pH: 0.1 M MES, pH 6.0 and 6.5; 0.1 M Tris-HCl, pH 7.0, 7.5, 8.0, 8.5, and 9.0; 0.1 M CAPS, pH 10.0 and 11.0. (○) UbL A; (△) UbL B_2; (●) UbL C. The dotted line represents the profile for the rate of Ub release by each of the Ub–protein ligase fractions. Ub–protein conjugates were formed in 10 mM Tris-HCl, pH 9, followed by a 1-min incubation with 5 mM 2-deoxyglucose and 0.5 μg/mL hexokinase to deplete ATP. After aliquots were adjusted to the above pH, the level of Ub–protein conjugates was measured following a 5-min incubation at 37 °C. The activities were expressed as percentage conjugates formed or hydrolyzed during incubations.

acceptors reached a constant level after 5 min while the conjugates with added proteins increased linearly with time. One pair of endogenous acceptors, labeled ep_1 and ep_2 in Figure 4, were present in UbL A. The concentrations of ep_1 and ep_2 could only be estimated because protein bands of the expected molecular weights would have migrated with the dye front. However, from the intensity of staining with Coomassie Blue, the protein in the dye front was less than 5% of the total in fraction UbL A (less than 3.8 μg). Even so, ep_1 and ep_2 competed successfully with excess amounts of added substrates (50 μg) so that 5 and 50 times more Ub–ep_1 and Ub–ep_2 were formed than with CM-BSA and α-lactalbumin, respectively (Table I). This specificity toward ep_1 and ep_2 suggests that they may be the natural substrates for UbL A.

Catalytic Properties of the Ligase Reaction. As shown in Figure 5, the optimal pH for three of the ligase fractions (UbL A, UbL B_2, and UbL C) was 8.5–9.0. The k_m for the Ub-activating enzyme at pH 9 was (1–2) × 10^{-3} unit/mL (0.3–0.6 μg/mL) for each of the three fractions.

The dotted line in Figure 6 shows a probable reason for alkaline pH optimum of the lipase reaction. The dotted line shows the rate of hydrolysis (or breakdown) of the Ub conjugates after removal of ATP with hexokinase and glucose. The conjugates were very stable at pH 9, but they were rapidly hydrolyzed around pH 7.5. Thus, the pH 9 optimum for the ligase probably reflects the decreased rate of hydrolysis of the conjugates, once formed, rather than the increased rate of conjugation. The Ub–protein ligases very likely have a neutral pH optimum but must compete with the rapid rate of hydrolysis.

The hydrolysis of the Ub conjugates appears not to be by reversal of the protein ligase reaction: Transfer of the Ub conjugate back to an E2–S–Ub or E1–S–Ub thiol ester followed by hydrolysis of the thiol ester. These thiol ester intermediates are more unstable at alkaline pHs (Gevers et al., 1969). Whether the hydrolysis of the conjugates is by protease digestion or by an isopeptidase reaction has not been established.

DISCUSSION

Human red cells contained multiple forms of Ub–protein ligase that catalyzed the formation of alkaline-stable Ub-conjugates with proteins in the presence of the Ub-activating

enzyme fraction which contained the thiol ester forms and MgATP. One fraction, UbL B_1, was active with only one of the four substrates tested, α-lactalbumin. A second fraction, UbL A, was active with CM-BSA and G-actin, with little or no activity toward the two remaining substrates. A third fraction, UbL B_2, was active with three substrates: CM-BSA, G-actin, and α-lactalbumin. The last fraction, UbL C, was active with all four substrates. Thus, the fractions contained different forms of Ub–protein ligase activity which had different substrate specificities to protein substrates.

There were protein contaminants present in enzyme fractions that were substrates for Ub conjugation. With the proper amount of enzyme added, the conjugates with these endogenous proteins were rapidly formed at pH 9 and reached a plateau. When an excess of CM-BSA was present, the conjugates with endogenous protein acceptors were still rapidly formed, and with SDS–PAGE one could observe the slower formation of the monovalent conjugate(s) with CM-BSA. Two low molecular weight acceptors (ep_1 and ep_2) were identified in the UbL A fraction purified on DEAE-Sephadex chromatography, which were far more efficient substrates than α-lactalbumin or denatured protein CM-BSA.

The formation of conjugates containing more than one Ub attached to a single substrate molecule was reported with lysozyme, α-lactalbumin, and globin in reticulocyte fraction II (Hershko et al., 1980). By contrast, monovalent Ub conjugates were usually the only conjugate formed with either single or mixed fractions of the purified Ub–protein ligase. In one exception, a variety of conjugates were formed when lysozyme was tested with UbL C. The conjugates with endogenous proteins appeared to be also monovalent as shown in Ub–ep_1 and Ub–ep_2. The ep_1 and ep_2 were very active substrates but did not form higher conjugates. Ub–ep_1 was not Ub–ep_2 conjugated to a second Ub molecule as judged from the difference in molecular weight.

Hershko et al. (1983) reported that three components (E1, E_2, and E3) of the Ub ligase system were involved in ATP-dependent proteolysis. All three fractions were purified by Ub–Sepharose affinity chromatography of crude fraction II from reticulocytes. E1 is the Ub-activating enzyme, and E2 is a Ub-carrier protein, which binds to Ub–Sepharose in the presence of E1 and MgATP. E2 binds to Ub through a thiol ester bond which is then a substrate for E3 in forming alkaline-stable amide linkages with protein substrates. E3 binds to Ub–Sepharose without addition of MgATP. Multiple forms of E2 were described (Pickart & Rose, 1985). Our results show that there are multiple forms of E3 that appear to have different specificities toward α-lactalbumin, lysozyme, G-actin, and CM-BSA.

ACKNOWLEDGMENTS

We express our appreciation to A. Rudner for her expert technical assistance and Dr. I. Listowsky for use of the densitometer. We thank Joan Byrne for her invaluable help in the preparation of the manuscript.

Registry No. Ub–protein ligase, 74812-49-0; lysozyme, 9001-63-2.

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