Auto-ubiquitination of ubiquitin-activating enzymes from chicken breast muscle

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A soluble ubiquitin-depleted fraction from chicken skeletal muscle (fraction II), when incubated at neutral pH for several hours with ^{125}I -ubiquitin and ATP, formed small amounts of a ubiquitin derivative (M_r 115000) of the ubiquitin-activating enzyme E1 as well as certain similarly modified E2 species (M_r 37000, 34000 and 24000). Treatment of such mixtures with NaOH during the incubations, even at early times, greatly enhanced the appearance of these entities; up to two-thirds of the thioesters of ubiquitin bound to these proteins before alkali treatment were thus converted. The bonds involved had properties compatible with their being peptidic in nature, suggesting that auto-ubiquitination had occurred in each case. The protease inhibitor and alkylating agent tosyl-lysylchloromethane ('TLCK'), when preincubated at 50 μM with fraction II for 2 h at 37 °C before the addition of ^{125}I -ubiquitin and ATP, promoted the subsequent auto-ubiquitination of E1 and inhibited its adenylate-forming and thioester-transferring activities. The findings have a bearing on the physiological substrate- and site-specificity of ubiquitin-conjugating reactions.

INTRODUCTION

Reversible auto-modification of regulatory proteins is a well-understood process for certain protein kinases, e.g. tyrosine-specific (receptor) kinases (Yarden & Ullrich, 1988) and Ca^{2+} /calmodulin-dependent protein kinases (Lickteig *et al.*, 1988). Ubiquitination of cellular proteins, like phosphorylation, is a general post-translational modification reaction, but there have been no reports to date of auto-modification of ubiquitin-transferring enzymes. Ubiquitin is a highly conserved protein of 76 amino acids present in all eukaryotic cells. Its multiple functions apparently all depend on its C-terminal *N*-conjugation to subsets of cellular proteins, in linkage arrangements which may take a variety of forms (mono-, poly- or multi-ubiquitination) [for reviews see Schlesinger & Hershko (1988) and Hershko (1988)].

Ubiquitin conjugation to target proteins requires the action of a ubiquitin-activating enzyme (E1), a dimer of M_r 210000, which in the presence of ATP and ubiquitin reversibly forms a bound ubiquitin C-terminal adenylate with the release of PP_i (Ciechanover *et al.*, 1982; Haas *et al.*, 1982). The activated ubiquitin moiety is transferred to a (presumably adjacent) thiol group on E1 with the release of AMP, after which a second ubiquitin adenylate is assembled in the active site to give the doubly charged form of the enzyme: this is probably the predominant intracellular species of the enzyme (Haas & Rose, 1982). It is important in the context of the present paper to note that, to date, E1 has not been shown to be capable of direct *N*-conjugation of target proteins. Instead, a cohort of up to five ubiquitin-carrier proteins (E2s, of M_r 14000–40000) is required for this to happen; each of these accepts carboxyl-activated ubiquitin from E1 into a thioester linkage to itself (Pickart & Rose, 1985; Haas & Bright, 1988). The charged carrier proteins act as direct ubiquitinating agents for a variety of protein targets (Haas *et al.*, 1988), but a subset of the latter appear to be selected for such an attack by ubiquitin–protein ligase, a protein of M_r 300000 called E3 (Hershko *et al.*, 1983, 1986). Recently obtained evidence suggests that simultaneous binding of the free *N*-terminal amino acid and a nearby 'mobile' lysine residue by

E3 is the basis of this selection mechanism (Bachmair & Varshavsky, 1989).

The above pathways for ubiquitination of proteins have been elucidated at the molecular level mainly in rabbit reticulocytes. Information gleaned from, among others, extracts of skeletal muscle and liver (Fagan *et al.*, 1987), as well as the heart (Gehrke & Jennissen, 1987), supports the notion that the same enzymes operate in a similar fashion in these tissues. More recently, a normal and a thermolabile E1 have been purified from a strain of mouse mammary tumour cells and a mutant derivative thereof (ts85) respectively (Mayer *et al.*, 1989). Multiple forms of E1 have been detected in wheat germ (Hatfield & Vierstra, 1989).

We have found that conjugation of ^{125}I -ubiquitin to endogenous proteins takes place in soluble ubiquitin-depleted fractions prepared from chicken skeletal muscle. This was catalysed by E1 in conjunction with one or more E2 molecules. During the incubations at pH 7.4, small amounts of stable entities were formed that appeared to represent mono-ubiquitinated forms of E1 and several E2 species. This reaction was greatly enhanced by treatment of the system with alkali, and appeared to represent self-ubiquitination of these proteins. Preincubation of the fractions with the alkylating agent tosyl-L-lysylchloromethane (Tos-Lys-CH₂Cl, 'TLCK') promoted the auto-ubiquitination of E1, but not that of the E2 isoforms. The mechanisms and functional consequences of these novel reactions have been explored.

MATERIALS AND METHODS

Materials

Adult Leghorn chickens were purchased from Golden Grove Poultry Farm, Cape Town, South Africa. Ubiquitin was either purified from outdated red blood cells (Western Province Blood Transfusion Service, Cape Town, South Africa) by heat denaturation by the method of Haas & Wilkinson (1985) or was kindly given by Dr. H. F. Deutsch (University of Wisconsin, Madison, WI, U.S.A.). Carrier-free Na ^{125}I was obtained from Amersham International (Amersham, Bucks., U.K.), and sodium [^{32}P]pyrophosphate from New England Nuclear (Boston, MA,

U.S.A.). DEAE-cellulose and glass-fibre filters were purchased from Whatman (Maidstone, Kent, U.K.). ATP, Tos-Lys-CH₂Cl, NEM, phenylmethanesulphonyl fluoride, chymostatin and hydroxylamine were obtained from Sigma (St. Louis, MO, U.S.A.). ¹²⁵I-ubiquitin was prepared by the chloramine-T method (Moore *et al.*, 1977).

Methods

Preparation of fractions. Chicken skeletal-muscle fraction II was prepared generally by the method of Fagan *et al.* (1987). Skeletal muscle (10 g) from exsanguinated chickens was homogenized for 1 min at 0 °C, by using an Ultra-Turrax homogenizer at 0.75 maximum setting, in 20 mM-Tris/HCl/1 mM-DTT/1 % (v/v) glycerol/1 mM-EDTA/1 mM-EGTA, all at pH 7.4. The homogenate was centrifuged at 30 000 *g* for 30 min and the supernatant applied in a cold-room to a DEAE-cellulose column (1 cm × 10 cm), which had previously been equilibrated in 10 mM-Tris/HCl/0.1 mM-DTT/1 % glycerol/1 mM-EDTA/1 mM-EGTA, pH 7.4. The column was washed until the *A*₂₈₀ readings had returned to baseline, after which the bound proteins were eluted with column buffer containing 0.5 M-NaCl. This material (fraction II) was dialysed overnight against a buffer containing 20 mM-Tris/HCl/0.5 mM-DTT/1 mM-magnesium acetate/0.1 mM-EDTA/20 mM-KCl/20 % glycerol, pH 7.4.

Formation of ¹²⁵I-ubiquitin conjugates. Samples of fraction II (100–200 µg of protein) were incubated with 1 µg of ¹²⁵I-ubiquitin (1 × 10⁵ c.p.m.) in a mixture (200 µl) also containing 2.5 mM-DTT, 5 mM-magnesium acetate, 2.5 mM-ATP and 25 mM-Tris/HCl, pH 7.4. After 2 h at 37 °C, the reaction was stopped by addition of 40 µl of 5 % (w/v) SDS and 5 µl of 2-mercaptoethanol, after which the mixtures were heated at 90 °C for 2 min (conditions known to destroy thioesters; Hershko *et al.*, 1983). The samples were applied to 5–20 %-gradient polyacrylamide gels and subjected to electrophoresis at 30 mA and room temperature (Laemmli, 1970). Ubiquitin conjugates were detected on dried gels by autoradiography on Kodak XAR-5 film by using Cronex intensifying screens. Radioactivity in specific conjugates was detected by excising the relevant Coomassie-Blue-stained lane areas and counting the ¹²⁵I-ubiquitin for radioactivity in a Packard Crystal II γ-radiation counter. For some samples, thioesters in the assay mixture were preserved by treating the samples with 40 µl of 2.5 % SDS without 2-mercaptoethanol, and avoiding the heating step; the gels were run at 4 °C.

NaOH treatment of incubated fractions. After incubating fraction II with ¹²⁵I-ubiquitin and ATP as described above, some samples were treated with enough 0.5 M-NaOH to give a final pH of 11.7 (10 µl). The mixtures were incubated for a further 10 min at 37 °C, and then reactions were terminated by addition of SDS and mercaptoethanol, followed by electrophoresis at room temperature, as described above. Variations in this protocol are mentioned in the text.

Assay of the adenylate-forming activity of E1 (ubiquitin-dependent ATP/[³²P]PP_i exchanges). Thioesters of ubiquitin, present in fraction II after its preparation, were cleaved by treatment with 1 M-hydroxylamine at pH 7.0 for 20 min at 37 °C, followed by rechromatography on DEAE-cellulose as described above. Samples of this material were assayed for adenylate-forming activity by measuring the rate of ubiquitin-dependent transfer of [³²P]PP_i to ATP as described by Calendar & Berg (1966). Briefly, the samples were incubated, with or without 1 µg of ubiquitin, for 20 min at 37 °C in the presence of [³²P]PP_i (1 × 10⁵ c.p.m.), 50 mM-NaF and 2.5 mM-ATP, pH 7.4, in a final volume of

140 µl. The reaction was stopped by addition of 300 µl of 2.5 % (w/v) acid-washed activated charcoal in 2 % (w/v) trichloroacetic acid containing 0.1 M-Na₄P₂O₇. Samples were filtered on 2.5 cm-diam. Whatman glass-fibre discs and the bound ATP was counted for radioactivity by Čerenkov radiation in a Beckman liquid-scintillation counter.

Turnover of auto-ubiquitinated proteins in breast-muscle extracts. NaOH-treated samples (prepared as described above) were neutralized to pH 7.5 with drops of 0.05 M-HCl. Samples containing 1 × 10⁵ c.p.m. were incubated for 6 h at 37 °C with 100 µg of fraction-II protein and 2.5 mM-DTT, with or without 2.5 mM-ATP. Protease inhibitors were added as indicated in the text. Samples were heated with SDS and 2-mercaptoethanol, and the loss of radioactivity from alkali-induced bands was assessed after SDS/PAGE and autoradiography as described above.

Protein determinations. Protein concentrations were assayed as described by Lowry *et al.* (1951), with bovine serum albumin as standard.

RESULTS

Ubiquitin conjugation in chicken skeletal muscle

The presence in muscle extracts of a ubiquitin-conjugating system active on endogenous proteins was shown by the formation of protein conjugates in an ATP- and time-dependent manner when crude fraction II was incubated with ¹²⁵I-ubiquitin at 37 °C for 3 h (Fig. 1, lane a). Apart from a smear of high-*M_r* material, most of the discrete bands detected had *M_r* values between 30 000 and 100 000; they were assumed to be isopeptidic

Fig. 1. Formation of ubiquitin derivatives of E1, E2 and the endogenous proteins

Lane a: fraction II (100 µg) was incubated at 37 °C for 3 h in the presence of 1 µg of ¹²⁵I-ubiquitin (1 × 10⁵ c.p.m.) and 5 mM-ATP. Samples were heated with 5 % 2-mercaptoethanol and 1 % SDS at 90 °C for 2 min and subjected to SDS/PAGE on a 5–20 % gel. Lane b: a separate sample was incubated in the same way, but thioester bonds were protected by omitting the reducing agent and the heating step and by performing SDS/PAGE at 4 °C. Lane c: fraction II was incubated as in lane a for 3 h, after which NaOH was added to a final pH of 11.7, followed by incubation for a further 10 min. Samples were treated as for lane a. Lane d: *M_r* markers for lanes a–c; phosphorylase *b* (94 000), bovine serum albumin (68 000), ovalbumin (43 000), carbonic anhydrase (30 000), soybean trypsin inhibitor (20 100), α-lactalbumin (14 400). Lane e: fraction II was incubated for 30 min and treated as in lane b. Lane f: *M_r* markers for lane e, as described in lane d. Lane g: fraction II was incubated for 2 h as in lane a, followed by NaOH treatment. Lane h: a further sample was treated as in lane g, but 50 mM-lysine was added before NaOH addition.

conjugates, because all thiolesters were destroyed before electrophoresis by the heating of the SDS-treated samples in the presence of 2-mercaptoethanol. One of the more obvious bands had an M_r of 115000, and we considered the possibility that this might be a mono-ubiquitinated form of E1; a few bands were also present at the M_r expected of similar derivatives of the E2s (Fig. 1, lane a). To ascertain whether these particular bands corresponded in position to ubiquitin thiolesters bearing E1 and E2 species, we omitted the heat and mercaptoethanol treatment; a heavy radioactive band appeared at M_r 115000, and a number of poorly resolved heavy bands were also visible in the M_r range 24000–45000, corresponding to E1 and several E2 species linked by thiolesters to single ubiquitin residues (Fig. 1, lane b). In order to investigate the possibility of auto-ubiquitination occurring as a result of nucleophilic attack on the thiolesters by a free amino group of these proteins, we treated samples of fraction II, previously incubated for 3 h under conjugating conditions with ^{125}I -ubiquitin, with NaOH for 10 min at 37 °C (pH 11.7). This resulted in the formation of large amounts of four ubiquitinated proteins, with M_r 115000, 37000, 33000 and 24000; these entities survived conditions known to cleave thiolester bonds (Fig. 1, lane c).

The appearance of the alkali-inducible bands was dependent on the presence of both fraction II and ATP, and hence was not due to the anomalous aggregation of ubiquitin (results not shown). The bands were identical in M_r value with ubiquitin-bearing E1 and three out of five E2 forms detected when the thiolesters had been preserved. Inclusion of L-lysine (50 mM) in the incubation mixtures at the time of NaOH addition severely depressed the formation of the stable bands (Fig. 1, compare lanes g and h).

The formation of the alkali-stable forms was dependent on the NaOH concentration used, and hence on the measured pH of the final mixture up to pH 11.7 (Fig. 2). Maximal conversion of the four bands occurred after 10 min post-incubation at 37 °C with

Fig. 2. Dependence of auto-ubiquitination of E1 and E2 on pH during post-incubation treatment with NaOH

Fraction-II samples were incubated for 10 min at 37 °C with 1 μg of ^{125}I -ubiquitin (1×10^5 c.p.m.) and 5 mM ATP. Various amounts of NaOH were then added to give the pH values indicated, and the mixtures were incubated for a further 10 min at 37 °C. Ubiquitinated E1 (○) and combined E2 species (●) were detected by autoradiography of SDS/PAGE gels and then quantified as described under 'Methods'.

25 mM-NaOH at pH 11.7. The amounts of the stable conjugates were then equal (with slight variations) to about two-thirds of the thiolesters present in the same mixtures before alkali treatment.

These results appeared to confirm the idea that the ubiquitin-containing bands appearing after NaOH treatment were the result of auto-conjugation of labelled ubiquitin to E1 and to several, but not all, of the available E2 species. The change to a more alkaline pH caused the thiolester linkage between ubiquitin and E1 or E2 to be susceptible to stronger nucleophilic attack by an amino group on the same protein: transacylation to this group would readily occur, with the formation of an isopeptide bond. The E1 and E2 products formed by NaOH treatment were termed E1-N-Ub and E2-N-Ub, as opposed to the thiolester forms, E1-S-Ub and E2-S-Ub.

Mechanism of the auto-ubiquitination reactions

Much shorter periods of incubation (5–20 min) were needed for fraction II to become capable of generating alkali-induced E1-N-Ub and E2-N-Ub bands than for the conjugation of other endogenous proteins (results not shown). This suggested that the bands were derived directly from the thiolester forms of E1 and E2 and were auto-conjugates. The thiolesters detected when incubations were terminated after only 10 min had M_r values of 115000, 45000, 37000, 33000, 28000 and 24000. Of these, alkali-inducible bands could be detected by NaOH treatment at all except the M_r -45000 and -28000 positions (Fig. 1, lane e).

The necessity for thiolesters being present before NaOH treatment was shown in two ways. Firstly, fraction II samples that had been incubated for 20 min with 1 M-hydroxylamine at pH 7.0, after incubations with ^{125}I -ubiquitin and ATP, did not form alkali-induced conjugates (Fig. 3, lanes a and b). Secondly, pre-treatment of fraction II with NEM at a concentration known to inactivate the thiol sites of E1 and E2 (Mayer *et al.*, 1989)

Fig. 3. Effects of hydroxylamine and NEM on the formation of the ubiquitin derivatives of E1 and E2

Lane a: fraction II (100 μg) was incubated with 1 μg of ^{125}I -ubiquitin for 10 min before addition of NaOH to give a final pH of 11.7. Samples were incubated for a further 10 min and submitted to SDS/PAGE (5–20 % gels) in the presence of 2-mercaptoethanol. Lane b: a sample was incubated as in lane a, but with hydroxylamine (final concn. 1 M) added 10 min before NaOH treatment. Lane c: a sample was incubated as in lane a, but for 2 h. Lane d: fraction II was preincubated for 20 min in the presence of 5 mM-NEM, before a 2 h incubation with ^{125}I -ubiquitin and ATP as in lane a.

Table 1. Stability of auto-ubiquitinated forms of E1, E2 and of ubiquitin-protein conjugates

¹²⁵I-auto-ubiquitinated E1 and E2 and ¹²⁵I-ubiquitin conjugates were prepared as described in Fig. 1. They were treated as indicated, and residual labelled material was quantified as described under 'Methods'.

Treatment	Radioactivity remaining (%)		
	¹²⁵ I-Ub-N-E1	¹²⁵ I-Ub-N-E2	¹²⁵ I-Ub-proteins
None	100	100	100
0.1 M-NaOH; 37 °C; 60 min	85	94	82
1 M-hydroxylamine, pH 7.0; 20 min; 37 °C	80	78	68
5% formic acid; 20 min; 37 °C	78	82	77

prevented the formation of both E1-N-Ub and E2-N-Ub (Fig. 3, compare lanes c and d), indicating that ubiquitin adenylate could not serve as the ubiquitin donor.

The properties of the NaOH-induced E1-N-Ub bonds were those of peptide bonds: they were stable to alkaline conditions and/or hydroxylamine treatment, and resistant to reductive cleavage by 2-mercaptoethanol at 90 °C (Table 1).

The question of the nature of the bonds was further addressed by testing their stability to enzymes present in fraction II. NaOH-treated mixtures were accordingly neutralized and then exposed to fresh fraction II. This showed that the ubiquitin-protein conjugates, E1-N-Ub and E2-N-Ub, were susceptible to breakdown even in the absence of ATP (Table 2). These losses were inhibited by NEM (5 mM), ZnCl₂ (125 μM) and phenylmethanesulphonyl fluoride (2 mM), the last feature reflecting the properties of an isopeptidase recently described by I. A. Rose (personal communication). No intermediates were detected, and this was also suggestive of isopeptidase activity. In reticulocyte extracts, studied in parallel, the E1-N-Ub and E2-N-Ub bands were also formed (results not shown). Affinity-purified mixtures of E1 and E2 also gave the alkali-induced bands after prior incubations with ¹²⁵I-ubiquitin and ATP (results not shown).

Activity of the adenylate- and thiol-forming site of E1 after auto-ubiquitination

The fact that the E1-N-Ub band appeared as one of many conjugated proteins after incubations of fraction II and ¹²⁵I-ubiquitin for several hours at pH 7.5 raised the possibility that enzyme preparations obtained by prior incubations at relatively mild pH, with unlabelled ubiquitin and ATP, contained varying numbers of auto-ubiquitinated E1 molecules. The effect of the modification on enzyme function could then be studied. Incubation of fraction II at pH values ranging between 7.5 and 9.0 revealed a steady increase in the amounts of E1-N-Ub and E2-N-Ub formed as a percentage of the total thiolester-forming capacity available (Fig. 4a). We assayed the adenylate- and thiolester-forming activities of such E1 preparations. Fig. 4(b) shows that the presence of a significant number of auto-ubiquitinated E1 molecules did not affect the ATP/[³²P]PP_i exchange reaction, even though the alkaline pH to which these molecules were exposed caused some loss of activity. This suggested that the covalently bound ubiquitin moiety did not interfere with the binding of a further ubiquitin molecule to the adenylate site.

Effects of Tos-Lys-CH₂Cl on ubiquitin-conjugation reactions

Tos-Lys-CH₂Cl is a chloromethyl ketone derived from *N*-α-tosyl-L-lysine. It is an irreversible inhibitor of trypsin and acts by alkylating a histidine residue in the active site of trypsin (Shaw *et al.*, 1965), as well as the thiol groups of cysteine proteinases (Barrett, 1973). When fraction II was preincubated for 2 h in the presence of 50 μM-Tos-Lys-CH₂Cl and then incubated with ATP and ¹²⁵I-ubiquitin, the banding pattern was dominated by a ubiquitin conjugate at *M*_r 115000 (Fig. 5, compare lanes a and b). This entity was identical in *M*_r with both the E1-N-Ub and E1-S-Ub forms, suggesting that Tos-Lys-CH₂Cl promoted the formation of the E1-N-Ub species even at pH 7.4. No E2-N-Ub forms were observed in this case. The bond between E1 and ubiquitin was of a peptide nature in this case also, being stable to 0.1 M-NaOH and 1 M-hydroxylamine at 37 °C (results not shown). The amount of E1-N-Ub material formed depended on the length of the preincubation time and on the concentration of Tos-Lys-CH₂Cl: the optimal Tos-Lys-CH₂Cl concentration for E1-N-Ub was 50–100 μM (Fig. 6). Concomitant with an increase in the Tos-Lys-CH₂Cl concentration was a decrease in general ubiquitin-conjugate formation, suggesting an impairment of the

Table 2. Effects of protease inhibitors on the degradation of the ubiquitin derivatives of E1 and E2 and of ubiquitin-protein conjugates

Fraction II (500 μg), 5 μg of ¹²⁵I-ubiquitin (5 × 10⁵ c.p.m.) and 5 mM-ATP were incubated at 37 °C for 2 h, followed by incubation for 10 min in the presence of 25 mM-NaOH. The samples were neutralized and samples incubated with 50 μg of fresh fraction II, in the presence of the indicated protease inhibitors, for a further 6 h at 37 °C. Loss of ¹²⁵I-ubiquitin was measured, after SDS/PAGE and autoradiography, as described under 'Methods'. Abbreviation: PMSF, phenylmethanesulphonyl fluoride.

Treatment	¹²⁵ I-ubiquitin derivative					
	E1		E2		Other proteins	
	(c.p.m.)	(% decrease in activity)	(c.p.m.)	(% decrease in activity)	(c.p.m.)	(% decrease in activity)
Zero time	5276	—	12923	—	15106	—
No inhibitor	2441	54	7787	40	9272	39
+ 5 mM-NEM	5240	0	12254	5	15319	2
+ 125 μM-ZnCl ₂	4430	17	11424	11	12162	20
+ 2 mM-PMSF	3288	38	9438	27	9952	34
+ 12.5 μg of chymostatin/ml	2975	44	8533	34	9064	40

Fig. 4. Effects of incubation pH on the formation of ^{125}I -ubiquitin derivatives of E1, E2 and other proteins compared with residual ubiquitin ATP/[^{32}P]PP_i-exchange activity

(a) Fraction II (100 μg) was incubated with 1 μg of ^{125}I -ubiquitin (1×10^5 c.p.m.) and 5 mM-ATP for 2 h at the pH indicated. E1-N-ubiquitin (○), E2-N-ubiquitin (●) and ubiquitin-protein conjugates (■) were quantified after SDS/PAGE and autoradiography, all as described under 'Methods'. (b) Fraction II (100 μg) was preincubated for 2 h in the absence (○) and presence (●) of 1 μg of unlabelled ubiquitin and 5 mM-ATP. After neutralization of all samples to pH 7.4, ubiquitin-dependent adenylate-forming activity was measured by ATP/[^{32}P]PP_i-exchange reactions, as described under 'Methods'.

overall conjugation pathway for other proteins (Fig. 5, lanes a and b, and Fig. 6).

Alkali treatment of fraction II preincubated with Tos-Lys-CH₂Cl provided information concerning its E2-S-Ub status. At 100 μM -Tos-Lys-CH₂Cl, the amount of ubiquitin associated with the M_r -37000 and -33000 forms of E2 was much decreased indicating that, although ubiquitin could be transferred from E1 to these E2s, this occurred at a lower rate under these conditions (Fig. 5, lanes d and e).

The action of Tos-Lys-CH₂Cl was not due to inhibition of a trypsin-like protease, as treatment of fraction II with 100 μM -

Fig. 5. Effect of Tos-Lys-CH₂Cl on ubiquitin-conjugate formation and on ubiquitin derivatives of E1 and E2

Lane a: fraction II (100 μg) was incubated for 2 h as in Fig. 1, lane a. Lane b: fraction II (100 μg) was preincubated for 2 h at 37 °C with 50 μM -Tos-Lys-CH₂Cl, before addition of 1 μg of ^{125}I -ubiquitin (1×10^5 c.p.m.) and 5 mM-ATP, and incubated for a further 2 h. Samples were then heated at 90 °C in the presence of 2-mercaptoethanol and run on 5–20% gradient SDS gels. Lane c: M_r markers, as described in Fig. 1, lane d. Lane d: samples were treated with Tos-Lys-CH₂Cl as in lane b and incubated for 30 min, followed by treatment for 10 min with NaOH at the end of the incubation. Lane e: sample was treated as in lane d, but Tos-Lys-CH₂Cl was omitted.

Fig. 6. Effect of Tos-Lys-CH₂Cl concentration at pH 7.4 on the auto-ubiquitination of E1 and the formation of ubiquitin conjugates

Fraction II was incubated for 2 h with Tos-Lys-CH₂Cl at the indicated concentrations before addition of 1 μg of ^{125}I -ubiquitin (1×10^5 c.p.m.) and 5 mM-ATP. Samples were then incubated for a further 2 h, and ^{125}I -ubiquitin conjugates (●) and auto-ubiquitinated E1 (○) were quantified after SDS/PAGE and autoradiography (see under 'Methods').

benzamidine, a trypsin-protease inhibitor, did not cause the E1-N-Ub band to form (results not shown).

The activity of the adenylate-forming site of E1 treated with Tos-Lys-CH₂Cl was next investigated to see if changes here were responsible for the decrease in conjugation activity. The ubiquitin-dependent ATP/[^{32}P]PP_i exchange reaction was

Fig. 7. Effect of Tos-Lys-CH₂Cl on the ubiquitin adenylate-forming activity of E1

Fraction II was incubated with 1 μg of unlabelled ubiquitin and Tos-Lys-CH₂Cl at the indicated concentrations for 2 h at 37 °C. Ubiquitin adenylate-forming activity was measured by ATP/[³²P]PP_i exchange, as described under 'Methods'.

markedly decreased as the Tos-Lys-CH₂Cl concentration was increased (Fig. 7). Thus Tos-Lys-CH₂Cl appeared to affect the adenylate site, possibly by the alkylation of an essential histidine residue or thiol group.

DISCUSSION

The novel phenomena reported in this paper arose from the observation that incubation at pH 7.4 of a ubiquitin-depleted fraction from chicken skeletal muscle with [¹²⁵I]-ubiquitin slowly led to the formation of auto-ubiquitinated forms of E1 and several E2 species. The reaction was greatly enhanced when the incubated extracts were adjusted to a more alkaline pH; under these conditions ubiquitinated forms of E1 and three of the five E2s appeared in much larger amounts. The formation under these conditions of peptide bonds between ubiquitin and E1 and E2s was shown by (i) the susceptibility of the covalently bound ubiquitin moieties to isopeptidases present in fraction II, (ii) the stability of the bonds to treatment with thiols, alkali and hydroxylamine, and (iii) successful competition for transfer of ubiquitin from a primary amine, L-lysine, added at a high concentration.

No primary or higher structures for E1 or any of the E2s are known, and we have accordingly not been able to determine whether a single residue is the ubiquitin acceptor in any of these cases. There is also no explanation yet for the fact that only three of the five E2s present in chicken muscle become auto-ubiquitinated. One may assume that a spatially adjacent lysine or N-terminal residue is involved in the transacylation reaction between ubiquitin and the E1 or E2 proteins, but this notion can be based provisionally only on the (slow) formation of the auto-conjugates at a non-denaturing pH (7.4). Mayer *et al.* (1989) have purified E1 to homogeneity both from the thermolabile mouse mammary-carcinoma cell line, ts85, and from the parental strain, FM3A. They found that E1 co-purified with a number of proteins of slightly higher *M_r* and suggested that these may be ubiquitinated forms of E1. Hatfield & Vierstra (1989), however, have purified several E1 species from wheat-germ extracts and reticulocytes, but have shown with antibodies to E1 and ubiquitin that these proteins are isoforms of E1 and do not contain any ubiquitin.

Both E1 and four of the five E2s each exist intracellularly in a weak subunit interaction as dimers and tetramers (Haas &

Bright, 1988). Auto-ubiquitination could thus involve inter-subunit as opposed to intra-subunit reactions, which may imply that these E2 forms that do not transacylate are monomeric species of E2.

At present, the physiological significance of auto-ubiquitinated forms of the ubiquitin-charging enzymes in regulating the ubiquitin pathway in living cells is unknown. We have shown that their formation is not unique to chicken muscle. Preincubation of the ubiquitin-depleted fraction with the protease inhibitor Tos-Lys-CH₂Cl decreased the adenylate-forming and the thioester-transferring activities of E1 and diminished ubiquitin conjugation to other endogenous proteins. At the same time auto-ubiquitination of E1 at pH 7.4 was greatly increased. This may have been due to alkylation of a histidine residue or critical thiol group on E1 accompanied by conformational changes that could have disrupted the ubiquitin-activation reaction and enhanced auto-ubiquitination of E1 at the expense of ubiquitin transfer to E2 species. Tos-Lys-CH₂Cl has been described by Schnebli (1975) as inhibiting the growth of virally transformed 3T3 fibroblasts (SV3T3) by blockage of their cell cycles in the G₂ phase. The thermolabile mutant, ts85, when grown at non-permissive temperatures is also arrested in this phase, an effect that is known to be caused by thermal inactivation of E1 (Finley *et al.*, 1984). The specific growth-inhibition response to Tos-Lys-CH₂Cl observed in the SV3T3 cells could thus have been due to decreased activity of E1 caused by intracellular alkylation of the protein. If this can be confirmed, the inhibition may prove to be a useful probe for the role of E1 in cellular processes.

The occurrence *in vitro* of these novel auto-modifying reactions of proteins bearing thioester-bound ubiquitin on specific active-site residues is a reminder of the incompleteness of our present understanding of the detailed mechanism(s) by which physiological ubiquitination of target proteins takes place (Haas & Bright, 1988). This applies especially to the apparent selectivity of such emplacements: only some proteins become ubiquitinated, and in each case the choice of free amino group is not random (Hershko, 1988). In one particularly significant instance, a ubiquitinating system achieves the selection of both a 'primary' target (a mobile lysine residue near a free N-terminal amino group) and repeated 'secondary' targets at lysine-48 positions of up to 20 ubiquitins forming a multi-ubiquitination chain (Bachmair & Varshavsky, 1989). It is thus clear that the different kinds of ubiquitin-transfer reactions require further molecular clarification and categorization.

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REFERENCES

- Bachmair, A. & Varshavsky, A. (1989) *Cell* **56**, 1019–1032
- Barrett, A. J. (1973) *Biochem. J.* **131**, 809–822
- Calendar, R. & Berg, P. (1966) *Biochemistry* **5**, 1681–1687
- Ciechanover, A., Elias, S., Heller, H. & Hershko, A. (1982) *J. Biol. Chem.* **257**, 2537–2542
- Fagan, J. M., Waxman, L. & Goldberg, A. L. (1987) *Biochem. J.* **243**, 335–343
- Finley, D., Ciechanover, A. & Varshavsky, A. (1984) *Cell* **37**, 43–55
- Gehrke, P. P. & Jennissen, H. P. (1987) *Biol. Chem. Hoppe-Seyler* **368**, 691–708
- Haas, A. L. & Bright, P. M. (1988) *J. Biol. Chem.* **263**, 13258–13267
- 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., Warms, J. V. B., Hershko, A. & Rose, I. A. (1982) *J. Biol. Chem.* **257**, 2543–2548
- Haas, A. L., Bright, P. M. & Jackson, V. E. (1988) *J. Biol. Chem.* **263**, 13268–13275

- Hatfield, P. M. & Vierstra, R. D. (1989) *Biochemistry* **28**, 735–742
- Hershko, A. (1988) *J. Biol. Chem.* **263**, 15237–15240
- Hershko, A., Heller, H., Elias, S. & Ciechanover, A. (1983) *J. Biol. Chem.* **258**, 8206–8214
- Hershko, A., Heller, H., Eytan, E. & Reiss, Y. (1986) *J. Biol. Chem.* **261**, 11992–11999
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Lickteig, R., Shenolikar, S., Denner, L. & Kelly, P. T. (1988) *J. Biol. Chem.* **263**, 19232–19239
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Mayer, A., Gropper, R., Schwartz, A. L. & Ciechanover, A. (1989) *J. Biol. Chem.* **264**, 2060–2068
- Moore, A. T., Williams, K. E. & Lloyd, J. B. (1977) *Biochem. J.* **164**, 607–616
- Pickart, C. M. & Rose, I. A. (1985) *J. Biol. Chem.* **260**, 1573–1581
- Schlesinger, M. & Hershko, A. (eds.) (1988) *The Ubiquitin System*, Cold Spring Harbor Laboratory, Cold Spring Harbor
- Schnebli, H. P. (1975) in *Proteases and Biological Control* (Reich, E., Rifkin, D. B. & Shaw, E., eds.), pp. 785–794, Cold Spring Harbor Laboratory, Cold Spring Harbor
- Shaw, E., Mares-Guia, M. & Cohen, W. (1965) *Biochemistry* **4**, 2219–2224
- Yarden, Y. & Ullrich, A. (1988) *Annu. Rev. Biochem.* **57**, 443–478

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