Purification and characterization of ubiquitin from mammalian testis

M. Loir°, A. Caraty, M. Lanneau, Y. Menezo*, J.P. Muh[+] and P. Sautiere[†]

*INRA, Physiologie de la Reproduction, 37380 Nouzilly, *INSA, Lab. Biologie 406, 69621 Villeurbanne, [+]ERA 697, CNRS, Faculté Médecine, 37032 Tours,* and *[†]U 124 INSERM, Place de Verdun, 59000 Lille, France*

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Ubiquitin was extracted from testis of 4 mammals and purified to homogeneity by gel filtration chromatography. Amino acid compositions and NH_2-terminal sequences were found to be identical in the 4 species and with calf thymus ubiquitin. Ubiquitin conformation was shown to be very sensitive to oxidation. Improved methods for radioimmunoassay of ubiquitin in tissue extracts are also discussed.

Ubiquitin *Radioimmunoassay* *Amino acid analysis* *Gel filtration chromatography* *Mammalian testis*

1. INTRODUCTION

Ubiquitin is a small protein (M_r 8500) which has been identifed in animal cells, higher plants, yeast and bacteria [1] and which has been purified from calf thymus [1–3], human thymus [4], and trout testis [5]. The wide distribution and highly conserved amino acid sequence of ubiquitin have suggested the importance of this protein in an, as yet, undefined but universal process. Ubiquitin has been identified as the ATP-dependent proteolysis factor required for protein degradation in reticulocytes [6]. Moreover, ubiquitin has been shown to exist as a free protein in chromatin [3,5] and as part of the chromosomal protein A24 [7]. In our previous investigations, we demonstrated that the mammalian testis often contains large amounts of ubiquitin, a protein which we designated P_2 [8]. This paper reports a preparative method for purification of mammalian testis ubiquitin and an improved radioimmunoassay useful for detecting ubiquitin in tissue extracts. Furthermore, data concerning the influence of oxidation on ubiquitin conformation are also reported.

° To whom correspondence should be addressed at: INRA, Physiologie des Poissons, Campus de Beaulieu, 35042 Rennes Cédex, France

2. MATERIALS AND METHODS

2.1. Purification of ubiquitin

All operations were performed at 2°C. Testes were removed from sexually mature rams *(Ovis aries)*, goats *(Capra hircus)*, boars *(Sus scrofa)* and Wistar rats *(Mus rattus)* and were used either immediately or after storage in liquid nitrogen or at −20°C. No difference was observed between fresh and frozen testes. For purification, 500 g of testis or the largest amount available, were homogenized for 2 min at top speed in a commercial Waring blender with 500 ml of 0.05 M Na bisulfite, 0.005 M EDTA in water adjusted to pH 7.0 with solid Tris. Subsequently, 500 ml of 0.4 M H_2SO_4 were added slowly with stirring and the homogenate was clarified by centrifugation at 15 000 × *g* for 30 min. Trichloracetic acid (20%) was slowly added to the supernatant with stirring to a final concentration of 2 or 3% (w/v). The precipitate was removed by centrifugation, as before, and the supernatant was then made up to 5, 7 or 9% (w/v). The final precipitate was recovered by centrifugation, washed once with acidified acetone (0.1 ml conc. HCl/100 ml), twice with acetone and dried under vacuum. The proteins thus obtained were fractionated by gel filtration on a Bio-Gel P10 column (100 × 3.2 cm)

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eluted with 0.01 M HCl [9]. The fractions containing ubiquitin were combined and lyophilized. When necessary, ubiquitin was further purified on CM–cellulose (Whatman, CM52) eluted with a linear gradient of LiCl in 0.01 M lithium acetate (pH 5.0) [10].

2.2. Oxidation of ubiquitin

Purified ubiquitin was oxidized either for 1 min in the course of the chloramine T procedure, the reaction being stopped by addition of Na metabisulfite [11] or for 1 h in 0.3% H_2O_2 [12]. Aliquots of oxidized ubiquitin were reduced by treatment for 22 h at 20°C with 0.5 M 2-mercaptoethanol in the presence of 8 M urea or 6 M guanidine–HCl. The reaction was stopped by addition of acetic acid to 0.9 M final concentration.

2.3. Polyacrylamide gel electrophoresis

Proteins were analyzed by electrophoresis in gels containing acetic acid and urea as in [13]. A modification of this method was developed to cast slab gels containing horizontal linear gradient of 0–8 M urea. Two-dimensional gels were run as in [14]. The percentage of ubiquitin in protein extracts was estimated after staining the gels with Coomassie brillant blue R250, scanning at 620 nm, and integrating the areas under the scanned peaks.

2.4. M_r determination

The M_r of testis ubiquitin was determined by gel filtration in the presence of 6 M guanidine–HCl on a column (100 × 2 cm) of Bio-Gel A-1.5 m [15] which had been calibrated with calf thymus histones and ram protamine (M_r 6600). The M_r (in parentheses) was also estimated by comparison of its mobility with those of histone 1: (21 000), ribonuclease A (13 700), cytochrome *c* (12 384) and insulin (6000) on SDS slab gels prepared as in [16] or [14].

2.5. Amino acid analysis

Protein samples were hydrolyzed in sealed evacuated tubes at 110°C for 24 and 72 h in 6 M HCl (1 ml/mg protein). One drop of 1% phenol was added to prevent excessive degradation of tyrosine. Amino acid analyses were preformed using a single column on a Beckman model 119 CL amino acid analyzer. Tryptophan was determined after hydrolysis with *p*-toluenesulfonic acid [17].

2.6. Structural studies

Automated sequencing of ubiquitin was performed on a Beckman model 890 C sequencer using a dimethyllallylamine program (102974) in the presence of polybren [18]. Phenylthiohydantoin derivatives of amino acids were identifed by high-pressure liquid chromatography on a column of C_{18} μBondapack [19]. For carboxy terminal analysis of ubiquitin, the protein (110 μg, 14 nmol) was dissolved in 0.5 ml of 0.2 M *N*-methylmorpholine acetate (pH 8.0) and digested at 37°C with carboxypeptidase B (2 μg). After 1 h, an aliquot was removed, acidified with glacial acetic acid and freeze-dried. Carboxypeptidase A (2 μg) was then added. An aliquot was removed at 1 h and treated as above. The released amino acids were analysed on the amino acid analyzer.

2.7. Ubiquitin radioimmunoassay

Ram ubiquitin was conjugated with bovine serum albumin (BSA) using either glutaraldehyde [20] or toluene diisocyanate (TDIC) [21]. Antisera were prepared by homogenizing ubiquitin conjugates with Freund's complete adjuvant and immunizing two New Zealand rabbits per conjugate. The first injection was made into the spleen (0.5 mg conjugated ubiquitin per rabbit); 3 booster intradermal injections were made after 1, 2 and 6 weeks; and a final intravenous injection without adjuvant was made 10 weeks after the original immunization. To carry out the radioimmunoassay, ubiquitin was labeled with ^{125}I by two different methods. This protein was either labeled directly by the chloramine T procedure [11] or coupled, via glutaraldehyde, to thyroglobulin or to pig luteinizing hormone (pLH) which had been previously labeled with ^{125}I by the chloramine T procedure [22].

3. RESULTS

3.1. Purification of testicular ubiquitin

Ubiquitin is present in the sulfuric acid extract of mammalian testis [8]. Addition of low concentrations of trichloroacetic acid to such an extract precipitates histones and other proteins, while higher concentrations of trichloroacetic acid are required to precipitate ubiquitin. The optimal trichloroacetic acid concentrations for these two precipitation steps were determined empirically for

each of the 4 mammalian species studied and found to be 3 and 7% for ram, 3 and 9% for rat, 2 and 5% for boar and 2 and 5% for goat. The yield in the second trichloroacetic acid precipitate was 200–800 mg/kg testis depending on the species.

These trichloroacetic acid extracts containing ubiquitin were fractionated on Bio-Gel P10. Ubiquitin was eluted as a symmetrical peak with $K_{av} = 1.8–1.9$. After electrophoresis on polyacrylamide gels containing acetic acid–urea or SDS, ubiquitin migrated as a single component, the mobility of which was identical in the 4 species. In goat the ubiquitin peak was slightly contaminated by minor components which were subsequently removed by ion-exchange chromatography on CM52. Goat ubiquitin was then eluted as a sharp peak with 0.25 M LiCl. After gel filtration chromatography, the yields of pure ubiquitin were 20–34 mg/kg testis.

3.2. *Partial characterization*

The M_r of ubiquitin purified from ram testis was 8400 as estimated by gel filtration in 6 M guanidine–HCl. By electrophoresis in SDS, ram and boar testis ubiquitin showed an M_r of 8100. As shown in table 1, the amino acid composition of testis ubiquitin was found to be identical in the 4-species studied and also identical to that of calf thymus ubiquitin. Similarly, the NH_2-terminal amino acid sequence of the 4 testis ubiquitins was found to be:

H-Met-Gln-Ile-Phe-Val-Lys-Thr-Leu-Thr-Gly-
1 5 10

a sequence which is also identical with that of calf thymus [2] and trout testis ubiquitin [5]. Digestion of ram testis ubiquitin with carboxypeptidase B and A established the carboxy terminal sequence as Arg-Leu-Arg (OH).

When pure ram testis ubiquitin was subjected to electrophoresis with histone 1 on a gel containing a horizontal gradient of urea, it exhibited R_{H1} values decreasing from 2.90 at 0 M urea to 1.41 at 8 M urea (fig.1). Histone H1 has been shown to remain in an extended configuration under these conditions [23].

When testis ubiquitin was oxidized either experi-

Table 1

Amino acid composition of ubiquitin from mammalian testes

Amino acid	Boar	Coat	Ram	Rat	Calf thymus
Aspartic acid	7.3 (7)	6.8 (7)	7.0 (7)	7.1 (7)	7
Threonine	7.0 (7)	6.5 (7)	6.7 (7)	7.2 (7)	7
Serine	3.4 (3)	3.2 (3)	2.9 (3)	3.5 (3)	3
Glutamic acid	12.4 (12)	12.1 (12)	12.1 (12)	11.8 (12)	12
Proline	2.7 (3)	2.7 (3)	3.0 (3)	2.7 (3)	3
Glycine	4.2 (4)	3.9 (4)	4.3 (4)	4.3 (4)	4
Alanine	2.3 (2)	2.1 (2)	2.3 (2)	2.1 (2)	2
Valine	4.1 (4)	3.9 (4)	3.9 (4)	3.9 (4)	4
Methionine	0.9 (1)	1.0 (1)	0.9 (1)	0.9 (1)	1
Isoleucine	6.5 (7)	6.7 (7)	6.6 (7)	6.7 (7)	7
Leucine	8.7 (9)	8.7 (9)	9.0 (9)	8.8 (9)	9
Tyrosine	1.3 (1)	1.1 (1)	1.2 (1)	1.2 (1)	1
Phenylalanine	2.0 (2)	1.9 (2)	2.0 (2)	2.0 (2)	2
Histidine	1.1 (1)	1.1 (1)	1.2 (1)	1.2 (1)	1
Lysine	7.1 (7)	7.1 (7)	7.6 (7–8)	7.3 (7)	7
Arginine	4.4 (4)	4.2 (4)	4.2 (4)	4.4 (4)	4
Tryptophan	0	ND	ND	ND	

Results obtained from duplicate analyses of 24 and 72 h hydrolysates are expressed as number of residues per mol protein. Values in parentheses are the nearest integers. Values for threonine and serine are zero-time extrapolations. Amino acid composition of calf thymus ubiquitin [2] is given for comparison. ND, not determined.

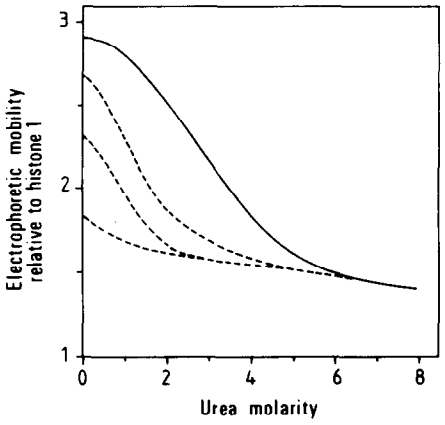

Fig.1. Relative mobilities of ram and boar testicular ubiquitin and of the 3 bands induced by chloramine T oxidation, as a function of urea molarity in acid gels.

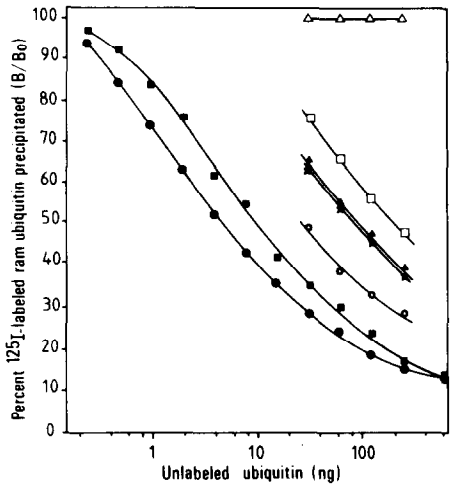

Fig.2. Curves of binding-inhibition for ubiquitin. Radioimmunoassay (RIA) was carried out using ram ubiquitin coupled to ^{125}I-labeled pig luteinizing hormone. Antiserum against ram ubiquitin conjugated to BSA by TDIC was at 1/32000 final dilution. Each determination was made in triplicate. (●) Purified boar ubiquitin; (■) purified ram ubiquitin; (□) 3% trichloroacetic acid-soluble testicular proteins of ram (3.9% ubiquitin, RIA = 2.0%); (▲) 3% trichloroacetic acid-soluble testicular proteins of stallion (4.1% ubiquitin, RIA = 4.7%); (×) 3% trichloroacetic acid-soluble testicular proteins of cat (4.9% ubiquitin, RIA = 4.7%); (○) 3% trichloroacetic acid-soluble, 7% trichloroacetic acid-insoluble testicular proteins of boar (7.7% ubiquitin, RIA = 14%); (△) 5% perchloric acid-insoluble proteins of ram testicular chromatin (0% ubiquitin, RIA = 0%).

mentally or by extended storage (3–5 years), 3 additional protein bands with slower mobilities were present on the acid gels containing no urea. The amount of 'native' ubiquitin was consequently reduced or even completely absent in the case of oxidation by chloramine T. The R_{H1} values of the 3 slower bands decreased with increasing molarity of urea so that in gels containing 6 M urea or more, only one band was present (fig.1). On SDS gels, samples of oxidized ubiquitin did not differ from those of native ubiquitin with only one band being observed. Treatment of oxidized ubiquitin with 2-mercaptoethanol did not reduce or suppress the 3 slower bands.

3.3. Radioimmunoassay for ubiquitin

Adequate titers of antibodies developed after 4 or 5 injections irrespective of whether the injected antigen had been conjugated to BSA by glutaraldehyde or by TDIC. When ram testis ubiquitin was labeled directly with ^{125}I by the chloramine T procedure, no binding was observed between the iodinated protein and the antiserum. On the other hand, when ubiquitin was coupled to iodinated thyroglobulin or pLH, there was binding. In the absence of unlabeled ubiquitin, the antisera bound more than 90% of labeled ubiquitin at a dilution of 1:16000 with antigen conjugated by glutaraldehyde and at a dilution of 1:1000 with antigen conjugated by TDIC. The antisera bound 50% (B/B_0 = 50%) of the labeled ubiquitin at dilutions of 1:128000 and of 1:32000, respectively. However, while 100 ng of unlabeled ubiquitin produced a substantial displacement of the labeled ubiquitin in the case of antisera obtained with antigen conjugated by TDIC (15–19% for B/B_0 = 50%), a smaller displacement was observed when the antigen had been conjugated with glutaraldehyde (3–6%). Fig.2 shows the binding-inhibition curve obtained with unlabeled ram and boar ubiquitin which produced substantial displacement of labeled ubiquitin in the range of 0.25–125 ng and with 5 different protein extracts from ram, pig, horse, and cat testes. The ubiquitin content of each extract was quantitated, by staining and scanning of acid–urea gels, and is noted in the figure legend. Each of the 5 displaced the labeled antigen by an amount proportional to its ubiquitin content as also noted in the figure legend.

4. DISCUSSION

Several methods [1,3,5] have been used for purification of ubiquitin, the more recent involving chromatin preparation and isolation of total high mobility group (HMG) proteins, and the yields were 12.5 and 17 mg per kg organ. The present method is much more rapid and resulted in protein yields approx. 2-fold higher than previously obtained. This simple one-step purification by gel filtration was made possible by the fact that ubiquitin was extremely retarded on the column of Bio-Gel P10, while most of the other acid-soluble testicular proteins were eluted early. The fortuitous retardation of ubiquitin was probably due to interaction between charged groups of the protein and those of the gel, since addition of 0.15 M NaCl to the elution buffer decreased the K_{av} of ubiquitin from 1.8 to 0.95 (not shown). This purification scheme has not been applied to tissues other than mammalian testis. However, if a tissue does not contain protein(s) eluting from a Bio-Gel P10 column with a K_{av} around 1.7–1.9, this scheme might be used.

The amino acid composition and carboxy terminal sequence analysis indicate that the ubiquitin which we have isolated terminates in Arg^{74}. Authors in [24] have shown that the ubiquitin active in stimulating ATP-dependent proteolysis possesses the carboxy terminal sequence Arg-Gly-Gly^{76}, and have suggested that the cleavage of the Gly-Gly terminus could be an in vivo control mechanism. Since the methods we have used exclude possible tryptic proteolysis of ubiquitin during purification our result suggests that most of the mammalian testicular ubiquitin should be inactive in situ in protein degradation.

An effect of oxidation on ubiquitin conformation has never before been reported. From our electrophoretic data (fig.1), we propose that oxidation induces 3 conformations less globular than native ubiquitin but less extended than the completely denatured protein. Because a reducing treatment did not suppress these conformations, oxidation of the methionine appears to not be responsible for the changes.

Authors in [1] have developed a radioimmunoassay for ubiquitin. The antisera which we obtained after 5 injections showed higher titers perhaps resulting from the immunization schedule which we used. In contrast to the results in [1] we did not obtain binding between the antisera and ubiquitin directly labeled with ^{125}I by the chloramine T Procedure. The effect of this strong oxidant on ubiquitin conformation could explain our result. Similarly, the different binding-inhibition curves (fig.2) which we obtained with ram and boar ubiquitin could be caused by the fact that the ram sample had been stored for 5 years and was more oxidized, as judged by electrophoresis, than the boar sample. When the antigen used for immunization was conjugated using TDIC, the antisera titers were somewhat lower than when glutaraldehyde was used; but, in the case of TDIC, unlabeled ubiquitin displaced much more efficiently the labeled ubiquitin. The hypothesis most likely to explain this last difference is that glutaraldehyde might cause a conformational change in ubiquitin and that antibodies directed against this antigen would have a higher affinity for the ubiquitin coupled to thyroglobulin or pLH via glutaraldehyde than for native ubiquitin.

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