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Ubiquitin found in the archaebacterium *Thermoplasma acidophilum*

Stefan Wolf, Friedrich Lottspeich and Wolfgang Baumeister

Max-Planck-Institut für Biochemie, Am Klopferspitz 18a, W-8033 Martinsried, Germany

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Systematic N-terminal sequencing of the low molecular weight proteins from *Thermoplasma acidophilum* separated by two-dimensional polyacrylamide gel electrophoresis led to the discovery of a polypeptide with an apparent M_r of 4.5 kDa identical as its first 18 amino acid residues to human ubiquitin. The occurrence of ubiquitin and proteasomes in an archaebacterium strongly suggests that ATP–ubiquitin-dependent proteolysis is a cellular function that developed early in evolution.

Ubiquitin; Archaebacterium (*Thermoplasma acidophilum*); Two-dimensional electrophoresis; Microsequencing

1. INTRODUCTION

Ubiquitin-dependent proteolysis plays a key role in the selective elimination of misfolded cytosolic and nuclear proteins, as well as in the spatially and temporally coordinated degradation of short-lived regulatory proteins (for recent reviews see [1–4]). Ubiquitin occurs in all eukaryotic cells; it is a highly conserved 76 amino acid residue polypeptide which is conjugated to its target proteins by means of a cascade of activating and ligating enzymes [5,6]. To our knowledge, all attempts made so far to detect ubiquitin to prokaryotic cells have been unsuccessful (see e.g. [7]).

In recent years, evidence has accumulated that the 26 S complex is the proteolytic system effecting the degradation of ubiquitinated substrate proteins (for recent reviews see [8–10]). The 26 S complex contains the 20 S proteasome, which is supposed to represent its catalytic core. Since proteasomes indistinguishable in their basic molecular architecture from eukaryotic proteasomes, albeit simpler in subunit composition (2 instead of 12–15 different subunits), have been found in the archaebacterium, *Thermoplasma acidophilum* [11,12], it was tempting to scrutinize this organism for the existence of ubiquitin.

Correspondence address: W. Baumeister, Max-Planck-Institut für Biochemie, Am Klopferspitz 18a, W-8033 Martinsried, Germany. Fax: (49) (89) 8578 2641.

Abbreviations: 2D, two-dimensional; IEF, isoelectric focussing; IPG, immobilized pH gradient; PAGE, polyacrylamide gel electrophoresis; NP-40, Nonidet P-40; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; PVDF, polyvinylidenefluoride; PTH, phenylthiohydantoin.

2. MATERIALS AND METHODS

2.1. *Sample preparation*

Thermoplasma acidophilum was grown at 59°C (pH 1.0) as described by Christiansen et al. [13]. 0.25 g of harvested cells (5,000 × *g*, 15 min) were suspended in 1 ml 20 mM Tris-HCl, pH 7.5, and were broken by ultrasonication (10 × 10 s, on ice). Nucleic acid digestion was performed by adding 10 μg DNase II plus 10 mM $MgCl_2$ and incubating for 1 h at 20°C. Particulate material was pelleted (16,000 × *g*, 5 min, 4°C) and the supernatant was re-centrifuged (100,000 × *g*, 1 h, 4°C). Protein concentration of the supernatant was determined with bicinchoninic acid (BCA) according to Smith et al. [14]. Soluble proteins were prepared for 2D polyacrylamide gel electrophoresis by adding urea (9 M), non-ionic detergent NP-40 (3% v/v), β-mercaptoethanol (5% v/v) and carrier ampholytes (0.8% v/v, pH 3–10).

2.2. *2D electrophoresis*

The 2D electrophoresis was performed basically as described by Görg et al. [15]. 400 μg of the total cell protein was applied in a volume of 20 μl to the gel for isoelectric focussing (IEF), run in the first dimension. For this purpose dried immobilized pH gradient (IPG) gel strips (5% acrylamide, 0.5 × 3 × 10 mm^3) with a linear gradient of pH 5.0–8.0 were rehydrated in an solution of urea (8 M), NP-40 (0.5% v/v), dithiothreitol (DTT, 10 mM) for 16 h at 20°C. The sample was focussed with at least 40 kV/h (1 h at 300 V, 16 h at 2500 V) on a horizontal Multiphor II apparatus (Pharmacia). For electrophoresis in the second dimension, gel strips were equilibrated in a solution of Tris-HCl (50 mM, pH 6.8), urea (6 M), EDTA (0.8 mM), SDS (2% w/v), DTT (1% w/v), glycerol (10% v/v) and Bromphenol blue (0.01% w/v) for 2 × 15 min and transferred to the top of a Tricine-SDS slab gel. The gel size and composition were 1.0 × 110 × 120 mm^3, 18% acrylamide and electrophoresis was carried out in the vertical orientation at 80 V for 1 h and 150 V for approximately 5 h.

Transfer of proteins from 2D gels onto a polyvinylidenefluoride (PVDF)-membrane (Fluorotrans, Millipore) was performed by horizontal semi-dry blotting in a buffer system consisting of 50 mM sodium tetraborate, pH 9.0, with 5% v/v methanol on the cathodic side and 20% on the anodic side, with a constant current of 200 mA for 4 h.

2.3. *Immunostaining*

After blocking non-specific binding sites with bovine serum albumine (BSA, 3% w/v) in 10 mM Tris-HCl, 150 mM NaCl, pH 8.0, proteins bound to the PVDF-membrane were incubated with anti-

ubiquitin antiserum (Sigma) developed in rabbit against native bovine ubiquitin (0.1% w/v dissolved in 50 mM Tris-HCl, 150 mM NaCl, 3% w/v BSA, 0.025% v/v NP-40) for 16 h at 4°C. The primary antibody was visualized by means of alkaline phosphatase-conjugated goat antiserum against rabbit IgG using the method of Blake et al. [16].

2.4. *N-Terminal sequencing*

The electrotransferred protein spots were visualized by staining with Coomassie brilliant blue R 250 (0.1% w/v) as described by Weber et al. [17]. Corresponding protein spots below 10 kDa from six identical blots were excised from the PVDF-sheet and combined in order to obtain sufficient material for amino acid sequence analysis. Edman degradation was done in an 477 A gas-phase sequencer from Applied Biosystems with identification of the phenylthiohydantoin (PTH) amino acid by a 120 A PTH-Analyser (Applied Biosystems).

3. RESULTS AND DISCUSSION

In a first attempt we have tried to detect ubiquitin in 2D gels of the *Thermoplasma acidophilum* total protein by immunostaining with rabbit antiserum against native ubiquitin from beef using alkaline phosphatase-conjugated anti-rabbit IgG for the visualisation of the primary antibodies. While control experiments, either adding bovine ubiquitin to the *T. acidophilum* proteins or subjecting HeLa cell proteins to high resolution 2D electrophoresis, gave clear signals with this antibody, no signal was obtained on blotted membranes of the *T. acidophilum* proteins.

Therefore, we took a 'brute-force' approach, subjecting all the spots in the 2D gels corresponding to molecular weights below 10 kDa to systematic N-terminal sequencing. Since all the eukaryotic ubiquitins described to date have isoelectric points (pIs) around 6.8, we performed IEF with a gradient ranging from pH 5.0–8.0. Several spots turned out to be *N*-terminally blocked and some yielded sequences which we were unable to assign to known proteins when checked against several protein databases using the FastP program [18] of the protein sequence query (PSQ) program

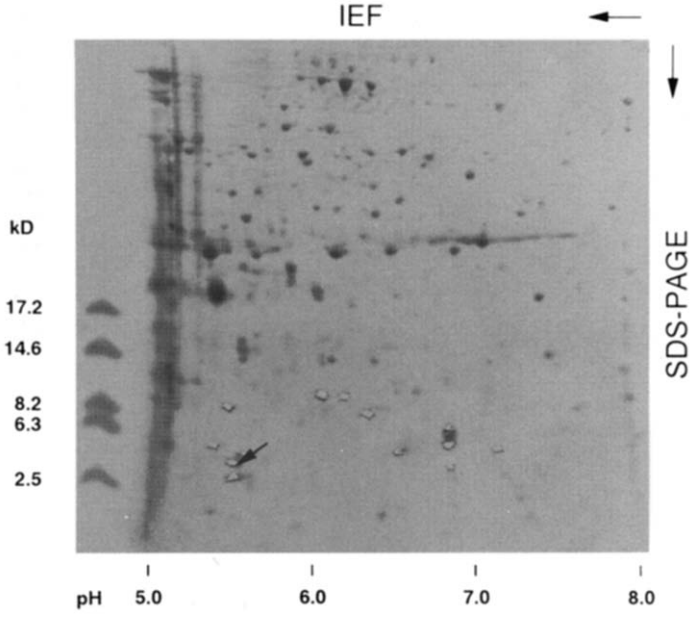

Fig. 1. 2D pattern of the *T. acidophilum* soluble proteins. A total of 400 μg of protein was subjected to 2D electrophoresis (1st dimension: IPG–IEF, pH 5.0–8.0; 2nd dimension: SDS-PAGE, 18% acrylamide), electroblotted onto a PVDF-membrane and stained with Coomassie brilliant blue R-250. Protein spots in the M_r range < 10 kDa were subjected to amino acid microsequence analysis. The spot marked with an arrow yielded the sequence shown in Fig. 2.

Thermoplasma acidophilum	MQIFV	KTLTG	KTITL	EVEA	[ref.]
Homo sapiens	- - - - -	- - - - -	- - - - -	- - - P	[19]
Saccharomyces cerivisiae	- - - - -	- - - - -	- - - - -	- - - S	[20]
Tetrahymena pyriformis	- - - - -	- - - - -	- - - - -	D - - -	[21]
Trypanosoma cruzi	- - - - -	- - - - -	- - - A -	- - - S	[22]
Dictyostelium discoideum	- - - - -	- - - - -	- - - - -	- - - G	[23]

Fig. 2. Comparison of the N-terminal sequence of *T. acidophilum* ubiquitin (top line) with ubiquitins from various eukaryotes. Only residues which differ from the *T. acidophilum* amino acid sequence are shown.

package. The spot marked with an arrow in Fig. 1, which corresponds to an apparent molecular weight of 4.5 kDa and a pI of 5.5, yielded the N-terminal sequence shown in Fig. 2. The first eighteen residues perfectly match the N-terminal region of human ubiquitin, and within the first nineteen residues there is only one exchange when compared with most eukaryotic ubiquitins. It should be noted in this context that under the same experimental conditions bovine ubiquitin also has an apparent M_r of 4.5 kDa, but a pI of 6.7; the abnormal electrophoretic mobility of the ubiquitins is either due to incomplete denaturation or to the binding of large amounts of SDS.

This is, to our knowledge, the first report on the existence of ubiquitin in a prokaryotic organism. The occurrence of both ubiquitin and proteasomes in *Thermoplasma acidophilum* makes it very likely that ubiquitin-dependent proteolysis is not a recent acquisition of eukaryotes but has an evolutionary antecedent in the archaebacteria. Whether or not ubiquitin conjugation involves a machinery of activating and ligating enzymes, as elaborate as in eukaryotic cells, remains to be investigated.

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