Crystal structure of the ubiquitin-like protein YukD from *Bacillus subtilis*

Fusinita van den Ent*, Jan Löwe

MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, UK

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Abstract The YukD protein in *Bacillus subtilis* was identified in a hidden Markov model (HMM) search as being related in sequence to ubiquitin. By solving the crystal structure we show that YukD adopts a fold that is most closely related to ubiquitin, yet has the shortest C-terminal tail of all known ubiquitin-like proteins. The endogenous gene of *yukD* in *B. subtilis* was disrupted without an obvious phenotypic effect and an inducible copy encoding a C-Myc and His-tagged version of the protein was introduced at the ectopic locus *amyE*. Conjugation assays performed both in vitro and in vivo indicate that YukD lacks the capacity for covalent bond formation with other proteins.

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1. Introduction

Ubiquitin is an abundant and conserved protein in eukaryotes that covalently binds to proteins, flagging them for protein degradation by the proteasome or for post-translational modification [1,2]. Once processed, ubiquitin’s C-terminal glycine bond covalently to its target substrate through an isopeptide bond, formed between the C-terminus and the ε-amino groups of a lysine residue in the acceptor protein. Ubiquitination requires ATP and a cascade of enzymes (E1, E2, and E3). Despite being ubiquitous in eukaryotes, no ubiquitin-like protein has been found in bacteria that signals proteins for degradation. It is still a mystery how in bacteria selective protein degradation is regulated and mediated by the energy-dependent proteases Lon, FtsH, HSLUV and the ClpA/XP complexes [3]. The half-life of a protein in bacteria is dependent on its N-terminal amino acid sequence (N-end rule), which is recognized by the ClpA/XP complex [4]. Small effector molecules regulate protein degradation, e.g. TraR [5], and also the association or dissociation of other proteins can control degradation of their binding partner (reviewed in [3]). Sometimes rapid, constitutive degradation combined with regulated gene expression directs protein levels, e.g. the SOS response protein SulA, which is degraded by LON protease [6]. In bacteria only one example is known where a covalently bound peptide tag targets proteins for degradation. The 11 amino acid co-translational SsrA-tag functions as a signal for degradation of proteins that are arrested in translation and form a substrate for ClpXP complex [7].

So far, the only known single-domain ubiquitin-like proteins in bacteria are the protein-based sulphur donor systems ThiS-ThiF and MoaD-MoeB. ThiS and MoaD have the same C-terminal double glycine motif as ubiquitin, which, upon activation, becomes covalently attached to a cysteine on their activating enzymes ThiF and MoeB, respectively. Following this, a sulphur transference step results in the formation of a thio-carboxylate, which acts as the sulphur donor in either thiamin synthesis or Molybdenum Cofactor biosynthesis.

Although the functions of these proteins is different from that of ubiquitin, the sulphur transference mechanism as well as their structural similarity points towards an evolutionary link between these proteins and ubiquitin [8–10]. Eukaryotes have single-domain ubiquitin-like (UBL) proteins (e.g. SUMO-1, RUB-1, NEDDS) that conjugate to other proteins using a similar chemistry as the sulphur donor-system described above, but rather than being involved in protein degradation, they are part of a wide variety of specific biological processes [11]. Most UBL proteins require an extra maturation step through C-terminal hydrolysis that makes the double glycine motif available for conjugation. The yeast UBL protein Hub1 lacks the C-terminal glycine motif usually used for conjugation to proteins and initial evidence suggested that Hub1 protein linkage might occur via a conserved tyrosine at the penultimate position of Hub1 [12]. Recent results, however, indicate that the detected protein interactions are of a non-covalent nature and furthermore the C-terminus of Hub1 is dispensable for its function [13].

In the past, several groups have searched for ubiquitin-like proteins in bacteria. Biochemical experiments were performed on protein extracts from the halobacterium *Natronococcus occultus* as well as the cyanobacterium *Anabaena variabilis* that showed cross-reactivity with antibodies raised against ubiquitin [14,15]. However, the identity of these proteins remained elusive. In a computational approach, sequence data were combined with structural information and three single-domain ubiquitin-like proteins were predicted to exist in Archaea [16]. No biochemical data are available that show ubiquitin-like conjugation of any form for these proteins.

The YukD protein in *Bacillus subtilis* was not identified as a potential candidate in the study mentioned above, but was found using a hidden Markov model (HMM) search with ubiquitin. YukD is not very widely conserved and its function is unknown. It is part of a large operon that constitutes the genes *yukABCDE* [17,18]. By solving the crystal structure we show that YukD adopts a fold that is most closely related to ubiquitin, yet has the shortest C-terminal tail of all known UBL proteins. The endogenous gene of *yukD* in *B. subtilis* was disrupted and an inducible copy encoding a C-Myc and His-tagged version of the protein was introduced at the ectopic locus *amyE*. Conjugation assays were performed both in vitro and in vivo.

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*Corresponding author. Fax: +44 1223 213556. E-mail address: fent@mrc-lmb.cam.ac.uk (F. van den Ent)."
2. Materials and methods

2.1. Bacterial expression and protein purification

All *B. subtilis* strains were grown in antibiotic medium No. 3 (Oxoid) supplemented with the required antibiotics.

The gene for the 79-amino acid protein YukD (Accession number BG123777; T4EMBL P71071) was amplified from *B. subtilis* genomic DNA (ATCC 23873D) by PCR. The N-terminus of YukD is preceded by HHHHHHGS, encoded by the forward primer (5'-AGTCTACCATATGCTACATCATCACACGGAAAGCTTATTGATAT-TACAATAGATTG). No extra residues were amended to the C-terminus (reverse primer: TGACTAGGATCCTATAATTTTCAACGCCGTCGCCGGTT). The PCR product was cleaved with BamHI/NdeI and ligated into BamHI/NdeI digested pHisBSD (B. Miroz, personal communication), generating pHisBSD. In a similar way, a flag-tagged version of the protein was made by introducing the flag-tag sequence with the forward primer (5'-AGTCTACCATATGCTACATCATCACACGGAAAGCTTATTGATAT-TACAATAGATTG). His-tagged protein was expressed in C41 cells [19] and purified using Ni²⁺-NTA affinity purification at pH 7.0, essentially as described [20]. After the protein was eluted from the column, the buffer was exchanged on a G25 column into TEN8.0 (20 mM Tris, 6.3, 0.45 M Na/K tartrate) and the crystals were grown in mother liquor (Sigma) and further purified over a superdex75 column in 50 mM potassium phosphate buffer supplemented with the required antibiotics.

2.2. Crystallisation and structure determination

YukD was crystallised at 10 mg/ml by sitting-drop vapour diffusion in 100 mM citrate 5.5, 1 M ammonium sulphate and the crystals were grown in mother liquor containing 12.5 mM EDTA, with 2 μl protein and 4 μl reservoir solution. The crystals were re-centred and indexed using SOLVE [23] and phases were calculated with SHARP [24]. The crystals belonged to space group P41212 with two molecules in the asymmetric unit. Cell constants were a = 50.32 Å, b = 50.32 Å, c = 174.93 Å (Table 1). Datasets were collected at beamlines SRS 14.2 (native dataset) and ESRF ID29 (selenomethionine derivatives).

2.3. Disruption of yukD and complementation by an inducible allele

The *b. subtilis* yukD gene was amplified using KOD hotstart DNA polymerase (Novagen) from pHisBSD with a forward primer including an Xba site, a RBS, an ATG and an SpeI site (5’-AGTCTACCATATGCTACATCATC-CAACGCCGTCGCCGGTT) and a reverse primer, including a stop codon and a NorI site (5’-TGACTAGGATCCTATA-ATTTTCAACGCCGTCGCCGGTT). The PCR product was digested with XbaI and NorI and ligated to the corresponding sites of pJPRI vector (J. Rawlins, J. Errington, pers. Communication), generating pFE188. pJPRI carries the 5’ and 3’ portions of the amy gene of *B. subtilis* flanking a neomycin resistance gene upstream of a xylose-inducible promoter (PxyI). Plasmid 3390 (gift from K. Nasmyth’s lab, IMP, Vienna) encoding 9 copies of the C-myc epitope between SpeI sites was digested with SpeI and the isolated c-myc region was ligated to SpeI-digested PFE188, generating pFE189. PFE189 was transformed into *B. subtilis* strain 168ED with selection for chloramphenicol (5 μg/ml) resulting in strain BS189 (bla amyE::cat PxyI-c-myc-his-yukD). Clones having an insertion in the amy locus were selected based on their inability to convert starch on a starch/chloramphenicol containing plate.

Subsequently, the wild type copy of the yukD gene was disrupted by inserting a neo cassette into the gene. A PCR product of 2 Kb of the upstream DNA sequence of YukD followed by 78 basepairs of the 5’ end of the gene was cloned into SpHl/PstI-digested vector derived from pBEST501 [27], generating pFE138. In the same way, 2 Kb of the downstream sequence of yukD preceded by 140 basepairs of the 3’ end of yukD was amplified and cloned into NorI/BamHI-digested vector derived from pFE138, resulting in plasmid pFE139. PFE139 was transformed into BS189 and transformants were grown on plates containing 0.5% xylose and 5 μg/ml kanamycin, generating strain BS139. All strains were PCR checked on genomic DNA and clones were verified by DNA sequencing.

2.4. Conjugation assay

In vitro reaction. Purified flag-tagged YukD (40 ng) was incubated in the presence of either 10 mM Mg-ATP or 12.5 mM EDTA, with or without *B. subtilis* extract (see legends to Fig. 4). For the latter, a 50 ml culture of wild type *B. subtilis* was grown until OD₆₀₀ = 0.5. The cells were lysed using 0.7 ml Y-PER (Pierce) and incubated for 20 min at RT, after which the extract was cleared by spinning at 13k for 10 min, at 4°C. Reactions were performed at 37°C for 20 min and were terminated by adding an equal volume of loading dye and heating for 5 min at 99°C.

In vivo reaction. *B. subtilis* strains 168ED, BS139 and BS139 were grown in an antibiotic medium No. 3 (Oxoid) supplemented with 5 μg/ml chloramphenicol (BS138) and 5 μg/ml kanamycin, 1% xylose (BS139) until late log-phase. Cells from 500 ml culture were lysed in 10 ml lysis buffer (8 M urea, 50 mM Tris 8.0, 20 mM imidazole) with two passes through a French press followed by 1 min sonication. Cell debris was removed by centrifugation and the supernatant purified over a Ni²⁺-NTA spin column. The columns were washed extensively
and the protein was eluted in lysis buffer supplemented with 600 mM imidazole. The eluent was precipitated with TCA, heated for 5 min at 99°C in 50 mM DTT. The samples were analysed on a 12.5% SDS–PAGE gel (Biorad) and immunoblotted using a monoclonal antibody against the C-myc epitope (9E10, Sigma).

3. Results and discussion

The structure of YukD shows the typical β-grasp fold of ubiquitin with two N-terminal anti-parallel β-strands connected to a α-helix, followed by a third β-strand that is linked via a helical turn to a fourth β-strand, which runs into a fifth β-strand via another helical turn (Fig. 1). The β-strands are arranged in the order S4pS3aS5pS1pS2a.

Structural comparison reveals that YukD and ubiquitin have the strongest resemblance among the single-domain ubiquitin-like proteins in the protein database, with an RMSD of 2.2 Å (Fig. 2) (using the DALI-web server [28]). However, a striking difference becomes apparent from a structure-based
sequence alignment showing that the C-terminal tail of YukD ends before the double glycine motif in ubiquitin (Fig. 3). The yeast protein Hub1 (with an RMSD of 2.5 Å) also has a much shorter C-terminal tail compared to ubiquitin (Figs. 2 and 3). Despite the absence of the double glycine motif at the C-terminus of Hub1, initial experiments suggested that Hub1 might conjugate with the conserved penultimate tyrosine [12]. However, recent evidence contradicted these findings, revealing that the conserved tyrosine residue is not required for formation of higher molecular weight complexes with Hub1 [13]. Precisely how Hub1 forms these SDS-resistant complexes is not clear at present. Small-ubiquitin-like modifier (SUMO-1) does conjugate to other proteins, although its role is to stabilize rather than to degrade proteins [29].

The striking structural similarity between YukD and ubiquitin prompted us to examine whether YukD would be able to conjugate to other proteins. Affinity purification and immunoblot analysis were combined to investigate whether any complexes were made either in vitro or in vivo. Purified flag-tagged YukD was incubated with extracts from B. subtilis in the presence or absence of Mg-ATP and reactions were analysed by SDS-PAGE and immunoblot (Fig. 4A). Clearly, flag-tagged YukD does not form higher molecular weight complexes under these conditions. To rule out the possibility of competition with endogenous YukD, the wildtype copy of yukD was disrupted by introducing a neo-cassette into the gene and a tagged version of YukD was expressed under the control of the Pxyl promoter at an ectopic locus (amyE) in B. subtilis (strain BS139; see Section 2). Since YukD was N-terminally tagged with C-Myc and 6 histidines, any putative complexes made by YukD in vivo should elute from a Ni^{2+}-NTA column, which could then be detected by immunoblot analysis using an anti-C-Myc antibody. The resultant strain BS139 was viable, even after extensive growth in the absence of xylose, indicating that YukD is not essential. This is consistent with a recent study describing the knockout of yueE and surrounding genes, including yukD [17]. Extracts from BS139 grown in the presence of 1% xylose were purified and analysed by immunoblotting (Fig. 4B). Even by increasing the sensitivity of the assay using 9 copies of the epitope C-Myc fused to YukD, only two very weak bands were visible on an overloaded gel. The nature of these bands is presently unclear, but are most likely not the result of conjugation, since they partially disappear after heating the sample in 50 mM DTT (compare lanes 4 and 5 in Fig. 4B). The band indicated by a single star is possibly a dimer of tagged YukD (Fig. 4B). The most likely explanation for the absence of a covalent interaction is the short C-terminal tail that lacks the double glycine motif, essential for conjugation of all known UBL proteins. Apparently, the ubiquitin-like fold of YukD is required for a different function for which the extended C-terminal tail is not necessary.

Fig. 3. Structure-based sequence alignment of the 15 C-terminal residues of YukD with Ubiquitin, SUMO1, Hub1, ThiS and MoaD proteins, shows that the C-terminal tail of YukD is the shortest of them all.
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References