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Auto-induction and purification of a *Bacillus subtilis* transglutaminase (Tgl) and its preliminary crystallographic characterization

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Abstract

Spores of *Bacillus subtilis* are covered by a multi-protein protective coat which is a key factor in their extreme environmental resilience. A fraction of the coat proteins undergoes covalent cross-linking following their assembly at the spore surface. Several types of covalent cross-links are found in the coat. These include ε-(γ-glutamyl)lysine bonds whose formation is catalyzed by a transglutaminase, Tgl, itself a coat component. Tgl is the smallest known transglutaminase. It bears no sequence resemblance to other proteins in databases, except for its counterparts in other *Bacillus* and related species, suggesting a highly specialized role in coat assembly. It is not known to what degree are the Tgl-like proteins structural and mechanistically related to other transglutaminases. Here, we have fused the His_6 tag to the C-terminal end of Tgl, and shown that the fusion protein is functional *in vivo*. We have overproduced *B. subtilis* Tgl-His_6 by auto-induction with high yield and purified the protein to nearly homogeneity in a single chromatographic step. The purified protein, active as it catalyzed the cross-linking of bovine serum albumin, behaved as a monomer of about 33 kDa in solution. Lastly, Tgl was crystallized and X-ray diffraction data were collected using synchrotron radiation to 2.1 Å resolution. Crystals of Tgl belong to the tetragonal space group $P4_{1,3}$ and contain two molecules per asymmetric unit.

Keywords: Transglutaminase; Spore surface; Spore coat; Macromolecular assembly; Cross-linking; Crystallization

Endospores formed by *Bacillus*, *Clostridia* and related genera of spore-forming bacteria are encased in a protective multi-protein structure called the coat. The coat is the outermost spore structure in many species, including *Bacillus subtilis* while in other species, as in the pathogenic organisms *B. anthracis* and *B. cereus*, the spore is covered by an additional glyco-protein layer, or exosporium. The assembly, structure and function of the spore coat have been extensively studied in the model organism *B. subtilis*. The coat contributes to spore protection against extremes of several physical and chemical insults, which include simulated extraterrestrial conditions, and against predation by unicellular microorganisms [1–4]. Hence, the coat has a role in the maintenance of viable spores in the environment for long periods of time, possibly in excess of millions of years [1–6]. In addition, the coat has also a key role in the ability of the spore to monitor its immediate environment, and to activate germination [1–4,7,8]. Mutations that interfere severely with the coat assembly process have a strong impact on the resistance of spores against harsh chemicals, bactericidal enzymes, predation, and also affect germination [1–4]. Some of the spore properties result from the assembly of enzymes that remain active within the coat lattice (see for example, [11–21]).

A key early event in the process of sporulation is the asymmetric (polar) division of the rod-shaped cell, which generates a smaller prespore, and a larger mother cell, each with a copy of the genome and able to deploy specific (although interconnected) programs of gene expression

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[9,10]. The prespore originates the spore, whereas the mother cell, which has a central role in spore morphogenesis, lysis at the end of the process to release the mature spore into the environment. Formation of the coat structure is mainly a function of the mother cell, which begins soon after its formation, and proceeds until its lysis some 6–8 h later [9,10]. During that period, genes involved in coat assembly are grouped in several temporal and epistatic classes, the precise deployment of which is important for the fidelity of coat morphogenesis [1–4]. However, proper assembly of the coat also depends on several other post-transcriptional mechanisms. A small group of morphogenetic proteins for instance, has a key role in the assembly process by guiding the assembly of the coat structural components [1–4]. In addition, the assembly process also involves post-translational modification of the coat structural components, including proteolysis, and protein–protein cross-linking. About 30% of the total coat protein is resistant to extraction and define a highly cross-linked insoluble fraction [1–4]. Some of the proteins in this fraction are cysteine-rich and undergo extensive cross-linking, but two types of irreversible covalent cross-links have also been detected in the coats: *o*,*o*-dityrosine bonds, presumably the result of an as yet unidentified peroxidase [1–4], and ε-(γ-glutamyl)-lysilne isopeptide bonds catalyzed by a transglutaminase [22]. Recently, a coat-specific enzyme, Tgl, has been identified [23–25] and its function in coat assembly investigated [5,26–29].

Transglutaminase-catalyzed ε-(γ-glutamyl)-lysilne cross-links are stable, protease resistant, and contribute to the rigidity and chemical, physical and mechanical resistance of several tissues or cellular structures [30]. All known TGases use a papain-like active site, in which a cysteine residue is essential for catalysis [30]. Remarkably, the Tgl-like proteins are not related to the mammalian or other microbial transglutaminases (or to other proteins in databases) except for their counterparts in *Bacillus* species and some other highly related spore-formers [27,31]. Despite the lack of sequence similarity to other transglutaminases, a conserved cysteine residue is required for the activity of Tgl *in vitro* and *in vivo*, suggesting the involvement of a papain-like active site in catalysis [24,27].

In vitro, Tgl is able to cross-link proteins such as BSA or α-casein [24]. *In vivo*, there is evidence for the existence of several polypeptides whose extractability is Tgl-dependent [26–29] and a coat-associated protease, YabG, appears to be involved in the formation of Tgl substrates from precursor proteins [14,28]. However, so far only the 20 kDa coat protein GerQ has been identified as a physiological substrate for Tgl [5,26,27].

To gain insight into the mechanism of action of the Tgl-like proteins as well as on their evolutionary relationships, we have initiated the structural characterization of the *B. subtilis* Tgl protein. Here, we report on the high yield production of Tgl in a soluble, active form, and on its crystallization and preliminary X-ray diffraction characterization.

Materials and methods

Over-production of Tgl-His$_6$

Plasmid pLOM4, a pET30a(+) derivative (Novagen) from which a Tgl-His$_6$ fusion protein can be produced from the T7*lac* promoter has been previously described [27]. The Tgl-His$_6$ fusion protein carries the sequence VDKLAAALEHHHHHH added following the last C-terminal residue of native Tgl. Derivatives of *E. coli* strain BL21 (DE3) (Novagen) bearing pLOM4 [27] or its parental plasmid pET30a(+) (strains AH4611 and AH4618, respectively) were constructed and used for the overproduction of Tgl-His$_6$ by either IPTG induction or by an auto-induction regime. For auto-induction, 100 ml cultures were incubated at 37 °C for 18 h with orbital shaking (190 rpm), using the Overnight Express Autoinduction System 1 accordingly to the specifications provided by the manufacturer (Novagen). For IPTG induction, AH4618 and AH4611 were grown in LB medium at 37 °C to an optical density at 600 nm of about 0.6, at what time IPTG was added to a final concentration of 1 mM, and incubation continued for 3 h. After the induction period, cells were collected by centrifugation (at 12,000*g*, for 10 min at 4 °C) and resuspended in one tenth of the culture volume of buffer A (20 mM CAPS, pH 10.0, 0.5 M NaCl and 10 mM imidazole). The cells were then lysed by passage through a French Press cell at 19,000 lb/in^2 as described previously [27]. A sample of 100 μl of the whole cell lysate of each culture was centrifuged at 3500*g* for 45 min at 4 °C to yield a sediment (resuspended in 100 μl of buffer A) and a soluble extract. Samples (2.5 μl) of the whole cell lysates, sediment and supernatant fractions were electrophoretically resolved on 12% polyacrylamide gels containing sodium dodecyl sulfate (SDS–PAGE).

Purification of Tgl-His$_6$

Lysates of induced cultures of AH4618 or AH4611 (cleared by centrifugation at 12,000*g* for 30 min at 4 °C), were applied on a His-Trap, Ni^{2+}-nitrilo-triacetic acid (NTA) affinity column (GE Health Sciences) pre-equilibrated with buffer A. The column was washed three times with 10 column volumes of buffer A containing 10% glycerol, then with 10 column volumes of buffer A, and finally with buffer A containing 40 mM imidazole. Bound proteins were eluted with buffer A containing 150 mM imidazole, and samples analyzed by SDS–PAGE (on 12% gels). The Tgl-containing fractions were pooled, dialyzed overnight against storage (S) buffer (10 mM CAPS, pH 10.0, 0.1 M NaCl) and concentrated to about 8 mg/ml using an Amicon ultra-4 cell (Millipore Corporation), with a molecular weight cut-off of 10,000. Purified Tgl-His$_6$ was stored in aliquots in S buffer at −20 °C.

Analytical size exclusion chromatography

For size exclusion chromatography Tgl-His$_6$ in S buffer was first dialyzed against 0.1 M Tris–HCl, pH 8.0 (sample