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# Structural characterization of the nonameric assembly of an Archaeal α-L-fucosidase by synchrotron small angle X-ray scattering

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**Abstract**

α-L-Fucosidase is a lysosomal enzyme responsible for hydrolyzing the α-1,6-linked fucose joined to the reducing-end *N*-acetylglucosamine of carbohydrate moieties in glycoproteins. The first α-L-fucosidase from Archaea was recently identified in the genome of the hyperthermophile *Sulfolobus solfataricus*; the enzyme is encoded by two open reading frames separated by a −1 frameshift. A preliminary biochemical and biophysical characterization of this extremophile enzyme has been carried out both in solution, through small angle X-ray scattering experiments, and in the crystalline state, showing an unusual oligomeric assembly resulting from the association of nine subunits, endowed with 3-fold molecular symmetry.


α-L-Fucosidases (EC 3.2.1.51) are exo-glycosidases that cleave α-linked L-fucose residues from glycoconjugates involved in a variety of biological processes. In plants, α-fucosidases that remove the α-L-fucosyl residues from fucosylated xyloglucans have been isolated from pea epicotyls [1] and from the leaves of *Arabidopsis* [2]. In mammals, α-fucosidases are lysosomal enzymes hydrolyzing the α-1,6-linked fucose moiety joined to the reducing-end *N*-acetylglucosamine, in glycoprotein carbohydrates. α-L-fucosidase deficiency results in the lysosomal storage disease fucosidosis. Alterations of the α-L-fucosidase levels have also been reported in patients affected by different cancer types. Thus, study of the general properties and of the activity of α-L-fucosidase is deemed relevant for an understanding of the molecular bases of carcinoma development [3].

The first α-L-fucosidase from Archaea was recently identified in the genome of the hyperthermophile *Sulfolobus solfataricus* [4], as an enzyme encoded by two open reading frames (ORF) separated by a −1 frameshift. The region of overlap between the two ORFs revealed the presence of the consensus sequence for programmed −1 frameshifting, a mechanism of gene expression regulated at translational level [5]. The maintenance of a correct reading frame is fundamental to the integrity of any translation process; nevertheless, localized deviations from the standard translational rules, named *recoding*, and including programmed ribosome frameshifting to a different reading frame, ribosome hopping over nucleotides, and reading of stop codons as sense codons, are used to regulate the correct expression of a minority of genes in Eukarya and Bacteria (for a review see [6]). Programmed −1 frameshifts are by far the most common recoding processes [5], and consist in the ribosomal backshift of a single base during translation. The shift is programmed to occur at a

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specific point by sequences in *cis* and, often, it is precisely regulated by metabolic conditions [7].

We inserted two specific mutations, designed on the basis of the programmed −1 frameshifting mechanism, in the region of overlap between the two ORFs encoding for the α-L-fucosidase from *S. solfataricus*, producing a full-length gene, and allowing the expression in *Escherichia coli* of a thermophilic and thermostable α-L-fucosidase [4,8]. In the following we report the biophysical characterization of the enzyme, in solution and in the crystalline state, as a first step towards its structural analysis. We show that *S. solfataricus* α-L-fucosidase adopts an oligomeric assembly resulting from the association of nine subunits, endowed with 3-fold molecular symmetry.

## Materials and methods

*Expression and purification of tagged α-L-fucosidase from S. solfataricus*. The full-length α-L-fucosidase from *S. solfataricus* (Ssα-fuc) was expressed in *E. coli* using the plasmid vector pGEX-frameFuc, as previously reported [4], Ssα-fuc was purified as fusion protein with glutathione *S*-transferase (GST) [4]. After the removal of GST by thrombin treatment, the enzyme sample was incubated at 80 °C for 20 min, followed by centrifugation at 10,000 rpm, for 30 min. Direct N-terminal sequencing of the purified Ssα-fuc yielded the sequence: Ser-Val-Gly-Ser-Met-Ser-Gln-Asn-Ser-Tyr-Lys-Ile-Leu-Lys-, where the underlined amino acids correspond to the Ssα-fuc N-terminal residues. Samples of Ssα-fuc that did not undergo heat treatment showed partial degradation. Accordingly, the protein bands were separated on a 10% SDS–polyacrylamide gel and blotted on a Problot membrane (Applied Biosystems); after staining with Coomassie brilliant blue R-250, the polypeptide bands were directly sequenced by an automatic protein sequencer (Applied Biosystems). The gel filtration experiments were performed on a Superose 6 column (Amersham Bioscience), as reported previously [4].

*Cloning, expression, and purification of the α-L-fucosidase from S. solfataricus*. Since crystallization trials based on the tagged enzyme preparations did not yield suitable crystals, an untagged version of the enzyme was later produced. The full-length gene encoding for Ssα-fuc, without GST, was cloned as follows. The Ssα-fuc gene was amplified from the *S. solfataricus* genome using the following primers: 5′-CTGGAGGCGCGCTAATACGACTCACTATAGGTCAGTTAAA TGTCACAAAATTCT-3′, and 5′-GACTTGGCGCGCCTATCTAT AATCTAGGATAACCCTTAT-3′, containing the T7 RNA polymerase promoter (underlined). The amplified fragment was cloned in the *Bss*HII site of the pBluescript II KS+ vector. Considering that the wild type Ssα-fuc gene is affected by a −1 frameshift, the full-length gene was obtained by subcloning in the corresponding sites the *Nco*I–*Kpn*I fragment, containing the insertional mutation described elsewhere [4]. The Ssα-fuc gene in the recombinant vector pBlueFrameFuc was completely re-sequenced.

The untagged Ssα-fuc was expressed in *E. coli* BL21(DE3) strain as described for the pGEXframeFuc plasmid [4]; the cell pellet was resuspended in 20 mM sodium phosphate, pH 7.3, 150 mM NaCl (PBS buffer), lysed by French Press (American Instruments, USA), and centrifuged at 30,000 rpm for 20 min. The cell extract was treated at 50, 65, and 75 °C for 20 min; after each heating step the sample was centrifuged at 30,000 rpm for 20 min, to remove the aggregate of *E. coli* proteins. The sample was equilibrated with L-fucose agarose matrix (Sigma), for 16 h at 4 °C, in batch; the matrix was washed with 30 volumes of PBS buffer and eluted with a solution of 50 mM fucose in PBS, at room temperature. The fractions containing α-fucosidase activity were collected and dialyzed for 16 h at 4 °C against PBS buffer, to remove fucose. The enzyme recovered was 95% pure, as evaluated by SDS–PAGE. This procedure yields 3 mg of pure protein from 6 g of wet cell pellet. The specific activity of the untagged Ssα-fuc, measured as described elsewhere [4], is 15 U/mg. Sequencing of the blotted (see above) untagged Ssα-fuc yielded the sequence Ser-Gln-Asn-Ser-Tyr-Lys-Ile, corresponding to the N-terminal segment of the protein, lacking the first Met.

*Crystallization and X-ray diffraction experiments*. Crystals of the recombinant untagged Ssα-fuc were grown using vapor diffusion techniques by equilibration of a 500 μl reservoir solution (1.4 M ammonium sulfate, 8%, glycerol, and 0.1 M Tris, pH 8.5) against a 2 μl droplet containing 1 μl of reservoir solution and 1 μl of 14 mg/ml protein solution, at 21 °C, for about 2 weeks. The cryo-protectant solution used for X-ray diffraction experiments was composed of 1.7 M ammonium sulfate, 20%, glycerol, and 0.1 M Tris, pH 8.5.

X-ray diffraction data were collected at 100 K, at the DESY-EMBL Outstation (Hamburg-D), beamline BW7B, $\lambda = 0.842$ Å. The diffraction pattern presented an anisotropic spread, with reflections along the equatorial zone of the detector reaching a maximum resolution of 3.5 Å; the maximum resolution along the meridian direction was 7.5 Å. The diffraction dataset was integrated using MOSFLM and scaled with the programs SCALA and TRUNCATE, from the CCP4 program suite [9]. A full list of data collection statistics is reported in Table 1.

*Solution scattering experiments and data processing*. The small angle X-ray scattering (SAXS) data were collected on the X33 camera [10,11] at the EMBL, Hamburg Outstation, on the storage ring DORIS III of the Deutsches Elektronen Synchrotron (DESY), using multiwire proportional chambers with delay line readout [12]. The scattering patterns were recorded at a sample-detector distance of 2.4 m, covering the momentum transfer range $0.15 < s < 3.5\,\mathrm{nm}^{-1}$ ($s = 4\pi\sin(\theta)/\lambda$, where $2\theta$ is the scattering angle and $\lambda = 0.15$ nm is the X-ray wavelength). The untagged Ssα-fuc solutions (in 0.02 M phosphate buffer, NaCl 0.15 M, and $NaN_3$ 0.02%, pH 6.5) were investigated at protein concentrations of 2, 5, and 10 mg/ml. The scattering data were collected in 15 successive 1-min frames, which were analyzed for the absence of radiation damage, averaged after normalization to the intensity of the incident beam, and corrected for the detector response; the scattering of the buffer was subtracted using the program PRIMUS [13]. The difference curves were scaled for the solute concentration and extrapolated to infinite dilution, following standard procedures [14]. The maximum particle dimension $D_{max}$ was estimated using the orthogonal expansion program ORTOGNOM [15]. The forward scattering $I(0)$ and the radius of gyration $R_g$ were evaluated using the Guinier approximation [16], assuming that at very small angles ($s < 1.3/R_g$) the intensity is represented as $I(s) = I(0)\exp(-(sR_g)^2/3)$. These parameters were also computed from the entire scattering

Table 1
Ssα-fuc X-ray diffraction data collection statistics

| Beamline | DESY/EMBL BW7B |
|---|---|
| Wavelength (Å) | 0.842 |
| Resolution range (Å) | 75.0–3.8 |
| (last shell) | 3.82–3.80 |
| Reflection observed | 147,275 |
| Unique reflections | 58,910 |
| $R_{merge}$ | 0.13 |
| (last shell) | 0.39 |
| Completeness (%) | 92.9 |
| (last shell) | 78.4 |
| Redundancy | 2.5 |
| $\langle I/\sigma(I)\rangle$ | 3.1 |

pattern using the indirect transform package GNOM [17], which provides also the distance distribution function $p(r)$ of the particle. The molecular mass (MM) of the solute was evaluated by comparison of the forward scattering with that from reference solutions of bovine serum albumin (MM = 66 kDa).

*Ab initio shape determination*. Low resolution models of the protein were generated ab initio using the program DAMMIN [18]. The program represents the particle as a collection of $M \gg 1$ densely packed beads inside a sphere with the diameter $D_{max}$. Each bead belongs either to the particle or to the solvent, and the shape is described by a binary string of length $M$. Starting from a random string, simulated annealing is employed to search for a compact model that fits the experimental data $I_{exp}(s)$ to minimize discrepancy:

$$\chi^2 = \frac{1}{N-1}\sum_j \left[\frac{I_{exp}(s_j) - cI_{calc}(s_j)}{\sigma(s_j)}\right]^2, \quad (1)$$

where $N$ is the number of experimental points, $c$ is a scaling factor, and $I_{calc}(s)$ and $\sigma(s_j)$ are the calculated intensity and the experimental error at the momentum transfer $s_j$, respectively. Prior to shape analysis by DAMMIN, a constant is subtracted from each data point to force the $s^{-4}$ decay of the intensity at higher angles, following Porod's law [19] for homogeneous particles. The resulting "shape scattering" curve (i.e., scattering due to the excluded volume of the particle with unit density), in the range up to $s = 2.0\,nm^{-1}$, was used for ab initio shape restoration. The outer part of the scattering pattern ($s > 2.0\,nm^{-1}$), dominated by the scattering from the internal structure, was discarded in the shape analysis. The excluded volume of the hydrated particle (Porod volume) was computed from the shape scattering curve using the equation [19]

$$V = 2\pi^2 I(0) / \int_0^\infty s^2 I(s)\,ds. \quad (2)$$

The ab initio shape reconstructions were performed both in the absence of symmetry restraints and assuming 3-fold symmetry.

*Molecular modeling*. Rigid body modeling of the quaternary structure of Ssα-fuc in solution was performed in the angular range $s < 2.5\,nm^{-1}$; no attempt was made to model the scattering at higher angles, since this is defined by tertiary rather than quaternary structure. The high resolution crystallographic coordinates of *Thermotoga maritima* α-L-fucosidase, displaying 33% sequence identity to Ssα-fuc, were kindly provided by Dr. G. Sulzenbacher (Marseille, France) [20]. Both the protein monomer and the trimer, assembled around a 3-fold crystallographic axis in *T. maritima* α-L-fucosidase crystals, were used as rigid bodies for modeling. A simulated annealing protocol was employed to construct an interconnected ensemble of nine crystallographic monomers without steric clashes, minimizing the discrepancy between the scattering curve calculated from the full nonameric assembly and the experimental scattering data. The procedure was restrained by the 3-fold symmetry, allowing for three monomers in the asymmetric part of the nonamer. The theoretical scattering pattern $I(s)$ was expressed in terms of spherical harmonics:

$$I(s) = 2\pi^2 N^2 \sum_{l=0}^{\infty}\sum_{m=-l}^{l}\left|\sum_n A_{lm}^n(s)\right|^2. \quad (3)$$

Here, $N$ is the number of symmetry mates, the complex functions $A_{lm}^n(s)$ are the partial scattering amplitudes of the subunits in their given positions and orientations, which depend on the scattering amplitudes in the reference positions calculated using the program CRYSOL [21], and on three rotational and three translational parameters. The summation runs only over the symmetry independent subunits, and over $m$ and $l$ values allowed by the selection rules for the spherical harmonics (for 3-fold axis, $m = 0, 3, \ldots, 3k$, where $k$ is an integer). The algorithm is a particular case of the program SASREF, which allows quaternary structure modeling of complexes and deletion mutants against the SAXS data (Petoukhov & Svergun, in preparation).

## Results and discussion

### *Ssα-fuc oligomeric structure*

The molecular mass of the tagged Ssα-fuc monomer, measured by SDS–PAGE, is 57 kDa, in good agreement with the deduced MM of 57,388 Da [4]. In a gel filtration experiment under native conditions the enzyme eluted as a single peak, showing thermophilic α-fucosidase activity, nestled between tyroglobulin (660 kDa) and ferritin (490 kDa) peaks, for a MM of 508 ± 22 kDa, indicating that the native oligomeric Ssα-fuc may result from the quaternary association of nine or 10 subunits [4]. Samples of Ssα-fuc, purified using the GST tag, but that did not undergo heat treatment, showed partial degradation, and yielded two extra bands of about 45 and 14 kDa, respectively, in SDS–PAGE (Fig. 1). This preparation revealed a specific activity of 4.2 U/mg; as a comparison, the tagged Ssα-fuc, yielding a single SDS–PAGE band, displayed a specific activity of 32 U/mg. To identify the fragmented polypeptides we analyzed their N-terminal after separation by SDS–PAGE, by direct sequencing of the blotted samples. The 45 kDa fragment displayed the sequence: Ser-Val-Gly-Ser-Met-Ser-Gln, that is identical to the N-terminal of the 57 kDa tagged Ssα-fuc. Instead, the 14 kDa polypeptide showed the N-terminal sequence Ile-Thr-Leu-Gly-Tyr, that corresponds to the amino acids Ile404–Tyr408 of the full enzyme (Ssα-fuc numbering, with Met in the sequence Ser-Val-Gly-Ser-Met-Ser-Gln counted as the first amino

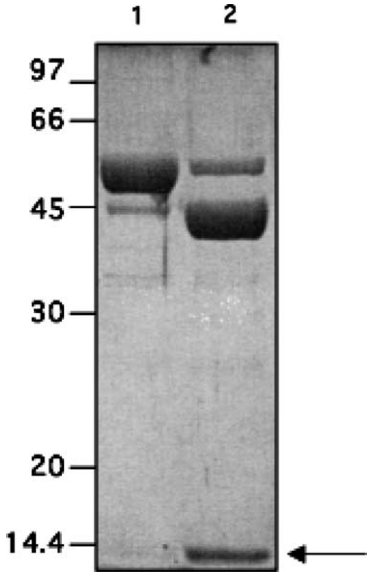


Fig. 1. Electrophoretic analysis of tagged Ssα-fuc. Lane 1: tagged Ssα-fuc; lane 2 partially degraded Ssα-fuc, the arrow indicates the 14 kDa polypeptide corresponding to the cleaved C-terminal domain. The molecular weight markers used are: phosphorylase b (97,000), bovine serum albumin (66,000), ovalbumin (45,000), carbonic anhydrase (30,000), trypsin inhibitor (20,100), and α-lactalbumin (14,400).

acid). These data show that the observed polypeptides were produced by limited proteolysis degradation of Ssα-fuc, presumably by trace amounts of contaminating *E. coli* proteases, which are inactivated by heat treatment in the normal purification procedure. Interestingly, inspection of amino acid sequences and of the *T. maritima* α-L-fucosidase 3D-structure shows that residues Ile404–Tyr408 of Ssα-fuc fall at the beginning of the C-terminal domain of the *T. maritima* enzyme, suggesting the presence of an interdomain protease sensitive site in Ssα-fuc.

A partially degraded Ssα-fuc sample was analyzed under native conditions in a gel filtration experiment, to test the apparent native molecular weight of the proteolyzed enzyme (Fig. 2). A single peak containing the thermophilic α-fucosidase activity eluted at a molecular weight of $473 \pm 26$ kDa (Fig. 2B), indicating that, despite proteolysis, the two domains are still held

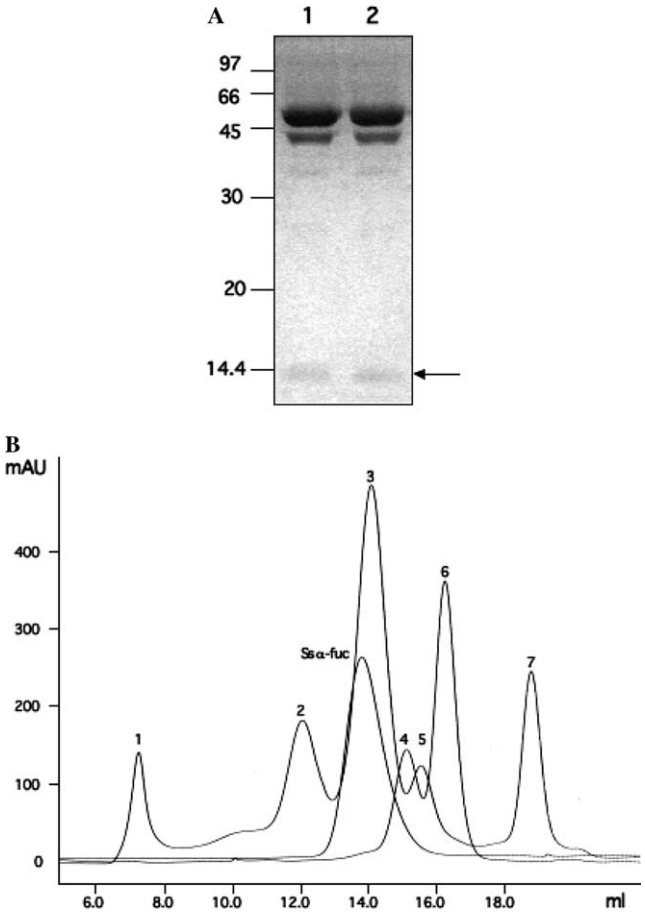


Fig. 2. Oligomeric structure of partially degraded Ssα-fuc. (A) Lane 1, partially degraded Ssα-fuc; lane 2, pooled single peak after gel filtration in native conditions (see Fig. 1, lane 2). (B) Elution profile at 280 nm from a Superose 6 column: peak 1, blue dextran (elution volume 7.4 ml); peak 2, tyroglobulin (669 kDa, 12.2 ml); peak 3, ferritin (490 kDa, 14.2 ml); peak 4, β-glycosidase from *S. solfataricus* [27] (240 kDa, 15.2 ml); peak 5, aldolase (158 kDa, 15.6 ml); peak 6, bovine serum albumin (67 kDa, 16.3 ml); and peak 7, ribonuclease A (13.7 kDa, 18.8 ml). Ssα-fuc eluted at 13.9 ml.

together by non-covalent interactions. The tagged Ssα-fuc proved scarcely useful for crystallization experiments. To avoid these problems, the untagged Ssα-fuc, prepared as described above, was used throughout the experimental approaches discussed below.

### *Crystallographic data*

Crystals of Ssα-fuc belong to the orthorhombic $P2_12_12_1$ space group, with unit cell constants $a = 173.0$ Å, $b = 229.0$ Å, $c = 245.8$ Å, $\alpha = \beta = \gamma = 90°$. Assuming a quaternary structure composed of nine subunits, the corresponding $V_M$ volume is 2.4 Å$^3$/Da, indicating 48% solvent content and 18 molecules (57.4 kDa each) in the asymmetric unit. The presence of 3-fold, but also of a 2-fold non-crystallographic symmetry axes, is recognizable in the protein self-rotation function [22], calculated at 4.5 Å resolution. Two strong peaks (correlation coefficient of 57.3) were promptly located in the $\chi = 119.7°$ and $\chi = 180°$ self-rotation function sections, respectively. While the 3-fold axis identified may be related to the nonameric Ssα-fuc internal symmetry, the 2-fold non-crystallographic symmetry may reflect pseudo-symmetry in the assembly of two oligomers in the crystallographic asymmetric unit. In order to solve the structure of the Ssα-fuc nonameric assembly, we performed several molecular replacement searches using different programs: AmoRe [22], EPMR [23], MOLREP [24], and Beast [25] and, as starting model, the structure of *T. maritima* α-L-fucosidase (PDB code 1HL8; [20]). Likely related to the unfavorable mass ratio between the search model and the asymmetric unit content, but also to evident sequence differences, particularly in the C-terminal regions of the search/target proteins, molecular replacement did not yield an evident structure solution. Different phasing methods are currently being tested [26].

### *Overall quaternary parameters of Ssα-fuc*

The processed scattering pattern obtained from the Ssα-fuc solution is presented in Fig. 3; the overall structural parameters are summarized in Table 2. The comparison of the estimated solute MM with the value calculated from the sequence of monomeric Ssα-fuc (57.4 kDa) indicates that the soluted protein aggregates into an assembly consisting of nine subunits. The accuracy of MM determination by solution scattering is limited, in particular, by the uncertainty in the measured protein concentrations required for the data normalization. Thus, as additional check, the excluded (Porod) particle volume was also analyzed, taking advantage of the fact that the Porod volume is calculated without model assumptions, and does not depend on data normalization [19]. For globular proteins, Porod (i.e., hydrated) volumes in nm$^3$ are about twice the MM in kDa.

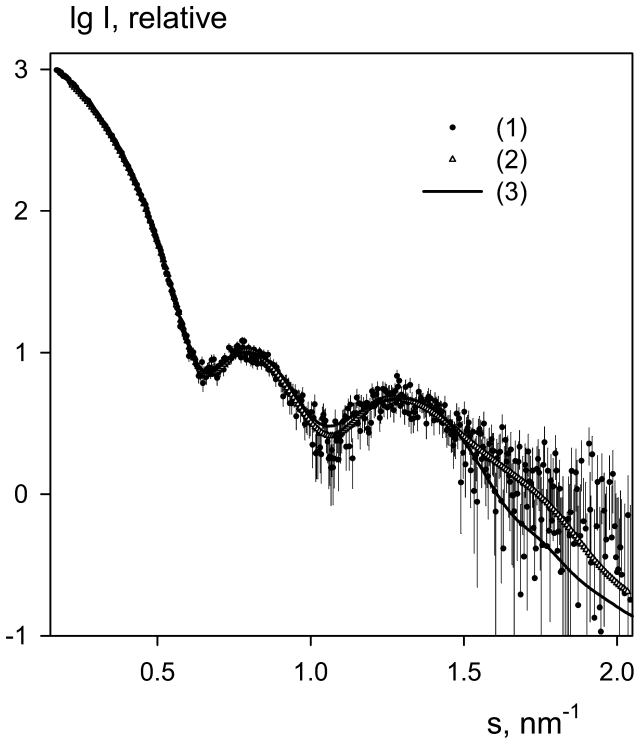


Fig. 3. Experimental and calculated scattering patterns of Ssα-fuc. (1) The experimental SAXS data; (2) scattering calculated from the typical ab initio model; and (3) scattering calculated from the best rigid body model.

As seen from Table 2, the Porod volume of the solute is in a good agreement with the estimated MM. The SAXS data therefore indicate that Ssα-fuc is nonameric in solution, at the protein concentrations used in the experiments. This finding is in agreement with the results of analytical gel filtration and dynamic light scattering experiments (our unpublished results). Furthermore, the nonameric assembly is compatible with crystal packing considerations and with the presence of 3-fold symmetry axes detected in the crystallographic self-rotation function.

*Molecular shape determination*

The low resolution shape of α-L-fucosidase was initially produced using the program DAMMIN, without symmetry restrictions. Several reconstructions were performed and a typical ab initio model is presented in Fig. 4, left panel. All models reconstructed in the absence of symmetry restraints provide good fits to the experimental data (Fig. 3), with $\chi s = 1.19$, and are similar in shape, displaying an oblate overall shape with *quasi* 3-fold symmetry along the short axis. To further improve the resolution of the ab initio shape determination, 3-fold symmetry and model oblateness restraints were introduced. A typical symmetric shape reconstruction (Fig. 4, central panel) is similar in the overall shape to the model obtained in the absence of symmetry restraints, fitting the experimental data with the same $\chi s$ value. Ab initio shape determination thus suggests that Ssα-fuc displays 3-fold symmetry in solution.

*Rigid body modeling*

The high resolution crystal structure of *T. maritima* α-L-fucosidase was employed for rigid body modeling of the Ssα-fuc oligomer against its solution scattering data using the program SASREF (unpublished). Modeling of the nonamer was performed in terms of positions and orientations of three independent monomers in point group 3; the remaining six monomers were then generated by the 3-fold symmetry. The best interconnected nonameric ensemble, free of steric clashes (Fig. 4, right panel), yields a good fit to the experimental data, with $\chi = 1.22$ (Fig. 3), being in good agreement with the ab initio Ssα-fuc molecular shapes described above. It should be noted that the monomer of *T. maritima* α-L-fucosidase has MM = 49.6 kDa, i.e., 13% smaller than Ssα-fuc, and the rigid body refinement based on *T. maritima* model can thus provide only an approximation to the subunit arrangement within the nonameric Ssα-fuc oligomer.

It was further attempted to perform rigid body modeling based on the *T. maritima* α-L-fucosidase crystallographic trimeric assembly. This attempt was, however, unsuccessful since the experimental data could be only poorly fitted by nonamer models built through the assembly of three such trimers, either with aligned 3-fold axes (the best model yielded $\chi = 4.0$, not shown) or in arbitrary orientations (best model $\chi = 1.77$, not shown).

As a whole, the results here reported indicate that α-L-fucosidase from the Archaeon *S. solfataricus*, while

Table 2
Summary of the structural parameters of Ssα-fuc computed from the scattering data

| $R_g$ (nm) | $D_{max}$ (nm) | MM (kDa) | $MM_{mon}$ (kDa) | $V_p$ (nm$^3$) | $\chi s$ | $\chi$ |
|---|---|---|---|---|---|---|
| 5.7 ± 0.3 | 150 | 495 ± 20 | 57.4 | 1000 ± 30 | 1.19 | 1.22 |

$R_g$, $D_{max}$, MM, and $V_p$ are the radius of gyration, maximum size, molecular mass, and excluded Porod volume, respectively, calculated from the scattering data. $MM_{mon}$ is the MM of the Ssα-fuc monomer calculated from the primary structure. Discrepancy between the experimental data and the scattering curves calculated from the model is denoted as $\chi s$ for ab initio shape determination, and $\chi$ for the model obtained by rigid body refinement, respectively.

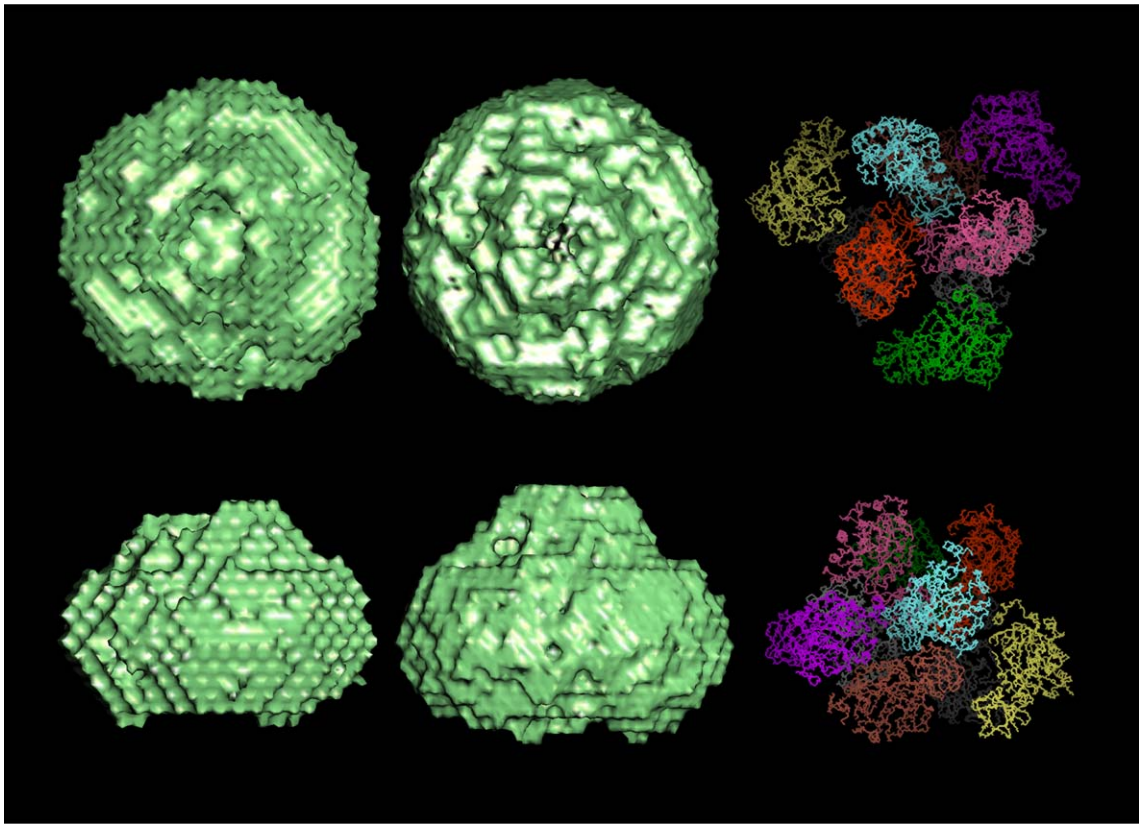

Fig. 4. Solution scattering models of Ssα-fuc. Ab initio models, as produced by DAMMIN, without symmetry restraints (left panel), and accounting for 3-fold symmetry (central panel); the bead model produced was rendered as a continuous surface using QUANTA. The best quaternary structure obtained by rigid body modeling is displayed as $C_\alpha$-trace of three *T. maritima* α-L-fucosidase model subunits (right panel). In all panels, the lower image is rotated by 90° around the horizontal axis, relative to the top figure.

maintaining a basic trimeric assembly loosely related to that of the homologous enzyme from the hyperthermophile *T. maritima*, adopts a more complex quaternary structure, where three trimers assemble into a nonameric homo-oligomer. It appears notable that the subunit packing geometry of such nonameric assembly is hardly matched by other protein quaternary structures so far reported.

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