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SHORT COMMUNICATION

Towards Atomic Resolution With Crystals Grown in Gel: The Case of Thaumatin Seen at Room Temperature

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ABSTRACT **One reason for introducing a gel in the crystallization medium of proteins is its ability to reduce convection in solution. This can lead to better nucleation and growth conditions, and to crystals having enhanced diffraction properties. We report here the X-ray characterization at room temperature of high-quality crystals of the intensely sweet thaumatin prepared in a sodium tartrate solution gelified with 0.15% (m/v) agarose. Using a synchrotron radiation, these crystals diffracted to a previously unachieved resolution. A diffraction dataset was collected from four crystals at a resolution of 1.2 Å with a R_{sym} of 3.6% and a completeness of 99%. Refinement was carried out to a final crystallographic *R*-factor of 12.0%. The quality of the electron density map allowed for the observation of fine structural details in the protein and its solvation shell. Crystallization in gel might be used more generally to improve the quality of macromolecular crystals. Advantages provided by the gelified medium in the frame of structural studies are emphasized. Proteins 2002;48:146–150.**

Key words: thaumatin; crystallogenesis; crystallization; gel; microgravity; X-ray structure; anisotropic refinement

INTRODUCTION

Hydrogels are known to be convenient, efficient, and inexpensive media for growing high-quality crystals of small molecules.[1] Although they were introduced for protein crystallization a few decades ago (for a review, see Robert et al.[2]), they are still seldom used despite their many advantages. Amongst their properties, they reduce convection in the mother liquor and favor diffusive transport of the molecules towards crystal nuclei and growing crystals, mimicking in some ways conditions occurring in a microgravity environment. The framework of the gels also traps crystal nuclei, prevents crystal sedimentation, and promotes their three-dimensional growth. In addition, gels may be used to control the number of nuclei.[2]

In the course of crystallogenesis investigations seeking to define growth conditions that lead to crystals of improved quality, we have designed a microgravity experiment in which an agarose gel was added to the protein solution. The test case molecule was thaumatin, an intensely sweet protein (for a review, see, e.g., Kinghorn et al.[3]) with monomeric structure encompassing 207 residues (Mw 22.2 kDa).[4] The gel was thought to play a role during and after crystallogenesis: it was destined to protect the crystals against vibrations and shocks upon landing and during subsequent transport prior to their analysis. Control crystallization experiments under normal gravity were performed in parallel in the laboratory under otherwise identical conditions. To assess the intrinsic quality of the gel-grown crystals, their diffraction properties were analyzed at a temperature close to that at which they were grown (and not under cryogenic conditions as is mostly done when using a radiation of high intensity). Consequently, possible effects due to cryo-cooling were eliminated.

Here, it is shown that gel-grown thaumatin crystals yield high-resolution diffraction data at room temperature, allowing full anisotropic refinement to be performed. The resulting high-quality electron density map facilitated model building at the level of protein backbone and of the amino acid side-chains and revealed a higher number of

Abbreviations: ADPs, anisotropic displacement parameters; B_{eq} and U_{eq}, isotropic *B* factor and isotropic mean-square displacement equivalent to a given set of ADPs ($B_{eq} = 8\pi^2 U_{eq}$).

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TABLE I. Data Collection Statistics

Space group	$P4_12_12$	
Unit cell dimensions (Å)	a = 58.53; c = 151.35	
Number of crystals	4	
Number of observations	594,523	
Number of unique reflections	82,240	
Resolution range (Å)	1.20–20	1.20–1.23
Completeness (%)	99.0 (84.3)[1]	97.8 (47.3)[a]
R_{merge} [b] (%)	3.6	52.2

[a]Values in parentheses are for reflections with $I > 2\sigma(I)$.
[b]$R_{merge} = \sum_{hkl}\sum_i | \langle I \rangle - I_i | / \sum_{hkl}\sum_i (I_i)$.

water molecules. Taken together, this study on thaumatin illustrates the positive contribution of gels in a biocrystallographic study. In a more general perspective, it highlights advantages of gels not only for crystallization but also for other steps that are crucial for structure determination.

MATERIALS AND METHODS

Crystallization and Data Collection

Thaumatin I, the major isoform of thaumatin, was purchased from Sigma (cat. no. T7638, lot 108F0299) and used without further purification. It was crystallized at 35 mg/ml in the presence of a final agarose concentration of 0.15% (m/v). Crystallization under microgravity at 293K was operated in dialysis or free interface diffusion reactors having 188-μl protein chambers that were installed in the Advanced Protein Crystallization Facility (APCF)[5] aboard the U.S. Space Shuttle during a 9-day mission (STS-95). Dialysis reactors for earth controls were installed in an in-house built facility.[6,7] The crystallizing agent was 0.52 M Na tartrate in 100 mM N-[acetamido]-2-iminoacetic acid adjusted to pH 6.5 with NaOH.[8]

Although crystals grown in gel either on earth or in a microgravity environment have similar diffraction properties (in terms of resolution and signal-to-noise ratio),[8] space-grown crystals were chosen for the present study because they yielded the most complete datasets. Four selected crystals measuring ~1 mm in length were mounted in glass capillaries. Data were collected at room temperature on the synchrotron beamline BW7B (λ = 0.8337 Å) at DESY-EMBL, Hamburg, using a 345-mm MAR research IP scanner. High (up to 1.2 Å) resolution and medium (2–20 Å) resolution datasets were recorded for each crystal to cover the whole range of intensities. Data were reduced using the *HKL* package.[9] Processing statistics are summarized in Table I.

Structure Refinement

The tetragonal structure of thaumatin (Protein Data Bank entry 1THW; Ko et al.[10]) deprived of its solvent molecules served as the starting model for molecular replacement. The refinement process utilized the programs CNS and SHELX97,[11,12] with stereochemical restraints based on the study by Engh and Huber,[13] and a bulk solvent correction. All reflections were included in the refinement (no σ cutoff was applied) and 5% of the data were randomly selected and omitted during refinement for cross-validation analysis by means of the free *R*-factor.[14]

Fifty steps of rigid-body refinement were first performed using CNS in the resolution interval 2–20 Å. Several rounds of conjugate-gradient least-squares (CGLS) minimization were done and resolution was increased stepwise from 2.0 to 1.2 Å. At this stage, individual isotropic *B*-factors were refined and water molecules developing sensible hydrogen bonds were added in the difference Fourier map (peaks > 5σ), leading to a model with *R*- and R_{free}-values of 21.1 and 22.0%, respectively.

Refinement was continued with SHELX97 based on intensities (i.e., squared structure-factor amplitudes). Each step consisted of 20 to 30 cycles of CGLS minimization. Default values of the program for distance, planarity, and chiral restraints were used throughout. The $3F_o$-$2F_c$ and F_o-F_c electron-density maps were calculated after each step and the model encompassing the protein and solvent was checked and rebuilt using the program *O*.[15] The *R*- and R_{free}-factors calculated after the first run treating *B*-factors isotropically were 20.6 and 22.8%, respectively. Refinement of anisotropic atomic displacement parameters (ADPs) of protein atoms dramatically decreased *R*- and R_{free}-values to 15.9 and 17.9%, respectively. The ADPs of solvent molecules were restrained to be approximately isotropic and an occupancy factor of 0.5 was applied to solvent atoms having high ADP-values ($U_{eq} > 0.7$ Å^2). At this stage, clear electron density was observed close to the side chains of 18 residues, which were subsequently modeled in two conformations. The presence of a tartrate ion in the crystal lattice was also clear at this step [see Fig. 1(B)]. In the latest steps of refinement, H-atoms were added to the model according to geometrical criteria. This yielded a gain of ~1% both on *R*- and R_{free}-factors without increasing the number of parameters. When the convergence of the CGLS minimization was achieved, all the data were included in the refinement leading to a final *R*-factor of 11.2% for 70071 reflections, with $F_{obs} > 4\sigma(F_{obs})$ and 12.0% for all data (82,240 reflections) in the resolution range 1.2–20 Å.

The final model consists of the 207 thaumatin residues with a total of 1,623 protein atom sites, one tartrate ion, and 197 water molecules. The final refinement statistics are indicated in Table II. The quality of the structure was assessed using several programs such as CNS, OOPS,[15] PROCHECK,[16] and SHELXPRO.[12] The protein backbone shows a good stereochemistry and all ϕ/ψ angles belong to the core region of the Ramachandran plot. The coordinates and observed intensities have been deposited with the Brookhaven Protein Data Bank (accession code 1KWN).

RESULTS AND DISCUSSION

Characterization of Thaumatin Crystals Grown in Gel

In a 0.15% (m/v) agarose gel, thaumatin crystallizes as individual and well-shaped tetragonal dipyramids in the presence of sodium tartrate. Inside the gel matrix, the crystals did not suffer from shocks during transport and their almost perfect morphology was unaltered [Fig. 1(A)].

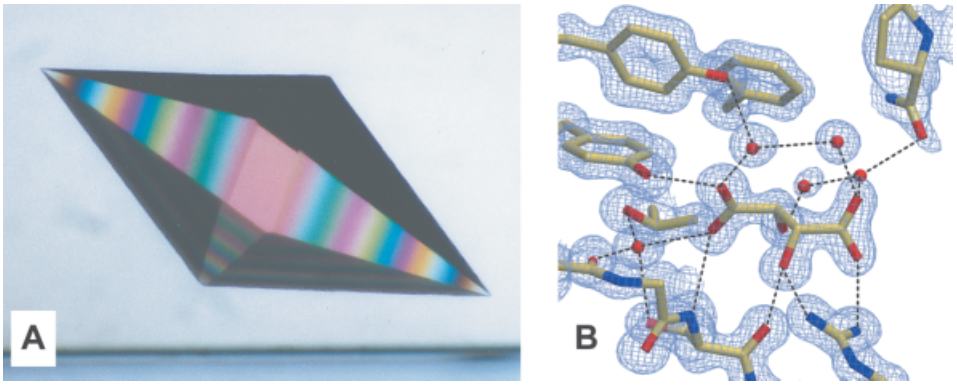

Fig. 1. High-quality crystals yield high-quality electron density maps. **A:** An almost perfect bipyramidal crystal of thaumatin (1 mm in length) with bright birefringence colors grown in 0.15% (m/v) agarose gel. **B:** Detail of the electron density map showing an ion of tartrate, the crystallizing agent, that connects three symmetry-related thaumatin molecules in the crystal lattice. Dashed lines highlight the main interactions between the tartrate molecule and protein residues. They consist of either salt bridges or hydrogen bonds (mostly mediated by a water molecule) with distances varying between 2.47 and 3.05 Å; they involve Arg29 and Ser36 of a first monomer (**bottom**), Tyr157, Tyr169 and Thr154 of a second (**left**), and Pro141 of a third molecule (**top right**). The $3F_o$-$2F_c$ Fourier map is contoured at a level of 1.8σ. The figure was prepared using the software SETOR.[28]

TABLE II. Refinement Statistics

Resolution range	1.2–20Å
Number of reflections in work and test sets	78,110/4,130
R_{all} (all reflections: work+test sets) (%)	12.03 (11.18)[a]
R_{work} (work set) (%)	12.71 (11.86)[a]
R_{free} (test set) (%)	14.52 (13.41)[a]
Observation-to-parameter ratio	5.1
Number of protein, solvent and tartrate atoms	1,623/197/10
Average B_{eq} (Å^2)	
Overall/protein/solvent/tartrate	23.5/22.0/36.7/19.4
R.m.s. deviation from ideal geometry[b]	
Bond length (Å) and bond angles (°)	0.016/2.6
Ramachandran plot statistics[b]	
Residues in most favored regions (%)	90.5
Residues in additional allowed regions (%)	9.5

[a]Values in parentheses are for reflections with $I > 2\sigma(I)$.
[b]Values determined using *CNS* and PROCHECK, respectively.

The crystals (~1 mm from apex-to-apex) used in this study were prepared by either free-interface diffusion (1 crystal) or dialysis (3 crystals) and removed from gel prior to mounting in glass capillaries. Diffraction data, collected at room temperature (293K) using synchrotron radiation, yielded after merging a 99% complete set of reflections at 1.2 Å resolution (Table I).

The structure of the protein was solved by molecular replacement and its model was refined by a series of well-defined steps (see, for example, the strategy used by Walsh et al.[17]). At 1.2 Å resolution, the model required only very few manual adjustments during the latest stages after imposing ADPs. Overall, the high quality electron density map allowed for the unambiguous identification of all atoms of the polypeptide backbone and the majority of the side chain atoms. The weaker density of a few residues located at the surface of the protein indicated some dynamic disorder. This was the case for the C-terminal Ala207 and for 15 other amino acid side chains. Alternate conformations were modeled for 18 side chains based on the residual density both in $3F_o$-$2F_c$ and difference Fourier maps. No difference was found with the thaumatin fold previously reported at ~1.7 Å resolution:[10,18] the r.m.s.d. on Cα positions (residues 1 to 205) after a least-square superposition of the present structure and those solved in the tetragonal (PDB id: 1THW) or in the orthorhombic packings (PDB id: 1THV) are 0.10 and 0.30 Å, respectively. Nevertheless, the quality of the electron density map allowed identification of a total of 197 ordered water molecules in the solvation shell, which is about double that seen in the 1.75-Å tetragonal structure (106 solvent molecules in 1THW). The present 1.2-Å structure model also shows in greater details the tartrate ion sitting at the interface of three symmetry-related monomers (it was previously identified by Ko et al.[10]). Tartrate is more than just a crystallizing agent for thaumatin; it has also to be considered as an additive that contributes to crystal cohesion [Fig. 1(B)]. Altogether, the example of thaumatin well illustrates how protein crystallography performed at atomic resolution using high-quality crystals can reveal details that are as accurate as those visible in small molecule structures.

There is evidence that protein crystals may incorporate the gel matrix in which they grow[19] but there is no convincing argument that the gel is ordered and observable by X-ray crystallography. Here, interactions between thaumatin and the agarose gel could not be clearly detected, even though unassigned weak density peaks were observed in solvent channels that could be due to the presence of the polysaccharide framework.

Crystallization in Gel: Facts and Perpectives

The present study demonstrates that a gel, like agarose, can be used to grow high-quality protein crystals suitable to solve structures at atomic resolution. For thaumatin, this high level of accuracy was obtained from diffraction

data collected at ambient temperature. Whether this feature can be generalized to other proteins is presently not known and awaits experimental verification. Nevertheless, the possibility to compute high-resolution structures of proteins from data obtained under non-cryogenic conditions opens interesting biological perspectives. Amongst them, information on the dynamics of surface residues, solvent shell and bound ions in conditions close to the physiological state may be obtained. In the present case, specific receptors were recently identified,[20] and the accurate knowledge at atomic resolution of the thaumatin fold in an ambient temperature environment may also provide new structural clues concerning its sweet-taste determinants.[21]

Surprisingly, usage of gels in crystallization is not widespread in biocrystallography. A reason might be that the potential advantages of gels have not been accompanied by real applications in structural biology. Further, the expected enhancements on crystal quality have until recently not been supported by strong experimental evidence in the macromolecular field. At present, it can be stated that gelified media indeed dramatically reduce convection in solution and the consequence is a better control of macromolecule transport during crystal growth.[22] Sedimentation is also hindered in gelified media, thus favoring three-dimensional growth (see, e.g., Lorber and Giegé,[7] García-Ruiz et al.,[22]; Fig. 2). Further, the combination of the two former properties with a reduced incorporation of impurities[23] is probably the reason why twinning has been suppressed when crystals of archaeal alcohol dehydrogenase were grown in agarose gel.[24] The decrease of convection in gelified media,[1] has been shown recently to yield macromolecular crystals of higher internal order. Indeed, X-ray topographic analyses have revealed more homogeneous lattices in gel-grown crystals.[25] Gel-grown crystals of thaumatin have a lower mosaicity and yield datasets with 20% higher signal-to-noise ratio than controls obtained by conventional convective solution growth.[8]

All the positive effects the gels have on crystals are expected to be reflected in the quality of the final crystallographic structures. A first answer came from studies on lysozyme that compared the structures derived from crystals obtained under four different growth regimes (microgravity, dialysis, liquid-gel diffusion, and microbatch). The structures derived from crystals grown under diffusive regime (either in gels or in microgravity) showed an improvement that was correlated to a higher number of well-ordered water molecules.[26]

From another viewpoint, gels also provide an efficient protection of samples during handling (mounting, soaking, seeding. . .) and transport without affecting their crystallographic analysis. In addition, their presence can be crucial for preserving the diffraction properties during cryocooling, as was shown for instance in the case of aspartyl-tRNA synthetase crystals.[27] In conclusion, from the above considerations and their successful application to thaumatin, we believe that the usage of gels should be encouraged amongst structural biologists and become an alternate means to prepare crystals of macromolecules with reproducible high quality.

Electronic Supplementary Material

Details on the crystallographic work have been included in Tables A and B found in the Supplementary Material. Table A gives a brief description of each step of the refinement (resolution range, model atoms, R- and R_{free}-factors) and Table B presents statistics on the data and the final model as a function of the resolution shells.

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