Goniometer-based femtosecond X-ray diffraction of mutant 30S ribosomal subunit crystals

E. Han Dao,1 Raymond G. Sierra,1 Hartawan Laksmono,1 Henrik T. Lemke,2 Roberto Alonso-Mori,2 Aaron Coey,3 Kevin Larsen,3 Elizabeth L. Baxter,4 Aina E. Cohen,4 S. Michael Soltis,4 and Hasan DeMirci1,4,a)
1 Stanford PULSE Institute, SLAC National Accelerator Laboratory, Menlo Park, California 94025, USA
2 Linac Coherent Light Source (LCLS), SLAC National Accelerator Laboratory, Menlo Park, California 94025, USA
3 Biophysics Program, Stanford University School of Medicine, Stanford, California 94305, USA
4 Stanford Synchrotron Radiation Lightsource (SSRL), SLAC National Accelerator Laboratory, Menlo Park, California 94025, USA

(Received 26 February 2015; accepted 20 April 2015; published online 30 April 2015)

In this work, we collected radiation-damage-free data from a set of cryo-cooled crystals for a novel 30S ribosomal subunit mutant using goniometer-based femtosecond crystallography. Crystal quality assessment for these samples was conducted at the X-ray Pump Probe end-station of the Linac Coherent Light Source (LCLS) using recently introduced goniometer-based instrumentation. These 30S subunit crystals were genetically engineered to omit a 26-residue protein, Thx, which is present in the wild-type *Thermus thermophilus* 30S ribosomal subunit. We are primarily interested in elucidating the contribution of this ribosomal protein to the overall 30S subunit structure. To assess the viability of this study, femtosecond X-ray diffraction patterns from these crystals were recorded at the LCLS during a protein crystal screening beam time. During our data collection, we successfully observed diffraction from these difficult-to-grow 30S ribosomal subunit crystals. Most of our crystals were found to diffract to low resolution, while one crystal diffracted to 3.2Å resolution. These data suggest the feasibility of pursuing high-resolution data collection as well as the need to improve sample preparation and handling in order to collect a complete radiation-damage-free data set using an X-ray Free Electron Laser. © 2015 Author(s). All article content, except where otherwise noted, is licensed under a Creative Commons Attribution 3.0 Unported License. [http://dx.doi.org/10.1063/1.4919407]

I. INTRODUCTION

In this work, crystals of a novel mutation of the *Thermus thermophilus* 30S ribosomal subunit were considered. *T. thermophilus* was genetically engineered to knock out expression of a 26-residue protein, Thx, which exists within the “head” domain of the 16S ribosomal RNA which comprises the skeleton of the 30S ribosomal subunit (Figure 1; Ref. 47). Choli et al., who first characterized this protein, gave Thx its name because it was found to be unique to several species of *Thermus*.1 An analysis of the Thx amino acid sequence (UniProt accession code P62612; sequence “GKGDRRTRRGKIKWRGTYGKYRPRKKK”) using ProtParam server suggests that Thx is positively charged at physiological pH, with seven arginine and six lysine residues within the 26 residue chain.1,2 Currently, the structure of the *T. thermophilus* 30S...
subunit without Thx has yet to be solved, and the precise contributions of this protein to the overall structure of the 30S ribosomal subunit are unclear. It is also not known how Thx influences or contributes to the structural dynamics of the 30S subunit in Thermus. The location of Thx within a pocket near the head of the rRNA, as ascertained by Wimberly et al. in their published structure of the wild-type *T. thermophilus* 30S ribosomal subunit, suggests ionic interactions between these amino acid chains with the electronegative phosphate backbones of the surrounding ribonucleotides. It is possible that because only *Thermus spp.* is known to possess this protein, Thx may be correlated to some aspect of thermo-stability. In order to better address these questions regarding the role of Thx in 30S ribosomal subunit structure and
dynamics, we purified 30S ribosomal subunits omitting this protein ("ΔThx" subunits) and grew crystals according to published conditions. We sought to ascertain the feasibility of using crystallography for these studies by screening crystals of the prepared ΔThx subunits using goniometer-based instrumentation. In general, ribosome crystals are a challenging crystallographic target due to their large solvent content, asymmetry and very large unit cell dimensions. Ribosome X-ray crystallographic analysis with synchrotron radiation has required substantial effort spanning many investigators and laboratories in order to produce high-resolution structures. Despite the substantial achievements and insights gained into ribosomal structure using synchrotron radiation, the application of macromolecular crystallography (MX) at a hard X-ray Free Electron Laser (XFEL) towards the study of ribosomal subunit crystals is appealing for several reasons.

Femtosecond X-ray crystallography (FX) presents new opportunities for the analysis of macromolecular crystals. The ability to collect diffraction data from crystals prior to the onset of radiation damage using femtosecond-scale pulses is a significant boon for MX. It provides the opportunity to circumvent the long-standing problem of introducing radiation damage to sample crystals and biasing the resulting structure. Initial modeling and experiments for parameters available at an XFEL (namely, the Linac Coherent Light Source; “LCLS”) have suggested that radiation-damage-free data collection is possible both in theory and in practice. Calculations, molecular dynamics simulations, and additional proof-of-concept work over the past decade and beyond involving numerous collaborators have predicted the possibility, modeled the nature of femtosecond diffractive imaging, and established the groundwork for femtosecond X-ray crystallography. For crystals, in particular, Barty et al. describe the instantaneous occurrence of Bragg diffractions right as the X-ray pulse hits the crystal, followed by the propagation of initial damage through the crystal within 10–100 fs of the incident pulse, during which time the lattice begins losing its periodicity and proceeds toward Coulombic explosion. In effect, the diffraction pattern is collected at the cost of destroying the analyzed sample, since the X-ray dose is higher than the calculated exposure limit for cryo-cooled crystals.

The ability to outrun radiation damage, whether induced proximally in the crystal by photoelectric absorption of X-rays or through the free radicals generated by the incident X-rays (“primary” and “secondary” damage, respectively), is particularly valuable for the study of biological macromolecules, ribosomes included. Previous work in characterizing radiation damage that occurs during crystallography, particularly to protein crystals, has shown that significant changes occur upon sample exposure to the X-ray doses available at synchrotron light sources. Decarboxylation of acidic residues and breakage of disulfide bonds are two such examples. MX experiments at synchrotron beamlines typically use beam exposures on the order of seconds, meaning modifications or damage to the crystal can be induced and then recorded during the exposure. The femtosecond time scale of XFELs means exposures of sufficient energy and intensity can be delivered and diffraction by the crystal induced even before the incident pulse terminates. This important capability offers the potential to reveal new structural insights which may have been previously obscured by the nature of synchrotron-based data collection.

The destructive nature of FX means experiments must rely on a serial approach, adding new crystals to replace destroyed sample in order to continuously collect data. Injector-based approaches have been the dominant approach thus far for several reasons. Under optimal conditions, samples can be flowed in a compatible medium (e.g., mother liquor) at ambient temperature. Consequently, data can be collected from fully hydrated nano- and microcrystals of biological targets such as wild-type ribosomes, photosynthetic centres, G-protein coupled receptors (GPCRs), and other biological targets at ambient temperature. The ability to collect data from crystals which do not grow to the larger sizes required for analysis at synchrotron light sources means more targets, which may have been previously off-limits or impractical to consider, can now be studied using Serial Femtosecond X-ray (SFX) crystallography. The main drawback of the injector-based approach is the inefficient hit-rate: a larger sample volume is required to collect a complete data set relative to the fixed-target analyses done...
at the synchrotron. For an injector-based SFX experiment operating at the 120 Hz pulse rate of the LCLS and assuming a crystal concentration of 10⁸ ml⁻¹ and a 10 μm diameter liquid jet flowing at a velocity of 10 m s⁻¹, 100 crystals are wasted per X-ray pulse. Because not every pulse hits a crystal, the overall efficiency is very low.

On the other hand, since our crystals typically grew to ~80 × 60 × 500 μm³ in size over 4–6 months, we chose the highly efficient fixed-target FX method for data collection. We had also considered crushing and filtering the samples to do injector-based SFX; however, the softness of the ribosome crystals (due to their large size unit cells and high solvent content) meant any harsh mechanical processing could have disrupted or destroyed the crystallinity of the sample in question. It is also unclear whether crushing reduces the diffraction quality of the ΔThx crystals. Additionally, the amount of available ΔThx crystals was limited and would have been insufficient to carry out an injector-based SFX experiment. Given the practical limitations involved with pursuing injector-based SFX for our sample, goniometer-based FX (GFX) was deemed as the preferable method.

GFX combines expertise in sample handling and data collection accumulated at synchrotron light sources with the ultrafast, ultra-bright peak power of pulses of the LCLS. Cohen et al. have described in detail their implementation of a goniometer-based system at the X-ray pump probe (XPP) end-station of the LCLS essentially similar to the arrangement available at the macromolecular crystallography beamlines of the Stanford Synchrotron Radiation Lightsource (SSRL). This approach results in a user experience that is highly similar between light sources, including the usage of small quantities of large macrocrystals. Parameters of the X-ray beam (e.g., attenuation and beam focus) are currently controlled by the beamline staff, but parameters related to the samples are user-controllable through the Blu-Ice control software and the functionality it affords. In addition to provide a familiar user experience for data collection, we were able to crystallize, cryoprotect, and store our samples as we would have done for an analysis at a synchrotron. The data processing methodology for SFX, whether injector-based or fixed-target, has seen a substantial development and improvement and continues to undergo further refinement and standardization.

II. METHODS

30S ribosomal subunits of the T. thermophilus ΔThx mutant strain provided to the authors by Dr. Steven Gregory and Dr. Albert E. Dahlberg (Brown University) were prepared and purified as previously described. Crystals were grown at 277 K by the hanging-drop method using 2-methyl-2,4-pentanediol (MPD) as the precipitant according to published conditions for the wild-type 30S subunit. Crystals of approximately 80 × 60 × 500 μm³ in dimension were harvested onto Hampton-style nylon cryo-loops, flash-frozen by plunging them into liquid nitrogen, placed into a SSRL sample cassette, and stored for data collection. Desired X-ray parameters were adjusted at the XPP beamline (50 fs pulses, 9.5 keV ± 60 eV photon energy, and 10¹² photons/pulse), and samples were manually selected for screening. Each ΔThx 30S ribosomal crystal was loaded via the Stanford Auto-Mounter robot controlled with the Blu-Ice software and aligned using the “click-to-center” functionality of the software. Helical data collection for each crystal was taken with 10–100 μm spacing between sites of exposure. Accordingly, the number of shots varied to the size of the screened crystal. This method allowed us to collect data semi-automatically by specifying the location of the crystal and automating the collection of exposures along the length of the crystal. Screening shots were collected in this way to maximize the number of shots collected per crystal. The resulting data were screened for resolution and diffraction quality using the Web-Ice software, which in turn used the LABELIT program SPOTFINDER for spot-finding and indexing. Subsequent diffraction pattern analysis, where warranted, was conducted using HKL2000 to obtain data regarding mosaicity and the unit cell parameters.

III. RESULTS

During our XPP protein crystal screening time, we experimented and adjusted the GFX experimental parameters for our 30S ribosome samples. A total of 45 crystals were screened
for diffraction quality during the 5 h allotted to the ΔThx 30S ribosomal subunit samples. The majority of crystals (35/45) were screened using a 50 × 50 μm² beam focus. Six and four exploratory shots at 11 × 11 μm² and 3 × 3 μm² beam foci were also conducted. Exposures using these smaller beam focuses instantly vaporized the site of the exposure and sometimes showed physical damage extending from the site, limiting the number of shots that could be recorded per sample.

The samples screened with a 50 μm focus diffracted to 4.5–6.0 Å, as determined by visual examination in the Web-Ice image viewer. The best diffracting image extended to 3.2 Å resolution as noted in Web-Ice and was observed using a beam focus of 3 × 3 μm². The first exposure caused physical damage to the remainder of the crystal and, as a result, further data could not be recorded from the crystal. Indexing and refinement of this chosen pattern in HKL2000 in the P4 space group resulted in calculated unit cell parameters of \( a = b = 402 \text{ Å} \), \( c = 175 \text{ Å} \), and \( \alpha = \beta = \gamma = 90^\circ \). Mosaicity was calculated to be 0.01 (Figure 2).

![A](image1.png)

![B](image2.png)

FIG. 2. Indexing and refinement of the crystal are performed by HKL2000. (a) Raw diffraction pattern prior to indexing and refinement. (b) Predicted spots after indexing and refinement of the crystal in the P4 space group. The unit cell parameters were determined as follows: \( a = b = 402 \text{ Å} \), \( c = 175 \text{ Å} \), and \( \alpha = \beta = \gamma = 90^\circ \). The mosaicity was calculated to be 0.010.
IV. DISCUSSION

The results show that our $\Delta Thx$ crystals, prepared according to published methods, were able to diffract to 3.2 Å resolution at an XFEL and to 3.7 Å resolution at the NE-CAT ID-24C beamline at the Advanced Photon Source (APS). Variations among the $\Delta Thx$ crystals were also observed: using a defocused beam of $50 \times 50 \mu m^2$, we were able to obtain diffraction up to 4 Å resolution. We obtained the best 3.2 Å resolution diffraction image using a $3 \times 3 \mu m^2$ focused beam. In addition, two other crystals diffracted to 4 Å, similar to the resolution observed with the defocused beam. Using a $3 \times 3 \mu m^2$ focused beam with ribosome macrocrystals mounted on cryo-loops was problematic given the observed sample degradation after 1–3 shots despite yielding higher resolution shots.

Collecting radiation-damage-free data for this mutant ribosomal subunit was paramount; collecting a full dataset at a synchrotron would not have sufficed. Considering the positive result of this crystal quality screening time, we believe it is feasible to pursue our goal of obtaining high-resolution, radiation-damage-free, and complete dataset for this target at an XFEL. As a reference, our best published cryo-cooled wild-type crystals are known to diffract to 3.2 Å resolution using synchrotron radiation. Additionally, recent XFEL SFX diffraction data for similarly prepared smaller microcrystals of wild-type 30S ribosomal subunits showed diffraction beyond 3 Å resolution (unpublished). High-resolution diffraction of $\Delta Thx$ crystals is possible provided that further improvements are made to our methodology.

For perspective, a recently published experiment using GFX was conducted by Suga et al. to collect a complete dataset for the dark state of photosystem II (PSII), which, like the ribosome, is a large, challenging, and radiation-sensitive target. In their experiment, more than 300 large crystals ($1200 \mu m \times 500 \mu m \times 200 \mu m$) were analyzed using a similar helical data collection approach: a $1 \times 1 \mu m^2$ beam focus and 50 μm spacing between exposure sites. Several beam time shifts were used in order to collect complete, high-resolution datasets for their target. While Suga et al. found success with macrocrystals, our empirical observations from this work with macrocrystals as well as unpublished data from wild-type microcrystals suggest that $\Delta Thx$ mutant microcrystals can yield a complete and higher-resolution dataset. This difference between 30S ribosome and photosystem II may exist due to numerous factors such as differences in unit cell packing, cryo-cooling method employed or beam quality, and intensity of the XFELs. In our case, using smaller crystals provides several benefits. An experiment with microcrystals would allow us to use a focused beam, whereas Suga et al. relied on multiple damage free shots per macrocrystal using a defocused beam. A focused beam appears to provide us with higher resolution at the expense of destroying the crystal. Thus with one image collected per crystal, using smaller crystals minimizes the crystal volume consumed per experiment. Additionally, the crystals used in our experiment took 4–6 months to grow to the large size used in this work; microcrystals could grow in one month’s time. Accordingly, sample handling can be modified to facilitate a rapid, serial analysis of numerous small microcrystals. Cohen et al. have proposed one such idea: pins with polycarbonate grids holding tens or hundreds of microcrystals each, a two order of magnitude increase in sample density over the typical one-crystal, and one-pin ratio of traditional sample pins. These improvements could make use of the intrinsic 120 Hz pulse rate of LCLS, thereby reducing the time needed to collect a complete, high-resolution data set.

Our results from this experiment also indicate the possibility of pursuing injector-based SFX study. Yet, relatively large sample volumes and high data redundancy required for an injector-based SFX experiment are not trivial: a sufficient quantity of microcrystals would still have to be grown. Recent development of liquid injectors for viscous media, however, has reduced the amount of sample necessary to obtain a full dataset from tens of milligrams to less than one milligram of protein microcrystals. Although it has not been applied to delicate and hard-to-grow ribosome crystals such as our $\Delta Thx$ mutant, it is a promising next step to try to address the problem of high sample consumption for injector-based SFX. If successful, a complete data set could be obtained within several hours using less than 100 μl of viscous media. This experiment would be a major effort requiring further testing of the compatibility of
ribosome microcrystals with viscous media along with additional LCLS beam time to evaluate the diffraction quality of that mixture.

Beyond the experimental conditions, ongoing and future developments in XFEL data processing such as improvement in post-refinement techniques will mitigate the need to collect highly redundant data. Recent work by Uervirojnakoon et al. has demonstrated a new post-refinement algorithm in which fewer images were needed than before in order to solve a structure using goniometer- and injector-based FX.\textsuperscript{45} Their data processing examples included: 100 out of 757 images needed to determine the structure of myoglobin,\textsuperscript{30,45} 100 out of 177 images used to determine the structure of hydrogenase,\textsuperscript{30,45} and 2000 out of 12,692 images for thermolysin were sufficient to obtain an high-quality structure by post-refinement.\textsuperscript{45,46} This method also improved the R and R\textsubscript{free} significantly despite the smaller number of images used for refinement. Whereas Suga et al. collected a minimum of 2058 still images (of which 1167 were used to determine their PSII structure),\textsuperscript{43} we expect that subsequent ribosome GFX experiments would need to collect a quantity of still images comparable with synchrotron cryocryostallography if this new post-refinement method is employed.

It is also interesting to note that modern synchrotron beamlines (e.g., SSRL beamline 12–2) with micro-focusing capabilities offer beam foci, photon energies, and photon flux that are competitive with current LCLS XPP capabilities. The ultrafast and ultrabright peak power of pulses of the LCLS provides a substantial leap in time resolution, but they also introduce new practical considerations. Still, hard-XFEL beam time is limited and in high demand across many fields of science, meaning opportunities for such radiation-damage-free studies, are limited until near-term improvements to XFEL capacity come to fruition. Fixed-target FX presents a new, practical, and important option for MX. Our findings with these $\Delta T\text{hx}$ crystals suggest that future crystallographic studies can use fixed-target FX for preliminary analysis, with subsequent data collection using either a more extensive, fixed-target strategy, or injector-based SFX if desired. Increased precision in the characterization of biological structures using the femtosecond pulses of hard X-ray FELs can potentially result in new significant insights for structural biology and could have further downstream implications for fields such as medicine. The ability to harness femtosecond time-scale pulses and obtain diffraction data from crystal structures prior to the induction of X-ray radiation damage is one of the most salient aspects of using scarce XFEL time for MX. Considering the quality of data that can be obtained, the ongoing methods development to improve FX methodology and throughput, as well as future increases in beam time capacity across new and existing XFEL sources, the outlook for FX is promising.

V. CONCLUSION

In this work, crystals of a novel $T.\ thermophilus$ 30S ribosomal subunit mutant were screened for diffraction quality using goniometer-based fixed-target femtosecond crystallography recently introduced at the Linac Coherent Light Source. The observed results suggest that ultra-bright femtosecond-scale XFEL pulses can be used to collect a complete, high-resolution, and radiation-damage-free data set for $\Delta T\text{hx}$ crystals. The findings presented in this work indicate that pursuit of a complete data set at an XFEL is feasible, but that additional improvements to sample preparation and sample handling are needed to make this goal a reality.

ACKNOWLEDGMENTS

The authors thank Dr. Steven Gregory and Dr. Albert E. Dahlberg for the $T.\ thermophilus$ $\Delta T\text{hx}$ 30S mutant strain. Portions of this research were carried out at the Linac Coherent Light Source (LCLS) at the SLAC National Accelerator Laboratory. LCLS is an Office of Science User Facility operated for the U.S. Department of Energy Office of Science by Stanford University. The LCLS was acknowledged for beam time access under Experiment No. xppe8814. E.H.D., H.L., R.G.S., and H.D. acknowledge the support of the Office of Basic Energy Sciences (BES) through the AMOS program within the Chemical Sciences, Geosciences,
and Biosciences Division of the Office of Basic Energy Sciences, and the Department of Energy through the SLAC Laboratory Directed Research and Development Program. E.H.D. acknowledges financial support from the Stanford University Dean of Research. H.D. and S.M.S. acknowledge support from the joint Stanford ChEM-H and SLAC National Accelerator Laboratory seed grant program. H.D. also acknowledges valuable discussions with Aiko Takeuchi, Kenji Dursuncan, and Emi Satunaz.

1T. Choli, F. Franceschi, A. Yonath, and B. Wittmann-Liebold, *Biol. Chem. Hoppe-Seyler* 374, 377 (1993).
2E. Gasteiger, C. Hoogland, A. Gattiker, M. R. Wilkins, R. D. Appel, A. Bairoch et al., *Proteomics Protocols Handbook* (Springer, 2005), pp. 571–607.
3B. T. Wimberly, D. E. Brodersen, W. M. Clemmons, Jr., R. J. Morgan-Warren, A. P. Carter, C. Vonhein, T. Hartsch, and V. Ramakrishnan, *Nature* 407, 327 (2000).
4A. Yonath, J. Harms, H. A. S. Hansen, A. Bashan, F. Schlünzen, I. Levin, I. Koelln, A. Tocilj, I. Agmon, M. Perez, H. Bartels, W. S. Bennett, S. Krumbholz, D. Janell, S. Weinstein, T. Auerbach, H. Avila, M. Piolleti, S. Morlang, and F. Franceschi, *Acta Crystallogr., Sect. D: Biol. Crystallogr.* 54, 945 (1998).
5M. S. Hunter, D. P. DePonte, J. D. Eland, R. B. Doak, J. C. H. Spence, and R. B. Doak, *J. Phys. D: Appl. Phys.* 31, 041706 (2015).
45. M. Uervirojnangkoorn, O. B. Zeldin, A. Y. Lyubimov, J. Hattne, A. S. Brewster, N. K. Sauter, A. T. Brunger, and W. I. Weis, *eLife* 4, e05421 (2015).

46. J. Hattne, N. Echols, R. Tran, J. Kern, R. J. Gildea, A. S. Brewster, R. Alonso-Mori, C. Glöckner, J. Hellmich, H. Laksmono, R. G. Sierra, B. Lassalle-Kaiser, A. Lampe, G. Han, S. Gul, D. DiFiore, D. Milathianaki, A. R. Fry, A. Miahnahri, W. E. White, D. W. Schafer, M. M. Seibert, J. E. Koglin, D. Sokaras, T.-C. Weng, J. Sellberg, M. J. Latimer, P. Glatzel, P. H. Zwart, R. W. Grosse-Kunstleve, M. J. Bogan, M. Messerschmidt, G. J. Williams, S. Boutet, J. Messinger, A. Zouni, J. Yano, U. Bergmann, V. K. Yachandra, P. D. Adams, and N. K. Sauter, *Nat. Methods* 11, 545 (2014).

47. S. T. Gregory and A. E. Dahlberg, unpublished results (2015).