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X-ray Structure of Native Scorpion Toxin BmBKTx1 by Racemic Protein Crystallography Using Direct Methods

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Recently, we determined the structure of the protein sfAFP by means of racemic crystallization.[1] In the current work, we additionally sought to explore the use of protein racemates to enable structure solution using direct methods; there are only a handful of native L-protein structures that have been solved by direct methods and the successful application of this approach is still a real challenge in modern crystallography.[2,3] Here we report the first crystallization and the X-ray structure determination of native scorpion toxin BmBKTx1. Using racemic protein crystallization, we obtained crystals of BmBKTx1 in the tetragonal centrosymmetric space-group *I*41/*a*, that diffracted to atomic resolution; this enabled the structure to be determined by the use of direct methods.

Difficulty in crystallization is often observed for proteins that contain numerous charged, surface-exposed amino acid residues. An example of one such protein is the lysine-rich scorpion toxin BmBKTx1, a 31 amino acid residue microprotein that is a high-conductance calcium-activated potassium channel blocker.[4–6] For BmBKTx1 it was reported that at 100 mg/mL protein concentration no crystal formation was observed over many weeks at room temperature, using extensive sparse-matrix crystallization searches.[6] In order to obtain diffraction-quality crystals, these authors found that it was necessary to use reductive dimethylation of lysine residues; this strategy resulted in an X-ray structure of methylated BmBKTx1 at 1.72 Å resolution.[6]

We explored the use of racemic crystallization for BmBKTx1; the protein contains six cysteine residues that form three disulfides in the folded molecule.[4] The mirror image isomer of the native protein required for racemic protein crystallization can only be prepared by chemical synthesis. We prepared the polypeptide chains of D- and L-BmBKTx1 in good yield and high purity by manual, Boc chemistry, stepwise solid-phase peptide synthesis.[7] The D- BmBKTx1 and L- BmBKTx1 polypeptides were synthesized at a scale of 0.1 and 0.2 millimole, respectively. The crude peptides were folded with concomitant disulfide formation, followed by HPLC purification; the LCMS of the folded and purified synthetic protein products are shown in Figure S1 (see Supporting Information). We obtained pure D- and L- proteins in multiple tens-of-milligram amounts.

Crystallization trials of L-BmBKTx1 in our hands were not successful, as previously reported.[6] In contrast, using a racemic solution containing equal amounts of D-BmBKTx1 and L-BmBKTx1 in the commercially available Hampton Research Index screen at 19 °C, crystals were obtained under multiple conditions; under some conditions, crystals appeared overnight. One set of conditions was further optimized to produce ultra-high quality crystals (Figure 1a). Diffraction data (Figure S2) were collected at the Advanced Photon Source to a resolution of

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1.1 Å, and diffraction intensity statistics revealed that the protein racemate crystallized in the tetragonal centrosymmetric space-group *I*41/*a*, with one enantiomer in the asymmetric unit. The {D-BmBKTx1 + L- BmBKTx1} crystals are the first example of a protein racemate crystallized in this high-symmetry space group; to the best of our knowledge, all other protein racemates reported to date have crystallized in space group P $\bar{1}$,[1,8–12] a preference predicted by Wukovitz and Yeates.[13]

With atomic-resolution diffraction data in hand, we explored the use of direct methods[14, 15] to obtain phasing information for structure determination of the BmBKTx1 protein racemate. Despite suggestions that direct methods should be more feasible with racemic crystals,[16, 17, 18] it is noteworthy that none of the three centrosymmetric racemic protein structures previously reported was solved using direct methods,[1, 8, 12] although three small peptide racemates have been solved by direct methods with some difficulty.[9–11]

Our initial attempt at direct methods using the program SHELXS[19] provided a clear solution (Figure S3a) from an 8-hour run on a 2.8 GHz dual-core Xeon processor. The best structure solution had a combined figure-of-merit (CFOM) of 0.23 which, after preliminary refinement with SHELXL,[19] resulted in a well-defined map of continuous electron density with the side chains easily identifiable (Figure 1c). After protein model building and placement of solvent molecules, the final model was refined to a crystallographic R-factor of 0.193 (R-free = 0.209) using REFMAC5.[20,19] A cartoon representation of the resulting X-ray structure of native BmBKTx1 is shown in Figure 1b. Both the solution NMR structure[5] and the X-ray structure of methylated[6] BmBKTx1 are generally similar to the crystal structure reported here, although significant backbone deviations were found in the β-turn region (Asn_{24}/Ser_{25}) between the X-ray structure of the methylated variant and the X-ray structure of native BmBKTx1 (Figure S5).

The packing of D- and L- enantiomers of BmBKTx1 in the *I*41/*a* unit cell is shown in Figure 2a. There are intermolecular hydrogen-bonding interactions (two direct inter backbone H-bonds and four water mediated H-bonds) between the anti-parallel C-terminal β-strands of the two enantiomeric molecules. A stereo pair representing this geometrical feature is shown in Figure S6. Interestingly, this interface is an achiral anti-parallel β-sheet (in natural L-proteins, all anti-parallel β-sheets are inherently twisted and are therefore chiral). Interactions in this region, as shown in Figure 2b and Table S2, contribute to the centrosymmetric arrangement of the D- and L-enantiomers.

In the case of the scorpion toxin microprotein BmBKTx1 reported here, racemic crystallization was used for the facile production of diffraction quality crystals for a target recalcitrant to crystallization in its native form. A significant increase in the ability to crystallize proteins from racemic mixtures was specifically predicted by Wukovitz and Yeates,[13] and previously demonstrated in one instance.[1] The BmBKTx1 protein racemate was observed to crystallize in the high-symmetry tetragonal space group *I*41/*a*. Additionally, direct methods were successfully used for the solution of the X-ray structure of native BmBKTx1 at atomic resolution of 1.1 Å; and, a novel β-strand-type intermolecular interaction at the centrosymmetric interface between L- and D-enantiomers was identified as a key feature of the BmBKTx1 racemate crystal.

To further explore the general utility of racemic crytallization, we are using total chemical synthesis to study proteins of ever-increasing size, which have been reported as difficult or impossible to crystallize, or for which no X-ray structure has been reported. To date we have found that racemic protein crystals form more readily and diffract to higher resolution. Racemic crystallography is proving to be a viable approach to obtaining structures of recalcitrant proteins.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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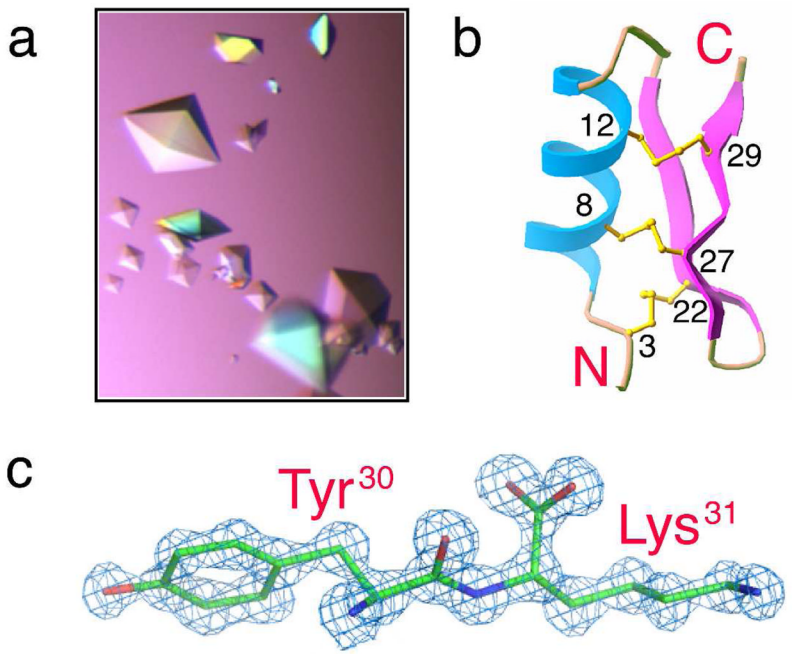

Figure 1.

Crystallization and X-ray structure of native BmBKTx1 obtained by the racemic method in the tetragonal centrosymmetric space group *I*41/*a*,. (a) Racemic crystals of D- BmBKTx1 and L- BmBKTx1grown at 19°C with a total protein concentration of 25 mg/mL using 0.1 M citric acid, pH 3.5 and 0.9 M ammonium sulfate as crystallization buffer. (b) A cartoon representation of the L-BmBKTx1 constituting the asymmetric unit. The structure was solved at 1.1 Å X-ray resolution by direct methods. The protein fold is shown as ribbons with three S—S bonds (labeled) shown in ball-and-stick mode. (c) SigmaA-weighted 2Fo-Fc electron density map, contoured at 2σ, showing residues 30–31 and derived from the best SHELXS solution (CFOM=0.23).

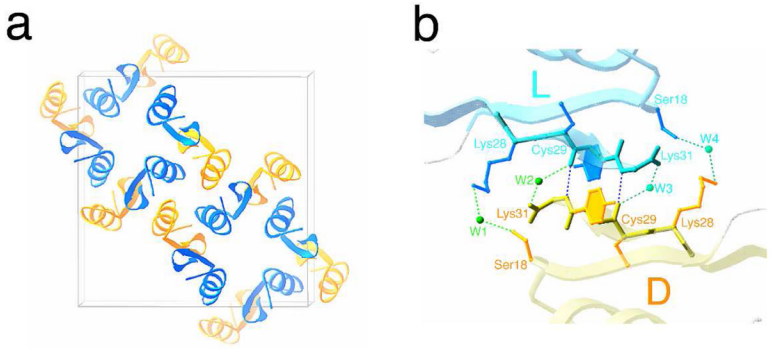

Figure 2.

(a) Unit cell composed of sixteen molecules, eight L-BmBKTx1 (shown as cyan ribbons) and eight D-BmBKTx1 (shown as gold ribbons) viewed along the 4_1 crystal axis. (b) Close-up view of the interface between L- and D- enantiomers related by an inversion center. Water molecules are shown as small green spheres, and hydrogen bonds are shown as dashed lines. H-bond lengths are listed in Table S2. The surface area buried at the interface is ~654 $Å^2$.