then transferred to this buffer prior to crystallization and simultaneously, the crystallization temperature was lowered to 4°C. This optimization resulted in formation of crystals with maximum dimensions of approximately 0.35 0.2 0.1 mm. which diffracted up to 2.5 Å and show significant anisotropic behaviour.

The structure of AHP2 was solved using SIRAS protocol using anomalous signal of the Lutetium. AHP2 protein represents α-helical bundle, comprising of four short and two long helices. Short helices form a central core of the protein with the conserved His residue carrying the phosporyl group situated in the middle of the third α-helix.

In order to gain insight into the structural features, underlying AHP2-CKI1rd interaction, molecular-dynamics simulations were carried out. Simulations were performed for 100 ns and show the stability of protein-protein complex. The key residues, responsible for the AHP2-CKI1rd interaction, were identified and reveal strong protein-protein binding. The analysis of the obtained data is currently in progress.

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S15

CRYSTALLOGRAPHIC STUDY OF HALOALKANE DEHALOGENASE DpcA FROM *PSYCHROBACTER CRYOHALOLENTIS* K5

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Introduction

Haloalkane dehalogenases (EC 3.8.1.5; HLDs) are microbial enzymes that catalyze the hydrolytic conversion of halogenated aliphatic compounds to their corresponding alcohols [1, 2], which is the hydrolytic dehalogenation accomplished by these enzymes is one of the most important steps in the biodegradation of 1-halo-n-alkanes and -dihalo-n-alkanes, serious halogenated pollutants [3]. HLDs have a broad substrate specificity [4] and a high enantioselectivity [5], which makes these enzymes applicable in bioremediation [6], in biosensing [7,8], biocatalysis [5, 9], cellular imaging, and protein analysis [10, 11] . Understanding of the structural bases of the enzyme extremophilicity allows for the construction of HLD variants with improved activity and stability at low and high temperatures and thus enlarges their applicability in environmental and biosynthetic applications.

Experimental details

A novel HLD enzyme, DpcA, exhibiting unique temperature profiles with exceptionally high activities at low temperature, isolated from Gram-negative psychrophilic bacteria *Psychrobacter cryohalolentis* K5 [12] was crystallized by sitting-drop and hanging-drop vapour-diffusion techniques. Crystallization drops were

prepared by mixing 2 μl of protein solution at the concentration 10 mg ml^{-1} in 50 mM Tris–HCl buffer pH 7.5 and 1 μl precipitant solution plus 0.3–0.6 μl of 0.1 M L-proline. Diffraction data were collected at the beamline 14.2, Helmholtz-Zentrum Berlin (HZB) (Germany) at the BESSY II electron storage ring, detector Rayonics MX-225 CCD [13] at wavelengths of 0.978 Å. All diffraction experiments were carried out in a liquid-nitrogen stream at 100 K using a Cryojet XTL system (Oxford Instruments). The diffraction data for DpcA were indexed,

integrated and scaled by HKL-3000 [15]. Matthews coefficient was calculated with MATTHEWS_COEF [15], using the the CCP4 software package [16].

Results and discussians

Crystals of DpcA enzyme diffracted to the 1.05 Å resolutions and belonged to $P2_1$ (primitive monoclinic space group) with unit-cell parameters a = 41.3, b = 79.4, c = 43.5 Å, = = 90.0 , = 95.0 and contained one molecule in the asymmetric unit [16].Structurally DpcA is a member of the superfamily of -hydrolase, molecular replacement with *MOLREP* [15] from the CCP4 software suite was used for structure solving.

Conclusions

The coordinates of *Xanthobacter autotrophicus* (PDB code: 1B6G; 40% sequence identities for 121 residues and 53% sequence similarity was used as search model for DpcA structure. DpcA protein has a globular shape and is composed of two domains. The core domain shows composed of eight -strands, within one is antiparallel (2). The central -sheet is flanked on both sides by -helices: four are on one side and two are on the other side of the sheet. The second domain, the cap structure is located at the C-terminal end of the -sheet and is composed of -helices and covers the active site, which will be more reviewed in the presentation. The structure of DpcA is consimilar to the others structurally known HLD.

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S16

USING IONIC LIQUIDS IN PROTEIN CRYSTALLIZATION

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Ionic liquids (ILs) are organic salts composed of separate cations and anions, which are liquid at room temperature and virtually have zero vapor pressure. ILs possess cationic and anionic components that can be tailored for different applications however the properties of ILs are not known well. ILs have been reported to influence on thermal stability, to stabilize protein structure and activity, to increase crystallization rates and crystal size, to enhance the kinetics of crystallization.

The investigation of using ionic liquids as additives for advanced crystallization was held. Twenty four ILs were incorporated in the experiment for their effect on the lysozyme and thaumatin crystallization using Ionic Liquid Screen kit. Experiments were set up with 50% w/v ILs added to the crystallization solutions at ration 1:9. Crystallization droplets were set up at four protein/precipitant ratios - 1:1, 1:2, 2:1, 3:1. Crystals were obtained under identical conditions that are optimal for common lysozyme and thaumatin crystallization.

Sitting drop vapor diffusion crystallization experiments showed that addition of some of the ILs led to less crystal polymorphism and larger the lysozyme crystals.

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S17

TOWARDS CRYSTAL STRUCTURES OF ANTIBODIES AND TRANSCRIPTION FACTORS

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Introduction

The knowledge of protein three-dimensional structure is important for understanding protein function. The three-dimensional structure can, among other things, provide information on regulation of protein function by interaction with ligands that can also be utilized in rational design of inhibitors or modulators. Structural information can also provide a basis for protein engineering approaches.

X-ray crystallography is the principal method of protein structure determination, close to 90% of all the protein structures deposited in the Protein Data Bank to date have been solved by this method. Crystallization of protein represents a common bottleneck in the process of structure determination as having sufficiently large monocrystal is the essential requirement for diffraction experiments. The crystallization process is influenced by large number of factors from which the protein sample properties are the most important variable. Therefore, pre-crystallization biochemical and biophysical characterization of the protein sample can help in successful crystallization. This analysis can also be beneficial for other methods of structure determination such as NMR.

The use of pre-crystallization analysis will be presented on an example of two case studies: antibody fragments and bacterial transcriptional regulator.

Recombinant antibody fragments

Monoclonal antibody MEM-57 recognizes CD3 antigen, which is expressed on the surface of T-lymphocytes in complex with the T-cell receptor. CD3 plays role in the transduction of activation signal after the antigen is recognized by T-cell receptor. Antibody MEM-57 shows similar properties to the therapeutic anti-CD3 antibody OKT3 used as an immunosuppressant in organ transplantation. Antibody MEM-57 could be used in diagnostics of autoimmune diseases, for T-cell lymphoma classification, or as an immunosuppressant in transplantation. Antibody MEM-57 could be also used in cancer therapy in therapeutic antibody format of Bispecific T-cell Engager (BiTE). BiTE molecule consists of an anti-CD3 antibody single chain variable fragment (scFv) linked to an anti-tumor antigen scFv. BiTE induces polyclonal activation of cytotoxic T-lymphocytes exclusively in the tumor site [1]. The structural information on scFv MEM-57 would enable humanization of the antibody for therapeutic applications.

Monoclonal antibody MEM-85 recognizes CD44 antigen, which is a cell surface receptor for hyaluronate and plays an important role in the immune system [2]. Some tumors exhibit CD44 overexpression and this is associated with bad prognosis. Antibody MEM-85 could be used in cancer diagnostics and classification or in cancer immunotherapy. MEM-85 blocks hyaluronate binding to CD44 and has been shown to induce CD44 shedding from the cell surface, similar to the shedding induced by hyaluronate binding. Thus, MEM-85 could also be used as a tool for analysis of the structural effects of hyaluronate – CD44 interaction on cellular events. Structural information on the antibody – receptor complex would allow rational design of potential hyaluronate binding inhibitors.

Deoxyribonucleoside regulator DeoR

In bacteria, transcription of metabolic genes is regulated by various catabolic repressors [3]. *Bacillus subtilis* can utilize deoxyribonucleosides and deoxyribose as a source of carbon and energy. The genes encoding the proteins required for their catabolism are grouped in the *dra-nupC-pdp* operon. Expression of this operon is repressed by binding of deoxyribonucleoside regulator protein DeoR. Expression of metabolic genes is induced by binding of small molecular effector to DeoR. The preferred effector molecule is deoxyribose-5-phosphate, but deoxyribose-1-phosphate was also described to act as an inducer. The DeoR repressor protein from *Bacillus subtilis* is homologous to bacterial regulator proteins of the SorC family. There is no sequence similarity between the DeoR regulators of *Bacillus subtilis* and *Escherichia coli*, even though they possess similar regulatory function. Structural information on DeoR free and ligand bound forms would elicit the regulation of DeoR function by a small molecular effector.

Experimental section

Proteins were prepared by heterologous expression in *E. coli* and purified by combinations of nickel chelation chromatography and ion-exchange chromatography. Protein pre-crystallization analysis employed size-exclusion chromatography, flow cytometry, dynamic light scattering, and thermofluor assay (also known as differential scanning fluorimetry, DSF). Thermofluor assay was used for the optimization of protein stability for crystallization, for optimization of protein oligomeric homogeneity, and for characterization of protein – ligand interactions. Crystallization screening was performed by the sitting drop vapor diffusion method; crystallization optimization employed a wide range of approaches using hanging drop vapor diffusion and counter-diffusion techniques. Diffraction data

were collected at beamline MX14.2 at BESSY, Berlin and processed using the HKL-3000 package. Structures of DeoR were solved by molecular replacement using MolRep program; model building was performed automatically by Buccaneer and manually by Coot programs. Structures were refined using Refmac program.

Results and discussion

Recombinant antibody fragments

Single-chain variable fragments (scFv) of the antibodies MEM-57 and MEM-85 were constructed: variable domains of the heavy and light chains were joined by a flexible Gly-Ser linker and further equipped with the N-terminal pelB leader sequence, and C-terminal c-myc tag and His_5 tag. The recombinant fragments were targeted into the periplasmic space of *E. coli* where they accumulated in a soluble form. Proteins were isolated from the host by osmotic shock. Two-step purification protocol employing nickel chelation chromatography and ion-exchange chromatography was developed to produce high yield of pure protein: 3 mg of scFv MEM-57 and 1.5 mg of scFv MEM-85 per 1l of bacterial culture. Antigen binding activity of both antibody fragments was confirmed by flow cytometry.

The ratio between monomeric and multimeric forms of the individual scFv fragments was determined by analytical size-exclusion chromatography. In case of scFv MEM-57, equilibrium established between monomer, dimer and higher oligomers with the majority of the protein being in the monomeric form. On the contrary, the monomeric and dimeric forms of scFv MEM-85 could be separated during the purification process.

Dynamic light scattering was used to evaluate the dispersity of the individual protein preparations at high concentrations used in crystallization experiments. Protein preparations of scFv MEM-57 which showed monomodal particle size distribution yielded crystals of better quality, unlike polydisperse protein preparations. All protein preparations of scFv MEM-85 were monomodal and monodisperse.

Thermofluor assay was used to screen for the composition of the storage buffer optimal for protein stability and to evaluate protein oligomeric homogeneity. For scFv MEM-57, the positive effect of the storage buffer composition (100 mM sodium phosphate pH 7.5, 200 mM NaCl) was confirmed by the results of initial crystallization trials. Crystallization screening in the original storage buffer did not yield any crystals of scFv MEM-57. After the buffer was changed to composition which showed to be optimal for protein stability in the thermofluor assay (100 mM sodium phosphate pH 7.5, 200 mM NaCl), protein crystals were obtained in 42 out of 96 screened conditions. Optimization of crystallization conditions by a wide range of approaches using hanging drop vapor diffusion and counter-diffusion techniques is now in progress.

For scFv MEM-85, thermofluor analysis showed high thermal stability of the antibody fragment in the majority of tested buffer systems (melting temperature Tm of 340 – 342 K). Crystallization trials were unsuccessful so far owing probably to the high stability of the protein.

Deoxyribonucleoside regulator DeoR

The C-terminal effector-binding domain of DeoR from *B. subtilis* (C-DeoR) equipped with N-terminal His_6 tag cleavable by tobacco etch virus protease was expressed in *E. coli* and purified using nickel chelation chromatography. Pre-crystallization analysis performed by size-exclusion chromatography and dynamic light scattering confirmed protein sample homogeneity and showed that a dimer is the biological unit of C-DeoR. The ligand binding activity of the recombinant C-DeoR was confirmed by thermofluor assay for deoxyribose-5-phosphate, but not for deoxyribose-1-phosphate. These results confirmed the role of deoxyribose-5-phosphate as a preferred inducer.

Crystallization screening trials in the presence of 50 mM deoxyribose-5-phosphate yielded needle-shaped crystals. Extensive crystallization optimization by a wide range of approaches using hanging drop vapor diffusion and counter-diffusion techniques together with protein re-purification by ion-exchange chromatography only yielded plate-like crystals of poor diffraction quality. Thermofluor assay was used to screen for the composition of the storage buffer optimal for protein stability and revealed specific thermal stabilization of C-DeoR by trisodium citrate The crystallization screening procedure was repeated in the optimized storage buffer (20 mM trisodium citrate pH 7.0, 150 mM NaCl, 0.02% (v/v) -mercaptoethanol) both in the presence and absence of 50 mM deoxyribose-5-phosphate, which yielded three-dimensional crystals. By optimizing the protein and precipitant concentrations large three-dimensional crystals with a good diffraction quality were obtained and diffraction data sets from three different crystal forms were collected at high resolution [4]. The structures were solved by molecular replacement using putative sugar-binding transcriptional regulator from *Arthrobacter aurescens* TC1 as a model. Structure refinement is currently in progress.

Conclusions

Analysis of proteins by combination of biochemical and biophysical methods such as size-exclusion chromatography, dynamic light scattering, and thermofluor assay can be successfully used to help protein crystallization. We have prepared scFv fragments of antibodies MEM-57 and MEM-85. We have performed protein pre-crystallization analysis and we have optimized protein stability and homogeneity for both NMR (MEM-85) and crystallization (MEM-57). Optimization of crystallization conditions is currently in progress. In order to gain information on the structure of the deoxyribonucleoside regulator from *Bacillus subtilis* in the ligand free form and in the complex with deoxyribose-5-phosphate, we have prepared the C-terminal effector-binding domain of DeoR (C-DeoR). We have performed protein pre-crystallization analysis and developed crystallization protocols which yielded monocrystals of three crystal forms suitable for diffraction data collection. Three complete high-resolution data sets were collected and processed, the structures were solved using molecular replacement; structure refinement is in progress.

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S18

CRYSTALLIZATION AND PRELIMINARY X-RAY CRYSTALLOGRAPHIC ANALYSIS OF RECOMBINANT ß-MANNOSIDASE FROM *ASPERGILLUS NIGER*

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-Mannosidase (-D-mannoside mannohydrolase, EC 3.2.1.25) is an important glycoside hydrolase (GH) specific for the hydrolysis of terminal -linked mannosides in various sugar chains. This enzyme plays an essential role in the complete hydrolysis of -mannans to mannose and therefore -mannosidases are very efficient in various industrial processes, such as hydrolysis of galactomannans used for the improved removal of drilled material in oil and gas drilling or coffee extraction, and as a bleach-boosting agent in the pulp and paper industries [1]. -Mannosidases are also used in the synthesis of oligosaccharides or alkyl -mannosides for medical and other purposes [2].

Here, we report the crystallization and preliminary X-ray crystallographic analysis of recombinant -mannosidase overexpressed in *Pichia pastoris*. The initial screening showed two conditions for obtaining the crystals: (i) 0.2 M magnesium chloride, 25%(w/v) polyethylene glycol 3350, 0.1 M bis-tris pH 6.5 and (ii) 0.2 M calcium chloride, 25%(w/v) polyethylene glycol 4000, 0.1 M Tris pH 8.5. The best crystals were produced by further optimization using the hanging-drop vapour diffusion method. The main precipitants PEG 3350 and PEG 4000 were changed in small concentration steps. The trials showed the best possible concentration was 20%(w/v) for PEG 3350 and PEG 4000. The long needles were observed to be more compact with a small increase in the salt concentration ($MgCl_2$ or $CaCl_2$); the effective range of the concentration is 0.2–0.4 M. The crystals of the -mannosidase were typically needles with dimensions 0.25x0.01x0.08 mm. Diffraction data were collected at BESSY II Berlin (14.1 and 14.2). The data were processed by XDSAPP [4]. The crystals belonged to space group P1. The -mannosidase in the native data set diffracted to 2.41 Å resolution and had unit-cell parameters $a = 62.37$, $b = 69.73$, $c = 69.90$ Å, = 108.20 , = 101.51 , = 103.20 . The calculated Matthews coefficient (V_M) of 2.56 $Å^3Da^{-1}$ with a solvent content of 52.06 % indicates the presence of one molecule in the asymmetric unit. The molecular-replacement method was performed with the structure of the -mannosidase from *Bacteroides thetaiotaomicron* (PDB entry 2JE8) [4] as a model, but a suitable solution was not found. The -mannosidase in the SAD data set diffracted to 2.44 Å resolution and had unit-cell parameters $a = 61.82$, $b = 65.23$, $c = 68.72$ Å , = 108.63 , = 101.06 , = 103.06 . The calculated Matthews coefficient (V_M) of 2.34 $Å^3Da^{-1}$ with a solvent content of 47.42 % indicates the presence of one molecule in the asymmetric unit. Experimental phasing, model fitting and refinement are in progress [5].

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