Automated matrix microseeding improves crystallization of FAD-dependent oxidoreductase

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Abstract

Microseeding is an important approach to the crystallization of biological macromolecules. Recent development of crystallization robots has allowed automation of the microseed matrix screening. Its remarkably positive impact was observed while using the MORPHEUS crystallization screen on the particular case of an FAD-dependent oxidoreductase. Using seeds, crystals were obtained in several conditions that previously contained only a light amorphous precipitate. After further optimization, promising diffraction patterns were collected.

Keywords: Macromolecular crystallography; Crystallization; Seeding; Diffraction

Introduction

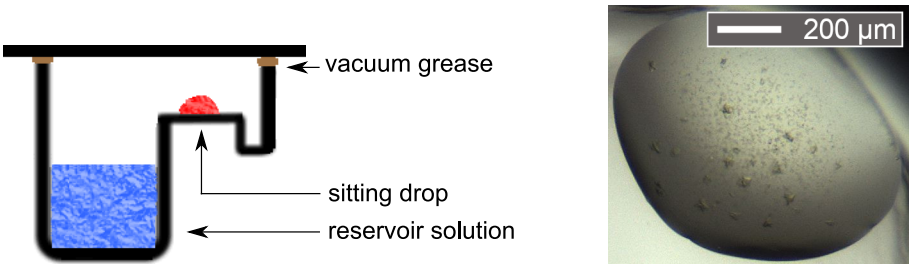
In order to investigate atomic structures of proteins or nucleic acids using diffraction techniques, a crystalline phase of a sample is required. The most common crystallization method is vapor diffusion in the setting of sitting or hanging drop. An individual enclosed experiment consists of a reservoir with a precipitant solution and the small drop, a mixture of the protein sample and the precipitant solution (Figure 1a). In most of the conditions water molecules move from the drop to the reservoir; thus, the system can reach a condition suitable for the crystal nucleation and subsequent growth [1].

One of the standard methods in crystallization is *microseeding*, *i.e.* addition of a very small amount of submicroscopic crystals into the drop with the same crystallization condition [2]. The provided nucleation centers prove to be beneficial in many cases for obtaining larger and better diffracting crystals. Moreover, the method was further extended to *matrix microseeding* where seeds are added into nonidentical conditions [3, 4].

In this work, we report the impact of automated matrix microseeding performed with a crystallization robot on the crystallization of a biotechnologically relevant enzyme belonging to FAD-dependent oxidoreductases [5].

Materials and methods

Sample. For the crystallization study, we used a freshly purified protein sample of the FAD-dependent oxidoreductase with a molecular weight of 41.3 kDa including His-tag,



(a) (b)

Figure 1: (a) Schematic diagram of the vapor diffusion method – sitting drop. [6]. (b) Crystalline material obtained in the MORPHEUS screen [7] in the condition C2 that was used for the preparation of seeds.

having a concentration of 10 mg/ml and stored in 20mM BIS-TRIS pH 6.5 and 50mM NaCl. The enzyme exhibits an intense yellow color that is caused by the presence of the FAD cofactor.

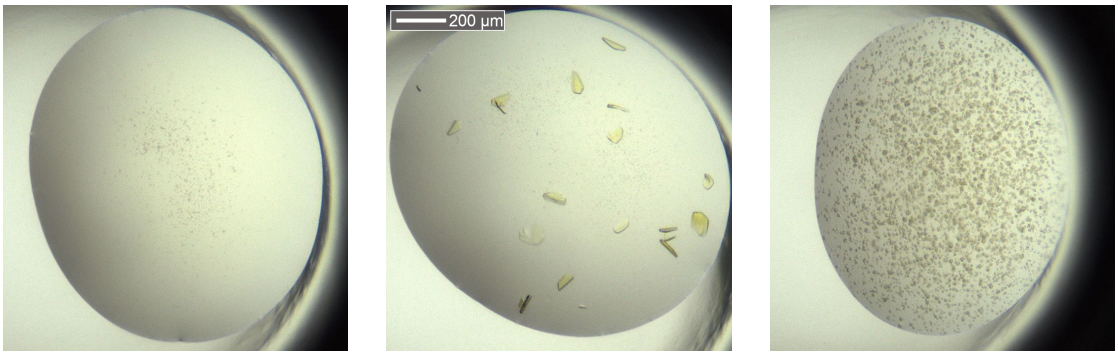
Seed stock. During previous crystallization trials with the FAD-dependent oxidoreductase, small crystals (Figure 1b) were obtained in the MORPHEUS screen [7] in the condition C2 (10% w/v PEG 8000, 20% v/v ethylene glycol, 0.03 M sodium nitrate, 0.03 M disodium hydrogen phosphate, 0.03 M ammonium sulfate, 0.1 M MES/imidazole pH 6.5) [7]. The drop (0.3 μl) with the crystalline material was diluted in 10 μl of the reservoir solution from the crystallization plate. Then the mixture underwent three times the following process: (i) crushing with a crystal crusher (Hampton Research) in a 200μl Eppendorf tube for 2 min, (ii) vortexing for 1 min, (iii) addition of further 10 μl of the reservoir solution and continuous mixing in a pipette for 1 min. Hence, the final volume of the seed stock was 40 μl.

Crystallization setting. The crystallization screen MORPHEUS [7] was set with an NT8 crystallization robot (Formulatrix) into two 96-well plates – a seeded plate and a control plate. Every condition was set into three sitting drops with a total volume of 0.3 μl varying in the ratio of protein sample and precipitant solution: 2:1, 1:1, 1:2; having a joint reservoir. The seed stock solution (50 nl per drop) was placed by the robot into drops of the seeded plate, whereas the control plate was left without seeds. Both plates were stored and monitored in a crystallization hotel RI1000 (Formulatrix) at 20°C. Crystals in the form of plates with a length of over 100 μm were obtained after an optimization process of condition C2, including seeding and changes in the concentrations of PEG, ethylene glycol and salts. The crystallization and diffraction experiments were performed at the Centre of Molecular Structure, Institute of Biotechnology of the Czech Academy of Sciences, BIOCEV.

Diffraction experiment. Diffraction patterns from these crystals were collected at 100 K using a D8 Venture diffractometer (Bruker) with a MetalJet D2 X-ray source (Excillum, gallium K_α), a 4-circle goniometer and a Photon III detector (Bruker). Collected data were processed with *XDSKAPPA* [8], *XDS* [9] and scaled using *AIMLESS* [10].

Results and discussion

After 60 days, the drops in seeded and control plates were scored into seven categories according to the nature of the resulting form. The typical examples of the categories “light



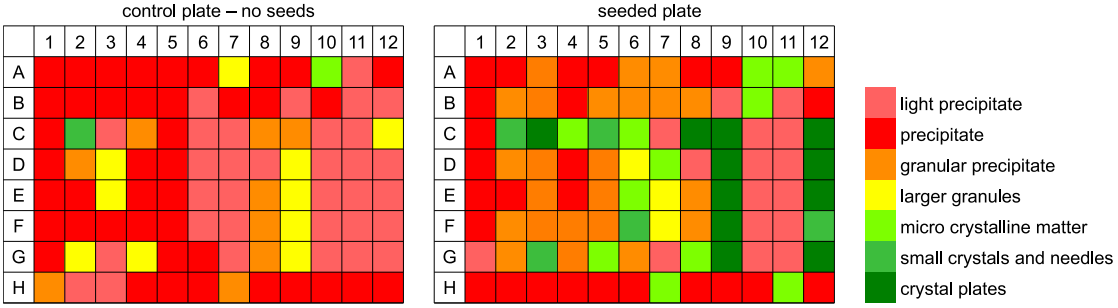
(a) E12, protein:precipitant 1:1, no seeds, light precipitate

(b) E12, protein:precipitant 1:1, seeded, crystal plates

(c) G9, protein:precipitant 1:2, no seeds, granular precipitate

Figure 2: Sitting drops of FAD-dependent oxidoreductase with MORPHEUS. Images are on the same scale. For the condition E12, despite only a light precipitate in the control plate (a), crystals were obtained in the seeded plate (b). The condition E12 consists of 12.5% w/v PEG 1000, 12.5% w/v PEG 3350, 12.5% v/v MPD, 0.1 M bicine/Trizma base pH 8.5 and 0.03 M of each ethylene glycol (di-, tri-, tetra-, penta-).

Table 1: Scored results of the crystallization experiments with MORPHEUS and FAD-dependent oxidoreductase for the control plate (left) and the seeded plate (right).



precipitate", "granular precipitate" and "crystal plates" are shown in Figure 2. The best result from three drops with the same reservoir solution was recorded in Table 1.

The comparison between the plates shows a strong positive effect of seeding on crystallization, similarly to D'Arcy *et al.* [3] and Ireton & Stoddard [4] for other proteins. Crystals were found in 16 conditions in the seeded plate, whereas there was a single hit (C2) in the control plate. Moreover, we did not observe the situation where a crystalline matter would be in the control and not in the seeded plate. In other words, the quality was only improved or remained similar with seeding but never not worsened.

In particular, very promising crystals were obtained in drops in rows C-G and columns 6, 9 and 12 in the seeded plate (Figure 2ab). Although the crystals grew without seeding in condition C2 (pH 6.5), the majority of the crystals in the seeded plate were found in the conditions with pH 8.5 – columns 9–12 (see the MORPHEUS formulation in Table 3 in Appendix). Surprisingly, crystals were obtained in all four different precipitant mixes used in MORPHEUS, representing polyethylene glycols (PEG) with a broad range of lengths from 1000 up to 20 000 daltons.

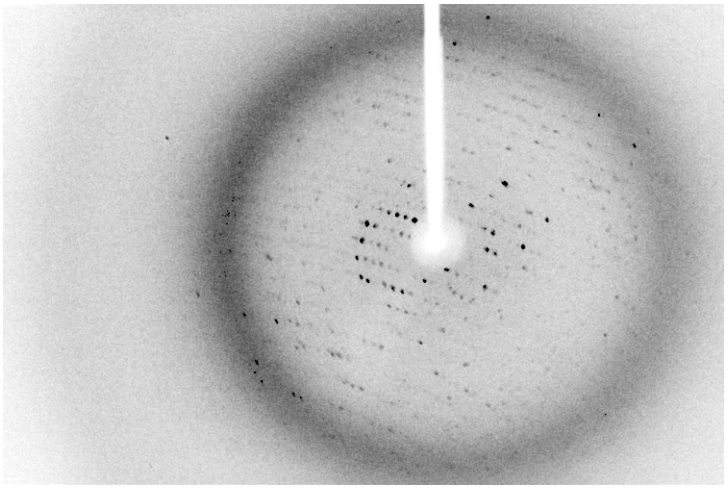


Figure 3: Diffraction pattern from a crystal that grew in the optimized and seeded MORPHEUS condition C2: 12% w/v PEG 8000, 24% v/v ethylene glycol, 0.06 M sodium nitrate, 0.06 M disodium hydrogen phosphate, 0.06 M ammonium sulfate, 0.1 M MES/imidazole pH 6.5.

Diffraction data from optimized crystals are promising, however, they are still too weak. The reflection spots are visible up to 2.9 Å (Figure 3). Nevertheless, the reflections are blurry, not well shaped, probably due to the plate shape of the crystal. Moreover, the diffraction exhibits severe anisotropy, resolution limits were estimated in the range from 2.7 Å to 2.9 Å [10]. Indicators of data quality have poor values (*e.g.* $R_{\text{meas}} = 0.215$); the data statistics are listed in Table 2 and attempts to build a structure model were not successful. Thus, further optimization of crystallization is necessary to collect diffraction data suitable for the solution of the phase problem and the structure determination.

Table 2: Statistics of diffraction data collected from the optimized crystal, a diffraction pattern from the related data set is shown in Figure 3.

Wavelength [Å]	1.3418		
Crystal-detector distance [mm]	158.0		
Number of images	3,103		
Oscillation per image [°]	0.2		
Space group	$P1$		
Unit-cell parameters [Å, °]	$a = 53.22$; $b = 95.91$; $c = 95.96$ $\alpha = 113.36$; $\beta = 102.90$; $\gamma = 103.08$		
Resolution range [Å]	46.74–2.90	46.74–9.61	3.04–2.90
Total observations	226,989	6,440	23,212
Unique reflections	35,245	959	4,621
Multiplicity	6.4	6.7	5.0
R_{merge}	0.198	0.073	0.798
R_{meas}	0.215	0.079	0.890
R_{pim}	0.083	0.029	0.385
$\text{CC}_{1/2}$	0.982	0.993	0.756
Mean $I/\sigma(I)$	7.4	16.3	1.6
Completeness [%]	99.9	99.2	99.4

Conclusion

Automated matrix microseeding [3, 4] was proven to be a technique dramatically improving the results of crystallization of FAD-dependent oxidoreductase using the MORPHEUS screen [7]. Crystals grew in conditions with different lengths of PEG: from 1000 up to 20 000 daltons, mostly in pH 8.5. However, the collected diffraction data from the optimized crystals exhibited multiple imperfections and could not be used for the solution of the protein structure. Further optimization of the crystallization of the FAD-dependent oxidoreductase, including seeding experiments, is required.

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Appendix: MORHEUS screen

Table 3: Schematic chemical formulation of the MORPHEUS screen [7].

Columns	Precipitant
1, 5, 9	10% w/v PEG 20000, 20% v/v PEG MME 550
2, 6, 10	10% w/v PEG 8000, 20% v/v ethylene glycol
3, 7, 11	10% w/v PEG 4000, 20% v/v glycerol
4, 8, 12	12.5% w/v PEG 1000, 12.5% w/v PEG 3350, 12.5% v/v MPD
Columns	**Buffer system**
1–4	0.1 M MES/imidazole pH 6.5
5–8	0.1 M MOPS/HEPES-Na pH 7.5
9–12	0.1 M bicine/Trizma base pH 8.5
Rows	**Salts or other additives**
A: Divalent cations	0.3 M magnesium chloride, 0.3 M calcium chloride
B: Halides	0.3 M sodium fluoride, 0.3 M sodium bromide, 0.3 M sodium iodide
C: NPS	0.3 M sodium nitrate, 0.3 M disodium hydrogen phosphate, 0.3 M ammonium sulfate
D: Alcohols	0.2 M 1,6-hexanediol, 0.2 M 1-butanol, 0.2 M (RS)-1,2-propanediol, 0.2 M 2-propanol, 0.2 M 1,4-butanediol, 0.2 M 1,3-propanediol
E: Ethylene glycols	0.03 M M of each ethylene glycol (di-, tri-, tetra-, penta)
F: Monosaccharides	0.2 M D-glucose, 0.2 M D-mannose, 0.2 M D-galactose, 0.2 M L-fucose, 0.2 M D-xylose, 0.2 M N-acetyl-D-glucosamine
G: Carboxylic acids	0.2 M sodium form., 0.2 M ammonium acet., 0.2 M trisodium citr., 0.2 M sodium potassium L-tartrate, 0.2 M sodium oxamate
H: Amino acids	0.2 M sodium L-glutamate, 0.2 M DL-alanine, 0.2 M glycine, 0.2 M DL-lysine HCl, 0.2 M DL-serine