Sveriges lantbruksuniversitet
Swedish University of Agricultural Sciences

The Faculty of Natural Resources and Agricultural Sciences

Optimization of CarG crystals

– a protein involved in the biosynthesis of 1-carbapen-2-em-3-carboxylic acid (Car); a β-lactam antibiotic.

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Department of Molecular Biology
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Biotechnology
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Abstract

Carbapenems are one of the most widely used groups of antibiotics because of their broad spectrum against both gram positive and gram negative bacteria. The biosynthesis of the simplest carbapenem, 1-carbapen-2-em-3-carboxylic acid (Car) is controlled by an operon of eight genes, *CarA-H*. CarG is believed to possess a function of self-resistance but the mechanism is not solved. The structure of CarG was solved a few years ago. The aim of this study was to improve the crystal quality to open up for further binding studies on CarG.

The protein was purified in a 4 step process; Ni^{2+}-column, desalting, MonoQ and gelfiltration. MonoQ was added to the previous protocol with hope to further purify the protein. Different screening protocols were used and as in earlier studies of CarG, $AmSO_4$ was a suitable precipitant. Crystals were obtained in a concentration range between 1.3-1.8 M $AmSO_4$ and pH 7.5 and 8, respectively. Using these conditions seeding techniques and cocrystallization with imipenem and meropenem were performed.

In this study I show that by adding MonoQ chromatography to the purification protocol bigger crystals and more single crystals can be obtained. Seeding, in drops with no substrate added, resulted in more square-shaped crystals but often much thinner crystals. Results from cocrystallization and seeding with imipenem show no improvement in crystal quality. Cocrystallization with meropenem gave clear crystals but was not further improved by seeding.

Content

1 Introduction

Antibiotic resistance is a huge topic in today's medical area. One of the biggest issues is the prescribing of antibiotic before a full diagnose and analysis of the cause of the symptoms has been performed. Antibiotics are sometimes given to patients with no bacterial infection or the wrong antibiotic is prescribed. This will lead to bacteria getting resistant to many antibiotics and these bacteria are easily spread causing minor infections to be life threatening. Carbapenems belong to a class of β-lactam antibiotics that are clinically used widely because of their broad spectrum activity against both gram positive and gram negative bacteria (Kropp et al., 1980) and also because of their resistance to β-lactamases. The first carbapenem to be isolated from *Streptomyces cattleya* was Thienamycin (Kahan et al., 1979) but as it turned out it was too unstable to be suitable for clinical use. Instead Imipenem, a derivative to Thienamycin, was chemically produced to be more stable and is today clinically used successfully (Kahan, 1983).

1-carbapen-2-em-3-carboxylic acid (Car) is the most simple carbapenem (figure 1a) and was first found and isolated from *Erwinia carotovora* and *Serratia marcescens* in 1982 (Parker et al., 1982). The biosynthesis is regulated by an operon of eight genes; *CarA-H*, which in turn are regulated by *CarR* (McGowan et al., 1995). These genes were sequenced and analyzed by McGowan and coworkers (1996) and revealed that *CarA* and *CarC* have homologues in the cluster of genes that are responsible for the expression of clavulanic acid in *Streptomyces clavuligerus*. Clavulanic acid has also a β-lactam core ring structure (figure 1b) and acts as an inhibitor of β-lactamases. *CarR* is a LuxR-type transcriptional activator that by concert with N-acyl homoserine lactones (N-AHL) induces the transcription of the carbapenem biosynthetic gene cluster (McGowan et al., 1995; Thomson et al., 2000). These N-AHL can act as signaling molecules and through cell density signal to neighboring cells and coordinate their gene expression. This is called quorum sensing control (Bainton et al., 1992; Thomson et al., 2000; Welch et al., 2000).

The structures of CarA and CarC have been solved to 2.3 and 2.4Å, respectively (Clifton et al., 2003; Miller et al., 2003). Both are required for the biosynthesis together with CarB (McGowan et al., 1997; Sleeman et al., 2005). CarD and CarE also have a function in the biosynthesis while CarF and CarG together seem to have a self-resistant function (McGowan et al., 1997). CarF and CarG knockouts show sensitiveness to carbapenem. The function of CarH is still unknown.

The structure of CarG has previously been solved to 2.3Å (K. Valegård, personal communication) but no binding studies, to investigate the self-resistance function of CarG have been done so far. Troubles with intergrown crystals in this study would be problematic in further binding studies. The three-dimensional structure contains two anti-parallel β-sheets

with a total of twelve β-strands and with one α-helix in the C-terminus (figure 2).

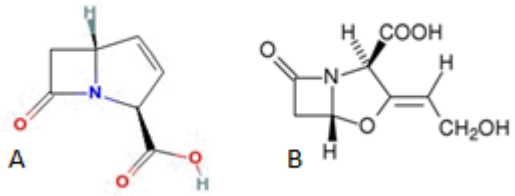

Figure 1. *Molecule structure of (A) Car and (B) Clavulanic acid*

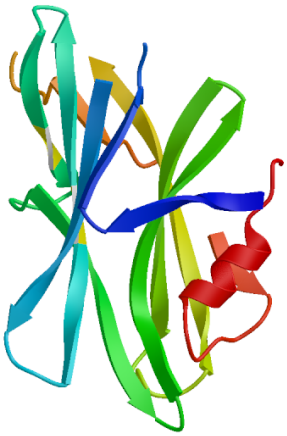

Figure 2. *Three-dimensional structure of CarG solved to 2.3 Å.*

Since carbapenems are important for clinical use it is of great importance that the production of the antibiotic is both cost and time effective. With more knowledge about the synthesis and function of the proteins encoded by the carbapenem cluster the production process can be developed to be more effective. It could perhaps also lead to the development of even more effective new antibiotics.

This study will focus on CarG and of improving the crystal quality. This will be done by refining the purification protocol and by adding possible substrates to the protein that could stabilize the protein. In this case crystal quality will be judged by eye.

2 Materials and Methods

2.1 Cultivation

CarG from *Erwinia carotovora* was cloned to the pET-30a-c(+) vector by Dr. Tom Taylor. The plasmid was transformed into *E. coli* BL21(DE3)

cells. Glycerol stock solutions with transformed *E. coli* BL21(DE3) cells were spread on LB plates with Kanamycin to grow. Several colonies were inoculated in an overnight culture (25ml 2TY media and 0.1 mM Kanamycin) at 37ºC with a shake of 170 rpm. Fifteen ml of the overnight culture were transferred to 500 ml 2TY media with 0.1 mM Kanamycin and placed on a shaker at 37ºC. When the culture had an OD_{600} between 0.6-0.8 the temperature was reduced to 18ºC. After 30 min IPTG (0.1 mM) was added to the culture to induce expression of CarG. The incubation continued for 16h at 18ºC.
The cells were harvested by centrifugation on a Sorvall SLA-1500 rotor for 15 min, 4ºC, 10000 rpm. The cells were divided in two parts. The first part, 6g cells, was used immediately and resuspended in 50 ml 0.02 M sodium phosphate pH 7.4, 0.5 M NaCl , one complete EDTA-free protease inhibitor cocktail tablet (Roche Diagnostics GmbH, Mannheim, Germany), 2 mM MgCl2 and 10 µl Benzonase Nuclease (Novagen, Denmark). Cell lysis was performed using a cell disrupter (Constant Cell Disruptor Systems, Daventry, UK) and diluting to approximately 70 ml with 0.02 M sodium phosphate pH 7.4, 0.5 M NaCl. The cell lysate was cleared by centrifugation in a Sorvall SS-34 rotor (30 min, 4ºC , 20000 rpm).
The cells that were not used immediately were centrifuged on Biofuge Primo at 6000xg in 30 min and the pellet was then frozen at -20ºC. When thawed, it was treated the same way as the fresh cells.

2.2 Purification

A 5 ml HiTrap Chelating HP column (GE Healthcare Bio Sciences AB, Uppsala, Sweden) loaded with Ni^{2+} was used for the first purification step. The cell lysate was applied and the column was washed with 0.02 M sodium phosphate pH 7.4, 0.5 M NaCl. A gradient of 0.0-0.5 M imidazole was used to elute the poly-His-tagged CarG protein. 2 ml fractions were collected and A_{260}/A_{280} was measured for the peak fractions. Fractions with values below one were pooled together. The sample was run through a 53 ml High prep 26/10 desalting column (GE Healthcare Bio Sciences AB, Uppsala, Sweden) with 0.1 M HEPES pH 7.5 buffer. The desalting column clears the sample from imidazole and salt. As an extra step, in the hope to improve the purification, MonoQ chromatography was performed. The protein fractions from the desalting process were pooled and loaded onto the MonoQ 10/100 GL column (GE Healthcare Bio Sciences AB, Uppsala, Sweden) and the column was washed with 0.1 M HEPES pH 7.5. The protein was eluted with a gradient of NaCl from 0.0-0.5 M. Fractions, corresponding to the eluted protein, were run on a SDS gradient gel (8-25%). The gel was stained with either Coomassie blue or AcquaStain. Fractions with satisfactory purity were then pooled together. The protein sample was applied to a HiLoad 26/60 Superdex 200 prep grade column (GE Healthcare Bio Sciences AB, Uppsala, Sweden) and washed through with 0.1 M HEPES pH 7.5. The purified protein was concentrated to approximately 10 mg/ml in a

Vivaspin20 (Sartorius Stedim Biotech, Germany) and stored at -20ºC for crystallization. The purification process was performed three times and each time the pooling of fractions was done a bit stricter. In the last purification the peak fractions from MonoQ chromatography were divided in two samples, 1 and 2. Both samples were preceded to the gel filtration separately. They were later compared in a crystallization experiment.

2.3 Crystallization

At first, different screening protocols were used such as JCSG , pH clear and $AmSO_4$ Suite from Qiagen, Crystal screen from Hampton and Proplex from Molecular dimensions. They were all performed with sitting drop vapor diffusion: 1µl protein (10 mg/ml) and 1µl reservoir solution were mixed.

From a previous experiment a set up of different pH and concentration of $AmSO_4$ were elaborated to grow crystals (K. Valegård, personal communication), see figure 3. These crystals were used for seeding. Two µl protein (10 mg/ml) were mixed with 2 µl reservoir solution using the hanging drop vapor diffusion technique.

When comparing the two separated samples from the last purification, the crystallization conditions in figure 3 were used.

	1	2	3	4	5	6
A	1.3 M $AmSO_4$ 0.1 M HEPES pH7.5	1.4 M $AmSO_4$ 0.1 M HEPES pH7.5	1.5 M $AmSO_4$ 0.1 M HEPES pH7.5	1.6 M $AmSO_4$ 0.1 M HEPES pH7.5	1.7 M $AmSO_4$ 0.1 M HEPES pH7.5	1.8 M $AmSO_4$ 0.1 M HEPES pH7.5
B	1.3 M $AmSO_4$ 0.1 M HEPES pH7.5	1.4 M $AmSO_4$ 0.1 M HEPES pH7.5	1.5 M $AmSO_4$ 0.1 M HEPES pH7.5	1.6 M $AmSO_4$ 0.1 M HEPES pH7.5	1.7 M $AmSO_4$ 0.1 M HEPES pH7.5	1.8 M $AmSO_4$ 0.1 M HEPES pH7.5
C	1.3 M $AmSO_4$ 0.1 M HEPES pH8.0	1.4 M $AmSO_4$ 0.1 M HEPES pH8.0	1.5 M $AmSO_4$ 0.1 M HEPES pH8.0	1.6 M $AmSO_4$ 0.1 M HEPES pH8.0	1.7 M $AmSO_4$ 0.1 M HEPES pH8.0	1.8 M $AmSO_4$ 0.1 M HEPES pH8.0
D	1.3 M $AmSO_4$ 0.1 M HEPES pH8.0	1.4 M $AmSO_4$ 0.1 M HEPES pH8.0	1.5 M $AmSO_4$ 0.1 M HEPES pH8.0	1.6 M $AmSO_4$ 0.1 M HEPES pH8.0	1.7 M $AmSO_4$ 0.1 M HEPES pH8.0	1.8 M $AmSO_4$ 0.1 M HEPES pH8.0

Figure 3. A scheme of conditions elaborated from earlier studies on CarG. These conditions were used as a first step in optimizing the crystallization and the crystals were also used for seeding.

2.3.1 Cocrystallization with imipenem and meropenem

Cocrystallization with 0.5 mM imipenem (Sigma-Aldrich, USA) and 0.5 mM meropenem (Sigma-Aldrich, USA) was carried out by hanging drop vapor diffusion with the same conditions as in figure 3 and using a protein concentration of 10 mg/ml.

2.3.2 Seeding

Seeding was performed in drops with no substrate added but also on cocrystallization drops with imipenem and meropenem. Crystals were collected, crushed and resuspended in 100 µl of the reservoir solution. This seeding solution was then diluted (10 & 100 times) and spread with a cat whisker on one day old hanging drops.

2.3.3 Additives

In one part of the experiment additives were added to the crystallization conditions hoping to get higher quality crystals. Additive Screen Formulation (Hampton Research) was used with sitting drop vapor diffusion with the reservoir solution 1.6 M $AmSO_4$, 0.1 M HEPES pH 8.

3 Results

3.1 Cultivation and Purification

E. coli BL21(DE3) expressing His-tagged CarG was cultivated in 2TY media and kanamycin. It was then purified in a four step process with a Ni^{2+}-column, desalting, MonoQ and gelfiltration.

After the first purification step (Ni^{2+}-column) the protein samples were run on a SDS-gradient gel to verify the purity. The wells clogged of all the protein and the migratory protein was just a smear, figure 4. Because of this it was difficult to judge the purity of the samples but since A_{260}/A_{280} was measured the fractions with most protein and less nucleotides could be pooled together. The ratio between A_{260} and A_{280} is a measurement of how much protein and DNA/RNA there is in a sample. The curve from MonoQ purification showed a split peak (figure 5). The split peak indicates that there is different folding of CarG with a slightly difference in charge and size. The peak-fractions were run on a SDS-gradient gel to verify the purity. Figure 6 shows one of these SDS-gradient gels where a thick band corresponding to the CarG proteins was seen at just below 30 kDa. Lane 3 and 4 seem to have one band while in Lane 5 a thinner band starts to be visible. Protein corresponding to Lane 3 and 4 were pooled (sample 1) while sample corresponding to Lane 5 was held alone (sample 2). By strictly pooling the highest peak the more rare folded proteins gets excluded. Nucleotide that is eluted later is also excluded.

Figure 4. *SDS-gradient gel analysis (8-25% acrylamide) after HiTrap Chelating HP chromatography. Stained with Coommassie Blue. Lane 1-7: Protein from peak-fractions. Lane 8: Low molecular weight marker. Size from the top: 97.0, 66.0, 45.0, 30.0, 20.1, and 14.4 kDa.*

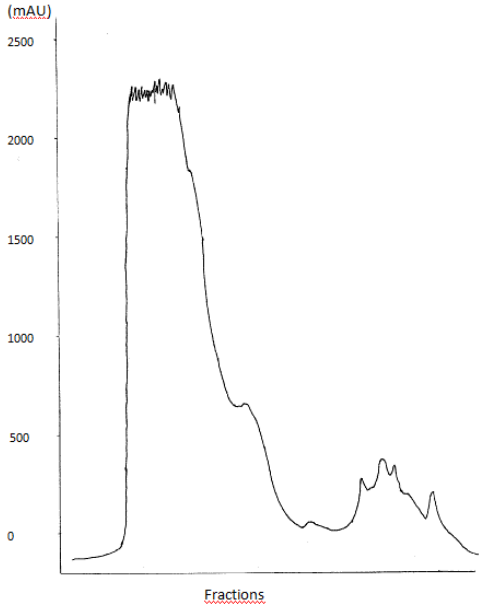

Figure 5*. Elution curve from MonoQ chromatography*

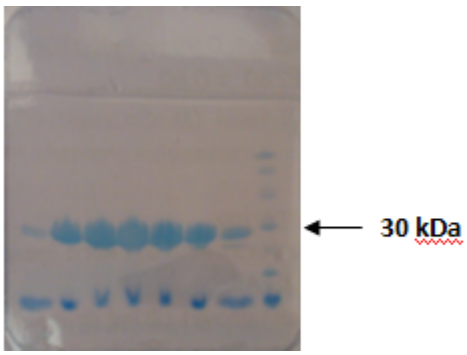

Figure 6. *SDS-gradient gel analysis (8-25% acryl amid) after MonoQ chromatography. Stained with AcquaStain. Lane 1-7: Continues samples from the elution. Lane 3 and 4 are the absolute peak fractions and 5 is the slope of the peak. Lane 8 is a low molecular weight marker (from the top 97.0, 66.0, 45.0, 30.0, 20.1, and 14.4 kDa). The arrow indicates the size of the marker-band closest to the expressed protein.*

3.2 Crystallization

A lot of different methods were tried to optimize and get better quality crystals. None of the big screening suites (JCSG, pH clear, AmSO4 Suite, Crystal screen and Proplex) gave any results that could be further used except $AmSO_4$ that was known from before to work for obtaining CarG crystals. The comparison of sample 1 and 2 from the last MonoQ purification did not show much difference in crystal appearance (figure 7A-B) but they both showed better crystals then from earlier purification processes (figure 7A-C). More single crystals were observed and they were also more square-shaped and bigger.

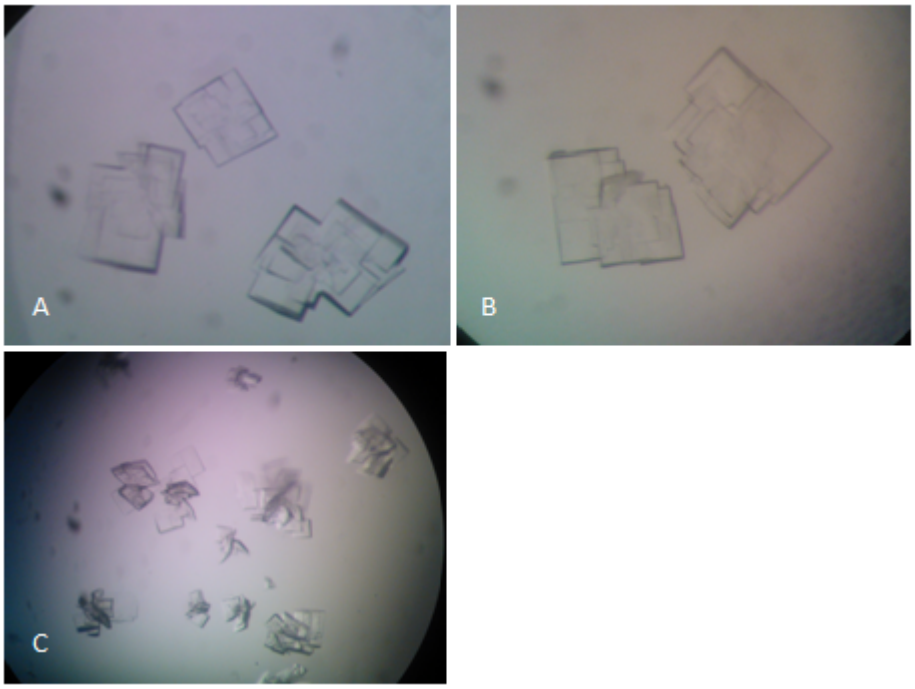

Figure 7. *Crystals obtained in $AmSO_4$.* ***(A,B)*** *These crystals are obtained from protein in the same purification process.* ***(A)*** *Protein from the top peak fractions with only one band visible in the SDS-gradient gel (sample 1). Reservoir solution: 1.6 M AmSO4, 0.1 M HEPES pH 7.5.* ***(B)*** *Protein from peak fractions but where two bands were visible in the SDS-gradient gel (sample 2). Reservoir solution: 1.6 M AmSO4, 0.1 M HEPES pH 8.* ***(C)*** *Crystals obtained from a second purification process where the pooling of peak fractions was made less strict. Reservoir solution: 1.6 M AmSO4, 0.1 M HEPES pH 7.*

3.2.1 Cocrystallization and Seeding

Crystallization protocol in figure 3 was used to grow crystals for seeding and it was also used as a control to see whether the seeding worked or not. It was clear that the protein needs higher $AmSO_4$ concentrations to form crystals without seeding since no crystals grew in $AmSO_4$ concentration between 1.3-1.5 M. After performing seeding, crystals grew even in the lower concentrations. When comparing non-seeded crystals with seeded crystals the seeded crystals are often more square-like without that lobed appearance but they are also much thinner, see figure 8A-C. Figure 8D is an enlargement of a crystal from the most diluted seeding solution.

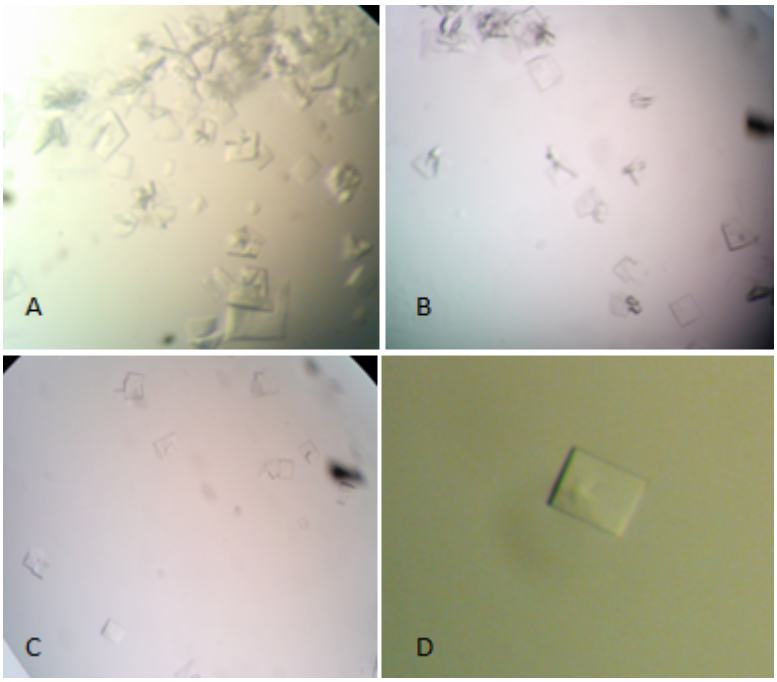

Figure 8. *Seeded crystals obtained in 1.6 M $AmSO_4$, 0.1 M HEPES pH8. (A) Seeded with stock solution. (B) Seeded with a 10^{-1} dilution. (C) Seeded with a 10^{-2} dilution (D) An enlargement of a crystal from figure 8C.*

Crystals co-crystallized with imipenem shows a mix of square-shaped single crystals and intergrown crystals (figure 9A). Using the seeding technique the crystals got smaller and less single crystals were observed (figure 9B). The cocrystallization with meropenem gave clear crystals but mostly non-single crystals, seen in figure 9C. After seeding (figure 9D-E) more crystals were obtained but mostly thinner and still not many single crystals.

Figure 9F shows what type of crystal that was used for seeding. This crystal was collected from condition: 1.6 M $AmSO_4$, 0.1 M HEPES pH 8.

Common for all crystallization methods the crystals with the highest quality were found, with few exceptions, in the conditions with 1.6 M $AmSO_4$ with no difference between pH 7.5 and pH 8.0.

3.2.2 Additives

What was notable was that with cesium chloride and guanidine hydrochloride needle-shaped crystals were formed. This has not been observed before for CarG. In other aspects it did not show any improvement.

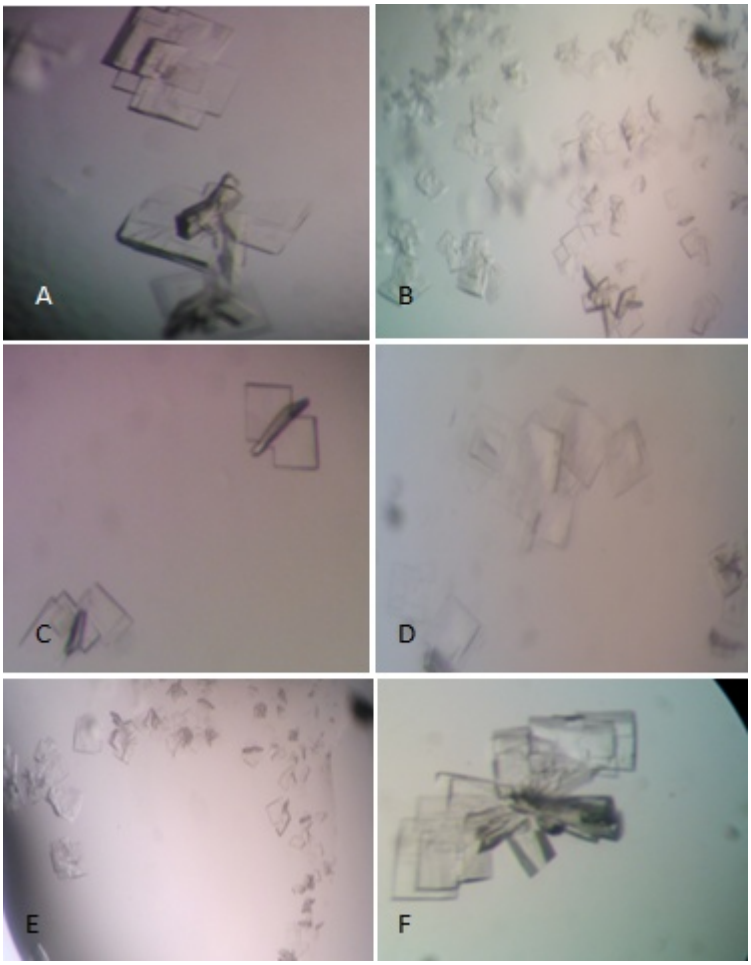

Figure 9. *Seeding and Cocrystallization with imipenem(0.5 mM) and meropenem (0.5 mM). (A) Cocrystallization with imipenem (1.6 M $AmSO_4$, 0.1M HEPES pH 7.5 (B) Seeding and cocrystallization with imipenem (1.6M $AmSO_4$, 0.1M HEPES) (C) Cocrystallization with meropenem (1.6M $AmSO_4$, 0.1M HEPES pH 8) (D,E) Seeding and cocrystallization with imipenem (1.3 M $AmSO_4$, HEPES pH 7.5; 1.6 M $AmSO_4$, 0.1 M HEPES pH 8). (F) Crystal that was used for seeding (1.6 M $AmSO_4$, 0.1 M HEPES pH 8)*

4 Discussion

To be able to perform binding studies on protein crystals they need to be of high quality. The structure of CarG was solved in 2008 but no binding studies have been performed. The purpose of this study was to increase the number of high quality crystals to get one step closer to solve the mechanism of CarG. Qualities that I looked at were the shape of the crystal, the thickness and that it is a single crystal.

When the three-dimensional structure of CarG was solved the protein was expressed in *E-coli* BL21(DE3) with pET-30a-c(+) vector (same as in this study) and was purified with Ni^{2+}-column, desalting-column and gelfiltration. The crystal were grown in 1.6 M $AmSO_4$, 0.1 M HEPES pH 8 using the hanging-drop vapor diffusion technique (K. Valegård, personal communication)

By adding MonoQ chromatography to the purification protocol I hoped to further purify the protein sample. MonoQ is a common method in purification of protein and has shown good results in other experiments (Joshi & Puri, 2005; Aggarwal *et al.*, 2011)
I was able to eliminate nucleotides by adding this step but looking at the SDS-gradient gel (figure 6) the band of CarG was smeared and it was hard to distinguish if there was more than one band. Since some of the peak fractions were separated after MonoQ chromatography I was able to see if crystals would appear different with the different fractions. But as it turned out the crystals appears similar in shape and in the speed of growing. However these crystals were of better quality than crystals grown from earlier purification where I was less strict when choosing what fractions to proceed with. This shows that purification step like MonoQ can improve the purity but also that the choosing of fractions are important.

The seeding worked in the way that crystals were formed even in the conditions where no spontaneous crystallization was observed. With the diluted seeding solutions single crystals where observed but they were often very thin. The seeding could be further explored by choosing a less intergrown crystal to crush. Many times the seeded protein forms crystal similar to the seeds. That might be counteracted if the seeds are as small as possible. Seeding has for some proteins shown to be a good alternative when improving crystal quality (McVey et al., 1997; Van de Water et al., 2011). The fact that seeding on drops with imipenem caused smaller and less single crystals could indicate that CarG crystals grows better without too much disturbance.

Often you start binding studies by trying substrates found in the biosynthesis pathway. An enzyme with the possibility to bind to a substrate homologue or an inhibitor could increase its stability. In this case the cocrystallization with imipenem and meropenem was an attempt to make CarG more stable and more easily form crystals. Meropenem is like imipenem a carbapenem but with differences in molecule structure, see figure 10.

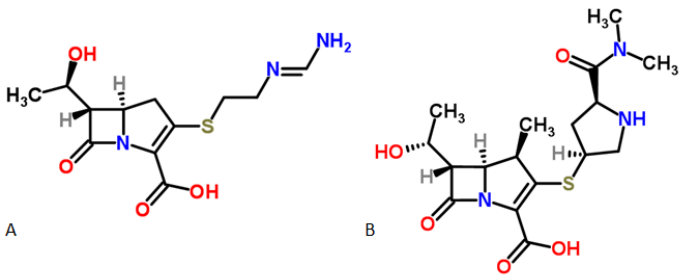

Figure 10. *The molecule structure of (A) imipenem and (B) meropenem.*

The cocrystallization with meropenem showed clear crystals but still some signs of intergrowing. The clear crystals could still indicate that a binding

has occurred. However, this could only be examined by structure determination work.

Crystallization is not an easy task and there are several parameters that have to be taken in consideration; purification protocol, temperature in which the purification is performed, the buffer that is used, storage temperature, how the sample is thawed after storage, different precipitants, what vector is used and many more. In this case where the protein is cloned into a vector with a His-tag one alternative to optimize for crystallization is to cleave the His-tag off after purification. Depending of the size of the His-tag it might disturb the crystallization by flipping back and forth or even disturb the active site of a neighboring protein (Tseng *et al.*, 2011; Darmon *et al.*, 2012). Another parameter that could be elaborated is the protein concentration. The protein might more easily form crystals in either higher or lower protein concentrations.

The needle shaped crystals from the additive screening is another part that can be further studied. Reservoir conditions can be optimized and single needles can be picked for x-ray diffraction.

Important to address is that the crystal quality in this case has not been examined by x-ray but only visually. Crystals that look fine by eye are not always the crystals that give the best diffraction. With a small x-ray beam it can be directed to a part of the crystal that is of better quality. Intergrown crystals can also be useful if it is possible to divide them in pieces and one single piece with high quality can be collected for x-ray analysis.

4.1 Conclusion and further work

An optimized purification protocol gave crystals with visually higher quality. Cocrystallization with meropenem improved the quality by generating clear and less intergrown crystals. In some cases seeding could add positive effect to the crystallization. The fact that less intergrown crystals were obtained opened up for binding studies. In further studies crystals will be soaked with meropenem, imipenem and acyl homoserine lactone. Diffraction data will be collected at European Synchrotron Radiation Facility (ESRF) in Grenoble, France, in June.

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