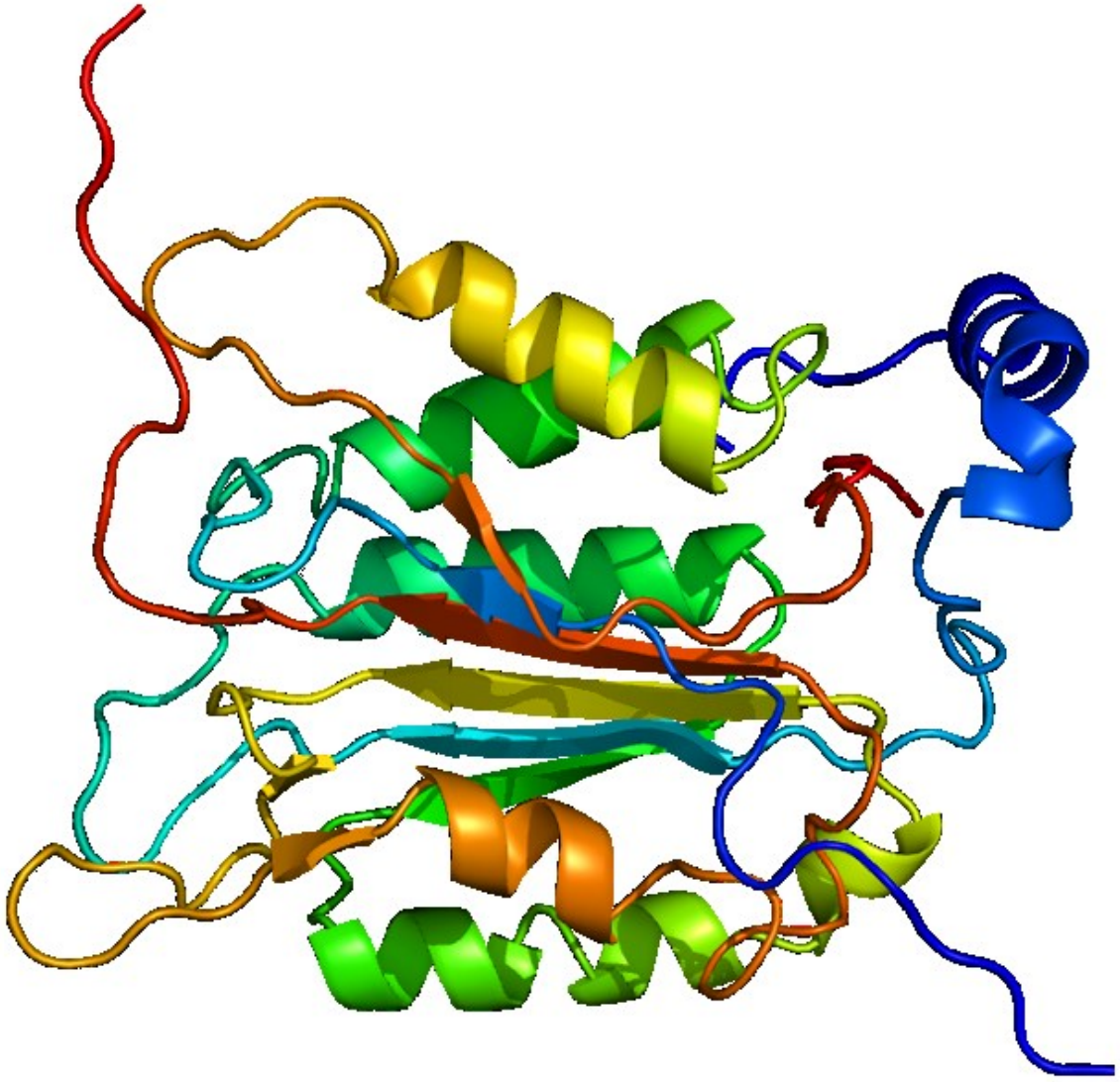
Experimental procedures for synthesis, refolding and crystallization of Caspase-1.



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Contents

Abstract

Caspase-1, also known as IL-1-converting enzyme (ICE), is a key protein in the inflammatory process, and an interesting target to prevent undesired inflammation. Caspase-1 is a protease responsible for activating pro-inflammatory cytokine IL1ß by cleaving pro-IL1ß into active inflammatory molecule IL1ß. In addition, Caspase-1 causes rapid cell death in macrophages that contain intracellular bacteria, which induces response against bacterial infections. In summary, Caspase-1 is a key inflammatory mediator for the host response to infection, injury and disease.

Though the inflammatory response is vital to the host, IL1ß-driven inflammation often has a disastrous effect during disease and/or brain injury, conditions that have limited clinical options. This makes Caspase-1 an interesting therapeutic target to mitigate autoimmune response[1]. Previously discovered inhibitors of Casapase-1 bind covalently to the protein. This covalent nature of binding resulted in toxicity and did not pass clinical trials[2].

To make the protein suitable for testing against possible leads to inhibit its function, Caspase-1 can be produced through protein expression, refolding and crystallization. Because conditions based on public protocols were hard to establish the goal of this project was to find a method to express Caspase-1 from its gene, refold it into its proper conformation and co-crystallize with a number of possible lead compounds (ALC150, ALC129, VX765 and AD4). Compounds used for co-crystallization have been produced by A. Chandgude in the drug design lab of Groningen University.

In this project Caspase-1 was expressed, purified and folded into its natural shape. Correct folding was demonstrated by circular dichroism. Caspase-1 was crystallized with & without compounds, diffraction data has been collected and structure solutions are underway.

Introduction

The Caspase-1 pathway

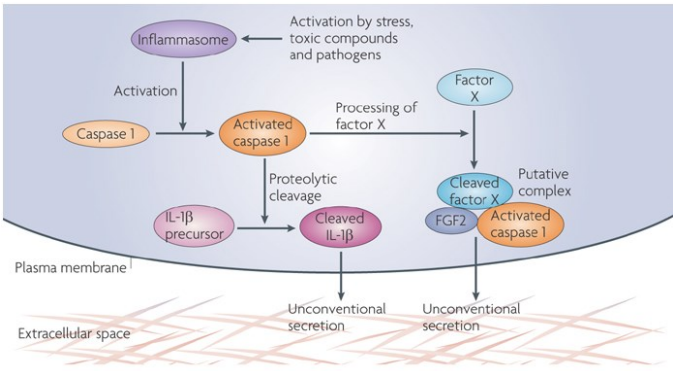


Figure 2: A schematic view of the activation of pro-IL1ß [4]

Caspase-1 is a part of the immune system that involves inflammation of cells after injury or disease. The cell inflammation is a part of the innate immune system in response to pathogen- and damage associated molecular patterns (PAMPs and DAMPs). PAMPs and DAMPs are mediated by pattern recognition receptors (PRRs) on macrophage membranes to control gene expression of inflammatory proteins. In order to respond quickly, pro-IL1ß and inactive Caspase-1 are already present in the cell. Caspase-1 is activated through the formation of a protein complex called the inflammasome; a protein complex formed by PRR receptors of the NLR (NOD-like receptor) or AIM2 (absent in melanoma 2) [3] [4] [5].

When PAMPs or DAMPs are present, they form complexes with ASC (apoptosis-associated speck-like protein containing a CARD)[6]. The CARD domain (caspase activation and recruitment domain) recruits pro-Caspase-1 and when formed to the complex ASC activates pro-Caspase-1 by binding the proteins together resulting in a cleaved portion of the protein that result in its activation. Once activated Caspase-1 cleaves pro-IL1ß into its active form IL1ß (Figure 1)[6].

The inflammatory process induced by IL1ß is vital to the host to provide protection from infection, injury or disease. However, during disease, in IL1ß induced immune response often has negative consequences. And undesired activation of Caspase-1 can lead to tissue damage and brain dysfunction[5] [6]. These reasons make Caspase-1 an interesting target for small molecules to inhibit.

Caspase-1 inhibitors have already been discovered. Examples are Pralnacasan, VX765, reversible inhibitors used for type II collagen-induced arthritis, and Emricasan an irreversible pan-caspase inhibitor investigated for the treatment of chronic HCV infection and liver transplantation rejection. Unfortunately these lead compound haven't passed clinical trials; Pralnacasan induced liver toxicity, VX765 has no recent development in treatment for inflammatory disorders, and Emricasan induced ameliorated liver fibrosis by inhibiting hepatocyte apoptosis[7].

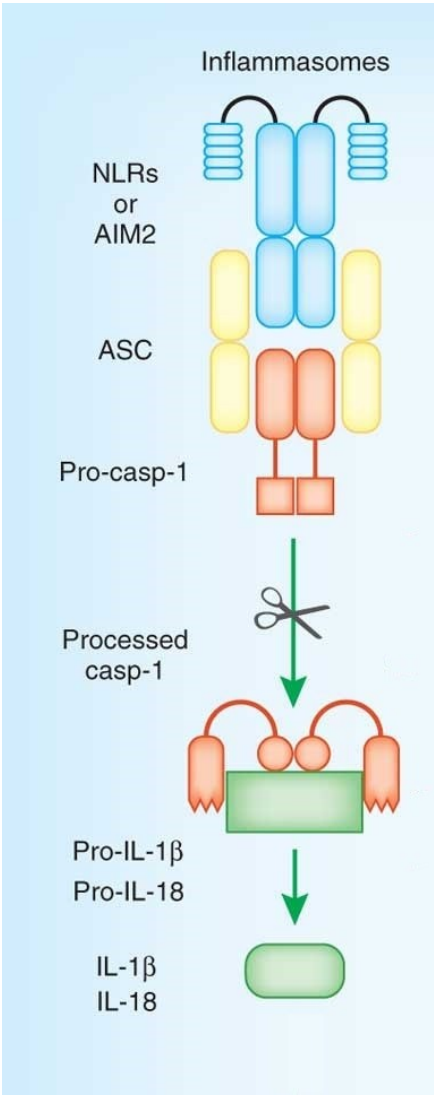


Figure 1: Pathway of Casapse-1 and IL-1ß[6]

Protein details

The gene

The location of the gene coding for Caspase-1 is located at human chromosome 11q22.2-q22.3. Six alternatively spliced forms of caspase-1 have been identified in Homo sapiens (Figure 3). Among these forms alpha, beta, gamma and zeta genes are able to form active caspase-1 proteins to induce inflammation[) 10]. In nature the most dominant form is the alpha variant containing 1364bp. After splicing the gene has a size of 404bp. Tumor suppressor genes like p53, p73 and SP1 activate transcription by binding to the promotor that is sited 550bp upstream of the chromosome. Pro-Caspase-1 is highly expressed in leukocytes, monocytes and epithelial cells. CASP-1 mRNA levels are high in ischemic tissue cells[) 11) 12) 13) 14) 15].

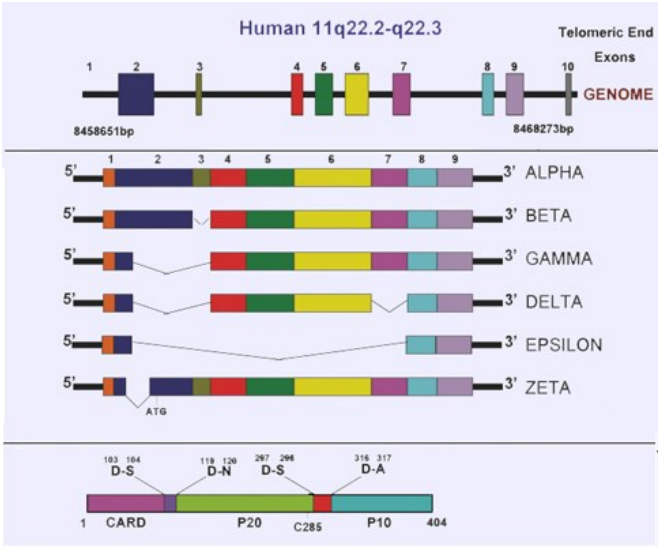


Figure 3: Schematic presentation of the gene coding (top), the splice variants (middle), and domain organization (bottom) of Caspase-1[) 8) 10]

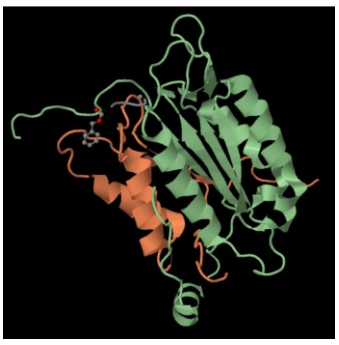


Figure 4: Casdpase-1 a heterodimer of p10 (orange) and p20 (green) subunits[) 11]

Expression in human cells

Caspase-1 is expressed in almost all cells, but is present at a higher concentration in innate immune cells, such as macrophages. The expression of pro-Caspase-1 is induced by various stimuli, eg. Microbial infections (*Mycobacterium avium, Salmonella typhimurium, Legionella pneumophila, Bacillus anthracis, Francisella tularenis* and bacterial LPS), cytokines (IFN-γ), growth factors (TGF-ß) and DNA damaging agents[) 10]. The expressed protein is cleaved into 2 subunits (p10 and p20) and folded together into pro-caspase-1. This proenzyme contains the active Capsase-1 with a CARD domain attached to it. This CARD domain can interact with other proteins that contain a CARD domain like ASC, RIP2 and NLRC4. These proteins are part of the inflammasome complex formed after activation of PRR receptors[) 10) 12) 13) 14]. A CARD-CARD interaction will take place between the inflammasome and pro-caspase-1, after the CARD domain is removed, a heterodimer of p10 and p20 subunits is formed. These heterodimers form an active homodimeric complex of 2 caspase-1 molecules (Figure 5).

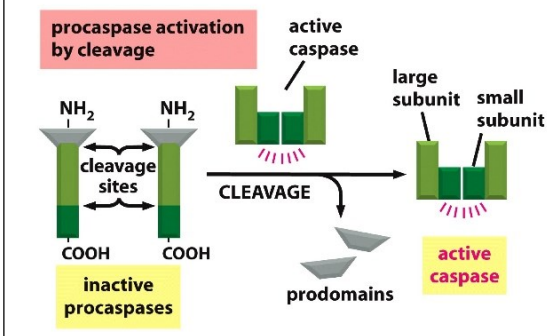


Figure 5: Acitvation of pro-Caspase-1, the protein get cleaved after CARD-CARD interaction. The CARD domain (grey) gets removed, the protein get cleaved into p10 (dark green) and p20 (light green) subunits and folded to an active complex of 2 caspase-1 molecules[) 12]

Activation

Once activated, the homodimeric caspase-1 complex is able to cleave several cytokines, mainly pro-IL-1ß and pro-IL-18. The catalytic site is formed by amino acid from both p10 and p20 subunits with the active cysteine located within the p20 subunit. Both are key proteins in several inflammatory

responses. Without Caspase-1, both IL1ß and IL18 cannot be activated, which could potentially dramatically suppress inflammation[11].

Approach

The catalytic site is formed by amino acids from both P20 and P10 subunits, with the active cysteine (Cys285) located within the P20 subunit. Molecules that have been discovered to inhibit Caspase-1 bind covalently to the Cys285 in the p20 subunit. These molecules could pass clinical trial due to this covalent nature which causes toxicity[2,16,17]. A noncovalent approach to Caspase-1 is used to avoid these problems. In this experiment a modified caspase-1 protein is produced that lacks the Cys285 amino acid in the p20 subunit preventing co-crystallization with molecules that rely on covalent binding with Cys285[18]. Molecules used for co-crystallization are known molecules ALC150 and VX765[18,19,20] and 2 new molecules, produced by A. Chandgude: ALC129 and AD4. All four molecules are displayed in Table 1. The chemical structures of ALC-129 and AD4 cannot be shown as these molecules under development by the Drug Design department of University of Groningen.

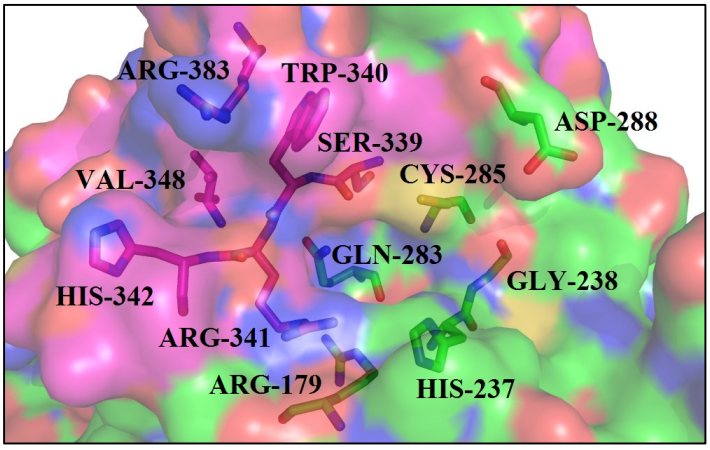
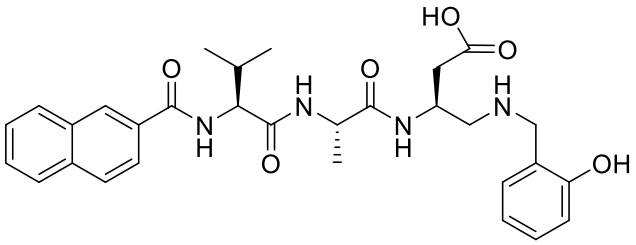
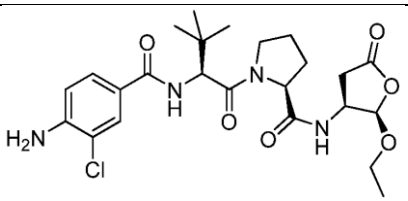


Figure 6: Pymol[21] image of the peptides around the active site of Caspase-1.

Table 1: Molecules used for co-crystallization with Caspase-1[18,19,20]

ALC-150	
ALC-129	Chemical Formula: C24H23ClN6O3 Molecular Weight: 478,93
VX765	
AD4	Chemical Formula: C21H26N6O4S Exact Mass: 458,1736

Synthesis

General procedure

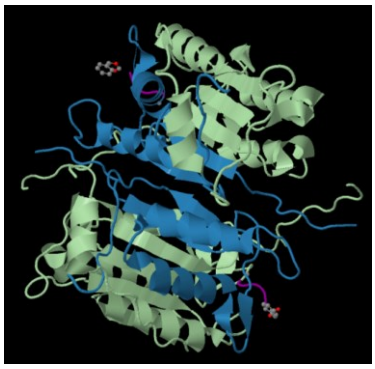


Figure 7: The homodimeric complex of 2 activated Caspase-1 molecules, showing both p10 subunits in blue and p20 subunits in green[11]

It is challenging for a bacterial cell line to make mature caspase-1n in the same way as the human cells. Recombinant expression of pro-caspase-1 would require subsequent treatment with purified Caspase-1 restriction enzyme and chaperone protein. However, the literature[14][15] has demonstrated that soluble procaspase-1 is available by combining denatured p10 and p20, followed by refolding it through shock dilution. To produce Casp-1 in vitro, 2 separate plasmids containing genes for subunit p10 and p20 were ordered and expressed separately. This way the Caspase-1 is available in its active form without the need to remove the CARD subunit by an inflammasome complex or compound with similar properties. Having both subunits produced separately also excludes the need of a restriction enzyme to cleave the pro-caspase into both subunits. Both subunits were merged together in a 1:1 molar ratio in denatured form and the refolding process was performed by shock dilution. Hanging drop crystallization methods were used in order to co-crystallize the protein with possible lead compounds[12][13][14][22].

Subcloning

Prior to protein expression the p10 and p20 genes were transferred from the commercially obtained pEX-A2 vector (MWG Biotech)[23][24] into a kanamycin resistant pETM11 vector[25] through digestion with HindIII[26] and NcoI[27] restriction enzymes (Figure 8). After ligation the gene was proliferated in competent DHT-Turbo cells in a LB-agar environment containing kanamycin. After ligation the gene was amplified in DHT-Turbo cells in a LB-agar environment containing kanamycin. After extraction the plasmid was stored at -20^0 C [28][22].

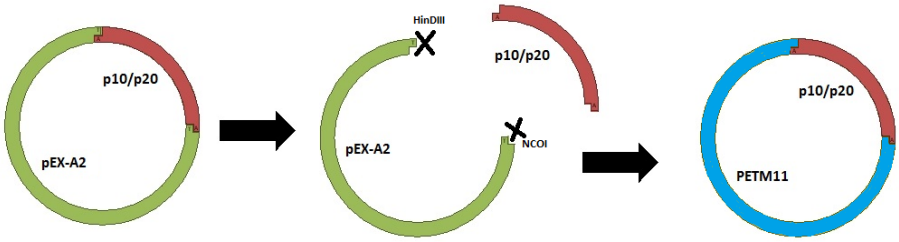


Figure 8: Visualization of the subcloning process, where the gene of both subunits get removed from pEX-A2 by digestion with HinDIII and NCOI restriction enzymes and ligated with the pETM11 vector

Expression

Competent BL21*(DE3) pRARE2[23] cells were used to express both proteins from the plasmid containing the genes in the pETM11 vector. To activate the expression of the protein Isopropyl β-D-1-thiogalactopyranoside (IPTG) is used as the inducer. IPTG binds to the lac repressor and allosterically releases the tetrameric repressor from the lac operator. This allows the transcription of genes in the lac operon and specifically the transcription of the p10 and p20 genes transformed into the cells (Figure 9)[29]. Both p10 and p20 are insoluble proteins captured in inclusion bodies in the cytosol of the cell. This means the expressed protein can be harvested from the insoluble pellet after lysis. To store the protein in denatured form a combination of urea and ß-Mercaptoethanol (BME) was used. BME is a reducing agent which will break the intra- and inter-molecular disulfide bonds in proteins and urea at high concentrations is a powerful protein denaturant, as it breaks non-covalent bonds in protein structures[30]. This prevents the protein from folding prior to the refolding procedure, which could cause the protein not being folded correctly.

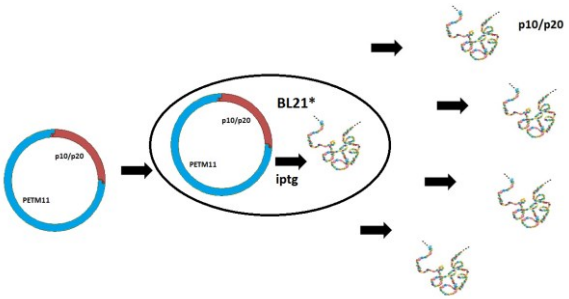


Figure 9: Expression through BL21(DE3)pRARE2 cells*

Refolding

Shock dilution

As both subunits are expressed and stored separately, Caspase-1 has to be refolded to regain its fold and activity. In order to achieve this the proteins were mixed together at a 1:1 molar ratio and diluted 100x in order to reduce the urea concentration. The mixture was added dropwise as it is diluted almost instantly. In order to prevent protein aggregation a detergent can be also added to the dilution buffer.

Dialysis

After refolding the detergent was removed through dialysis by adding the sample into a semi permeable membrane with a pore size that retains the protein but allows the detergent to diffuse to the outer environment. The outer environment is a buffer of a significantly higher volume than the sample volume. This action will shift the concentration of the detergent to equalize with the outer buffer (Figure 10). After several repetitions the concentration of detergent drops to levels approaching the protein concentration[29].

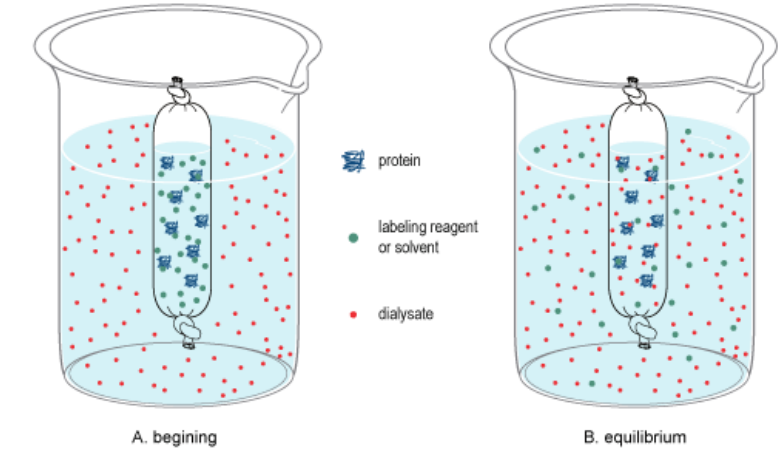


Figure 10: A standard setup of membrane dialysis

Crystallization

The eventual goal of the experiment is to get the protein into crystals. The method used is crystallization by hanging drop vapour diffusion. The protein hangs in a drop above a buffer with certain conditions sealed off from the outside environment (Figure 11). The drop itself is a mixture of protein sample and buffer in 1:1 ratio. Because the concentration of precipitant is higher in the basin, water vapour from the drop will be transported to the buffer in the well (vapour diffusion). The decreasing of volume of the drop causes the protein to become supersaturated in the drop. In these conditions, the proteins can become packed in a repeating array held together by non-covalent interactions. These crystals can be used to study the molecular structure of the protein[31].

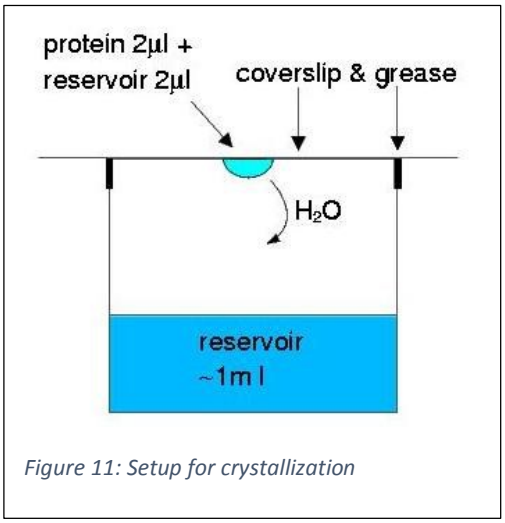


Figure 11: Setup for crystallization

X-Ray diffraction

Determination and interpretation of the crystals was done through X-ray diffraction; an analysis of the crystals by the scattering of X-rays by the electrons in the molecules. When the protein is stacked into a crystal, the scattering of X-radiation is enhanced in selected directions while extinguished completely in others. As the intensity is dependent on the geometry of the crystal and wavelength of the X-ray, which should be in the same range as the interatomic distances the crystal can be decoded from the diffracted X-rays[32].

Experimental Procedures

Reference

Previous results from Cheneval et al.[12) 13] and Datta et al.[14] have been used as guideline to find the right conditions for refolding, dialysis and crystallization. Unfortunately, we were unable to reproduce soluble material following the published protocols, necessitating the establishment of refolding protocols contained within this thesis.

Subcloning

Separate plasmids containing the gene for the p10 and p20 subunit for caspase-1 were obtained from MWG Biotech (Appendix 2). The pEX-A2 ampicillin resistant vector was replaced by a kanamycin resistant pETM11 vector by digesting the commercial plasmid with HindIII and NcoI restriction enzymes. The digestion products were separated by electrophoresis 1% (w/v) agarose gel[33) 34) 35] and extracted with GeneJet gel extraction kit (Appendix 3). The purified plasmid was ligated with predigested pETM11 vector with matching sticky ends (NcoI/HindIII, a kind gift from S. Lunev) using T4 DNA ligase and transformed into DHT turbo cells for DNA amplification. Identification of correctly formed pETM11-p10 and pETm11-p20 was performed by colony PCR. The DNA plasmid was extracted with the GeneJet plasmid miniprep kit (Appendix 4) and was stored at -20°C.

Protein expression

Both subunits were acquired by transforming the gene with pETM11 vector into competent BL21*(DE3)pRARE2 cells and inoculating the culture in 1L LB-medium with kanamycin and chloramphenicol (Appendix 6) incubated at 37°C (180rpm) until an OD measurement of 0,6 was reached. The expression of the protein was induced by adding 1mL 1M IPTG[33] to the culture (final concentration of 1mM) and cultured overnight at 18°C (120rpm). The inclusion bodies containing protein were harvested by centrifuging the culture with a Sorvall RC6 plus centrifuge for 15 minutes at 5k rpm. The supernatant was removed and the pellet was washed by adding 45 mL of Ni-NTA buffer pH 8.0 (Appendix 7), 4.5 mg Lysosome and 1,2mg $MgSO_4$. The inclusion bodies were incubated for 15 minutes at 20°C. The samples were centrifuged and washed 3x with the Ni-NTA buffer (Appendix 7) and finally 2x with Ni-NTA buffer without Triton X-100. The purified proteins were stored separately in 10mL 8M urea buffer (Appendix 8) at -20 °C. After each washing step the sample was centrifuged with a Sorvall RC6 plus centrifugation for 30 minutes at 19k rpm. The inclusion bodies were dissolved in 10-20mL 8M urea and 20mM BME (Appendix 8). To visualize the protein SDS-PAGE was used with 12.5% polyacrylamide[35) 36) 37]. Protein measurements were performed on a Biodrop-duo spectrophotometer. The protein signal was read on λ=279nm, impurity with DNA was read from λ=260nm.

Refolding procedure

Concentrating procedure

Concentrating the sample was done by 2 methods: By centrifugation and by a stirring cell. The centrifugation method was done by a Vivaspin 15R (Sartorius) with a 5kDa cut-off membrane[) 38]. The stirring cell method was performed using an Amicon stirred cell model 8050 (Millipore) placed in an ice bath at an air pressure of 18psi[) 37) 38) 39].

Shock dilution

For the shock dilution 100mL of different buffers were tested: Phosphate buffer solution (PBS, Appendix 9), Tris buffer solution (TBS, Appendix 10) and a buffer containing 1M of Non detergent sulphobetain (NDSB-201, Appendix 11). All buffers were tested with and without the presence of ß-Mercaptoethanol (BME). Prior to the shock dilution both proteins were mixed together in a 1:1 molar solution containing a total amount of 30mg protein (3,3mg p10 and 6,6mg p20). The shock dilution was performed by adding mixture drop-wise to the buffer while stirring 750rpm at room temperature. After stirring overnight (room temperature), the aggregates in the sample were removed through centrifugation with an Eppendorf 5810R centrifuge (5 minutes at 5k rpm) and the remaining solution was concentrated to 30mL. Concentrating the samples was both done by centrifugation and the stirred cell model to compare which procedure works most efficiently.

Dialysis

Membrane dialysis

The shock diluted and concentrated sample was placed into a 10kDa cutoff dialysis membrane and dialyzed against a 10mM HEPES buffer (Appendix 12) for 8 hours at 4°C. This procedure is repeated in order to remove all NDSB-201 and urea.

Dialysis through concentration

An alternative way to remove the detergent from solution was performed by concentrating the sample with the stirred cell model until the volume dropped to 4-5mL, addition of 50mL dialysis buffer (Appendix 12), and concentration again to 4-5mL of volume. This procedure was repeated until the signal of NDSB-201 could not be detected on the spectrophotometer (Biodrop Wavescan).

Storing the protein

After dialysis the sample was concentrated by the stirred cell method to 3-5mg/mL. The sample was concentrated further to 0.5-1mL with a Vivaspin concentrator. Once the detergents were removed the protein became temperature sensitive and had to be stored at 4°C conditions at all times. NDSB-201 has a UV maximum at λ=259nm (Appendix 14). After dialysis the decrease of NDSB-201 was determined by a spectrophotometer (Biodrop Wavescan).

Crystallization

The crystallization conditions where derived from Cheneval et al.[12] [13] and Datta et al.[14] reporting a condition containing pH 7.4, 2M $(NH_4)_2SO_4$, 25mM DTT and 0.01% Triton-X 100.

Table 2: setup of the crystallization plate with the changing concentrations of ammonium sulphate horizontally and and pH vertically in each well.

column	row	1	2	3	4	5	6
A	Ammonium sulphate (M)	1,4	1,6	1,8	2	2,2	2,4
	pH	6,5	6,5	6,5	6,5	6,5	6,5
B	Ammonium sulphate (M)	1,4	1,6	1,8	2	2,2	2,4
	pH	7	7	7	7	7	7
C	Ammonium sulphate (M)	1,4	1,6	1,8	2	2,2	2,4
	pH	7,5	7,5	7,5	7,5	7,5	7,5
D	Ammonium sulphate (M)	1,4	1,6	1,8	2	2,2	2,4
	pH	8	8	8	8	8	8

The concentrated sample was crystallized by hanging drop vapor diffusion against reservoirs containing 25mM DTT, 0,01% Triton X-100. The reservoirs had a concentration of ammonium sulfate ($(NH_4)_2SO_4$) between 1,4-2,4 M and a pH range of 6,5 to 8,0. To acquire pH=6,5, a 0,1 M MES buffer solution used. For mixtures containing pH 7,0-8,0 0,1M HEPES was used. All buffers used for crystallization are summarized in Appendix 15. Table 2 shows the plate setup with ammonium sulfate concentrations varying horizontally and varying pH vertically. A specific table of volumes of all compounds added to the wells are shown in an extended table at Appendix 16. The drops hanging above the plates consisted of 2µL protein sample mixed with 2µL crystallization buffer taken out of the well. The crystallization took place during overnight at 4°C. The crystals were diffracted by the synchrotron beamline P11 at PETRA III, DESY, Hamburg and diffraction analyzed with XDS software[40] [41].

Results

Sub cloning

Figure 12 shows the agarose gel of p10 and p20 genes amplified through PCR from the DHT-Turbo cells. A 1kb DNA ladder from Axygen was used as marker (Figure 12, left band). The p10 gene had a size between 500 and 600bp and p20 between 700 and 800bp. The encircled bands were extracted with GeneJET DNA extraction kit (Appendix 3). After extraction the genes were ligated with pETM11 vector and transformed into DHT-turbo cells. After incubation overnight the plasmids were harvested from the cells with GeneJET miniprep kit (Appendix 4).

Figure 12: Agarose gel showing the size of the DNA amplified with DHT-Turbo cells. The plasmids used for PCR amplification are marked in red circles

Stocks of 50µL of both pETM11-attached genes were acquired and stored under -20°C conditions.

Protein expression

From the DNA stock 1µL was added to 100µL competent BL21*(DE3)pRARE2 cells. After plating, inoculation, protein expression and washing, the protein concentration was measured and the samples were stored separately in 8M urea. An SDS-page[36][37] gel of both stocks are shown in Figure 13. The concentrations of both proteins solved in urea were 16,7mg/mL for p10 and 28,8mg/mL for p20 (Figure 14). Both proteins were dissolved in 10mL. This is equivalent to a total yield of 167mg p10 and 288 mg p20. Prior to shock dilution 0,6mL p10 (10mg) was added to 0,7mL (20mg) p20 to obtain a 1:1 molar ratio mixture of both proteins containing 30mg of total protein mass. Figure 14 shows the Uv-Vis absorption spectrum of the samples of both subunits. Because there is no additional signal at 260nm there can be concluded that the amount of DNA present in both solutions are negligible and that the samples contain pure protein.

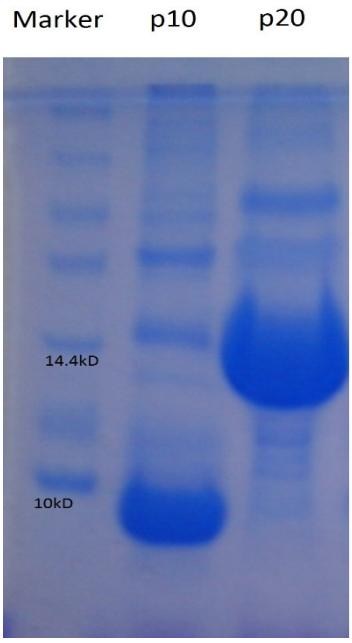
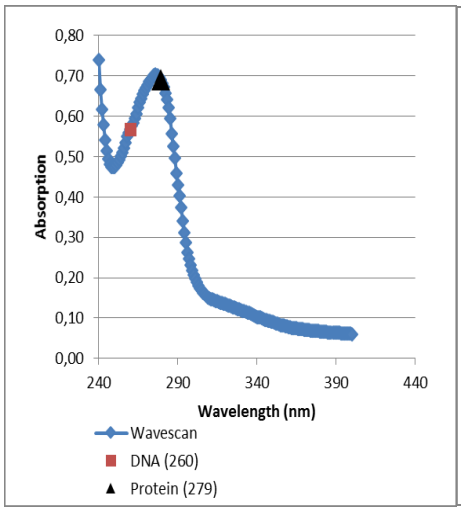
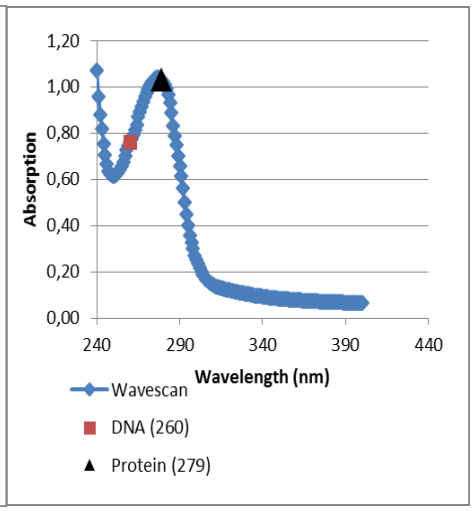


Figure 13: SDS-page gel with p10 and p20 subunits after expression by BL21 cells*





DNA (260)	0,568	DNA (260)	0,760
Protein (279)	0,690	Protein (279)	1,031
Ratio Prot/DNA	1,2	Ratio Prot/DNA	1,36
Concentration p10 (mg/mL)	16,7	Concentration p20 (mg/mL)	28,8

Figure 14: UV spectra and acquired concentration of harvested p10 (left) and p20 (right) subunits.

Shock dilution

The initial step was to establish conditions that yield a soluble form of Caspase-1

30mg of a 1:1 Molar ratio of both subunits was added to 100 mL shock dilution buffer. In order to find the right environment for refolding 3 different buffers were used separately: Phosphate buffer solution (PBS, Appendix 9), Tris buffer solution (TBS, Appendix 10) and NDSB-201 shock dilution buffer (Appendix 11). After an overnight stirred incubation at room temperature the samples were centrifuged and the supernatant was analyzed by SDS-PAGE [36] [37]. It was expected that a band around 20kDa range (p20) and 10kDa (p10) range would appear if both subunits were folded into a soluble protein shape. The shock dilution executed in Tris- or Phosphate buffer solution showed a 20kDa band only (Figure 15), indicating that the p10 subunit was not visible on the gel.

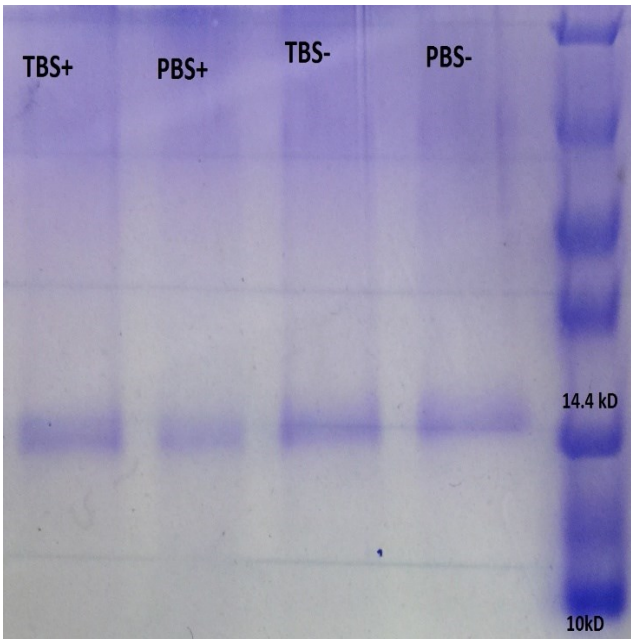


Figure 15: SDS-Page gel of shock dilution with Tris and phosphate with 5mM BME (lane 1 & 2) and without BME (3 & 4)

As a control the p10 subunit was shock diluted in the absence of p20. These separate samples are shown in Figure 16. It appears that, when the p10 subunit is refolded independently, it appears at the gel in the 20kDa region, similarly to the p20 subunit. A possible explanation is that p10 forms a homodimeric protein when it doesn't fold together with p20 in solution. This probably means that in Figure 15 p10 *is* present in solution but the signal comes from the misfolded homodimeric stateshowing up in the same region together with the p20 band.

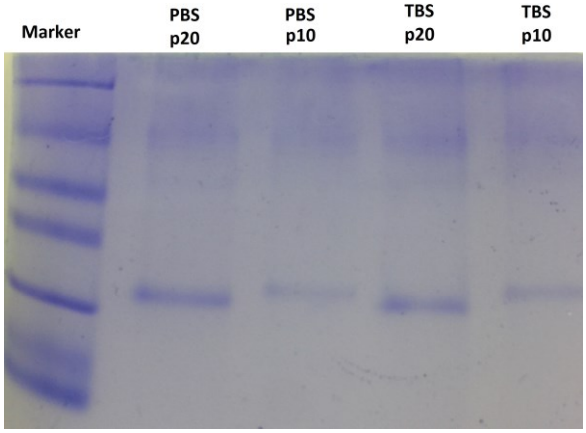


Figure 16: SDS-page gel of a shock dilution in PBS and TBS with p10 and p20 subunits separately

Figure 17 shows the SDS sample taken from a shock dilution with NDSB-201 (Appendix 11). It is clearly shown that the p10 subunit now appears in the 10kDa range. This indicates that the protein in the gel has not folded as a p10-p10 homodimer but comes from the correctly folded protein. The buffer was performed optimally was a buffer containing 1M NDSB-201 pH8.0 buffer, 5mM BME (Appendix 11). Determining concentration of protein by UV-measurement on a spectophotometer could not be done due to the presence of NDSB-201, which has a λ_{max} of 259nm. To illustrate this effect, a UV-Vis spectrum of a shock dilution with NDSB-201 present is shown in Appendix 14. The concentration of the protein could eventually be determined after removing the NDSB-201 by dialysis.

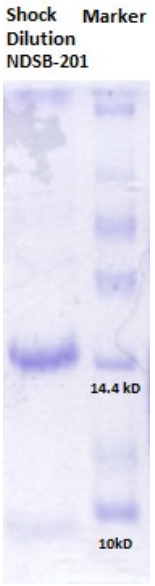


Figure 17: Shock dilution of 30mg of p10 and p20 mixture of 1:1 M ratio in the NDSB-201 buffer.

Figure 17 shows that we have isolated a soluble form of p20-p10, although the correct folding state needs to be demonstrated.

Dialysis

The next key step is the removal of the refolding buffer reagents, specifically the detergent NDSB-201

Dialysis by membrane

After each dialysis a UV-measurement was done until the absorption peak from NDSB-201 at 259 nm (Appendix 14) did not interfere with the protein signal anymore. The original dialysis buffer from Journal papers from Cheneval et al[) 12) 13] and Datta et al[) 14], containing sodium acetate, glycerol and with a pH of 5.9 resulted in precipitation so a different dialysis buffer had yet to be determined. To establish the cause of precipitation 4 different samples were dialyzed, each having one variable changed (Appendix 13). None of the variables caused precipitation by itself, but the combination of pH-drop and removal of NDSB-201 caused precipitation. In addition, the removal of NDSB-201 caused the protein to become temperature sensitive. After dialysis the protein had to be kept at 4°C. To make the solution suitable for crystallization a dialysis buffer containing 10mM HEPES and pH=8 is used (Appendix 12).

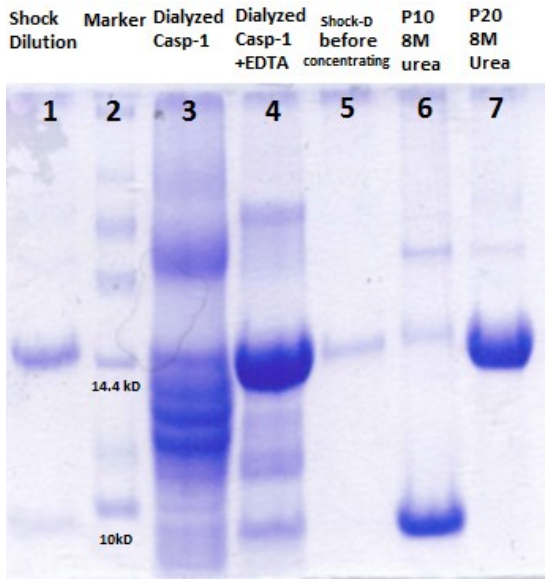
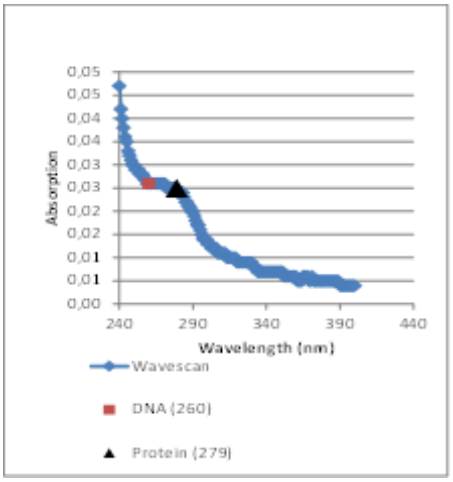


Figure 18: SDS gel containing samples of shock dilution, the protein before dialysis and showing the effect of EDTA

EDTA

Even when the protein was correctly refolded and did not precipitate during dialysis, a final problem remained: Analysis on SDS-PAGE indicated that the protein was degraded. This can clearly be seen in Figure 18 on a SDS gel showing the dialyzed sample in lane 3. A possible explanation is that there was some minor protease contamination present in the sample. The digestion of the enzyme was inhibited in the presence of EDTA (Figure 18, lane 4). To successfully reduce the NDSB-201 concentration to levels that didn't interfere with the protein absorption signal and considered to be low enough to avoid influence on crystallization, the sample had to be dialyzed 5 times with an inner membrane volume of 15-20ml placed in a container of 1L dialysis buffer. The concentration of the protein could now be measured in a spectrophotomer shown in Figure 19 without interference of NDSB-201. The protein concentration was 0,67 mg/mL.



DNA (260)	0,026
Protein (279)	0,025
Ratio Prot/DNA	0,96
C Protein (µg/mL)	666,7

Figure 19: Biodrop UV-wavescan of refolded Caspase-1 after 5x dialysis where the NDSB got removed

Dialysis by concentrating

According to the procedures from previously published articles the protein is separated from the NDSB-201 detergent using a dialysis membrane[12][13][14]. Another possible approach was to concentrate the sample from 100 mL to 5-10mL, add dialysis buffer to 100mL (Appendix 12) and repeat these steps until the NDSB-201 concentration was reduced solution to a level in which the detergent's signal at 259nm (Appendix 14) was negligible compared to the protein's signal at 279nm. After 5-6 times of concentrating the NSDB-201 could not be determined anymore. Figure 20 shows a comparison between the sample after dialysis by membrane (lane 5) and dialysis through concentration (lane 3). Although both methods gave similar results the advantage of the concentrating method lies in the efficiency in time and resources. Whereas membrane-dialysis takes 3-4 days (2cycle/day) and 5L of dialysis buffer, the concentration-dialysis could be done in 1 day (45 min/step) using around 600mL of dialysis buffer. When both samples were concentrated from 20mL to 1mL, samples with a concentration of 5-9 mg/mL could be acquired. The first sample used for crystallization had a concentration of 4.45mg/mL (Figure 21), the second one 8.34 mg/mL (Figure 22). This is higher than articles from Cheneval et al.[12][13] and Datta et al.[14], who report a concentration of 3-5mg/mL.

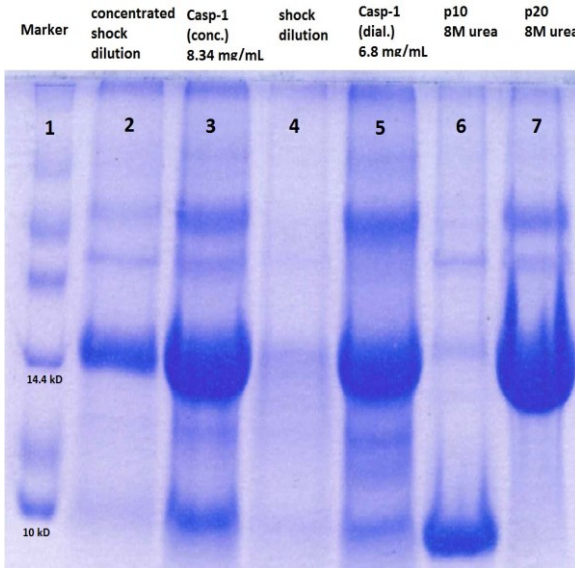
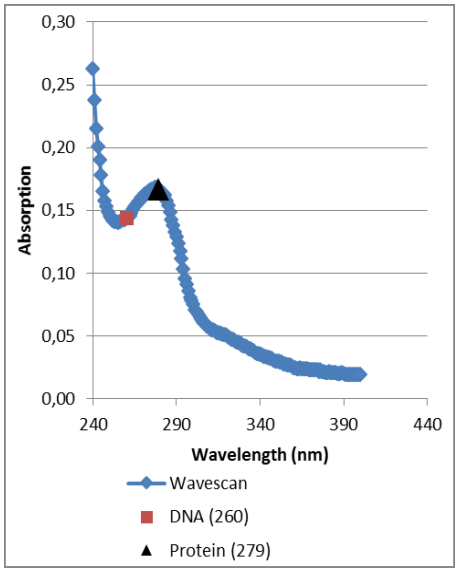
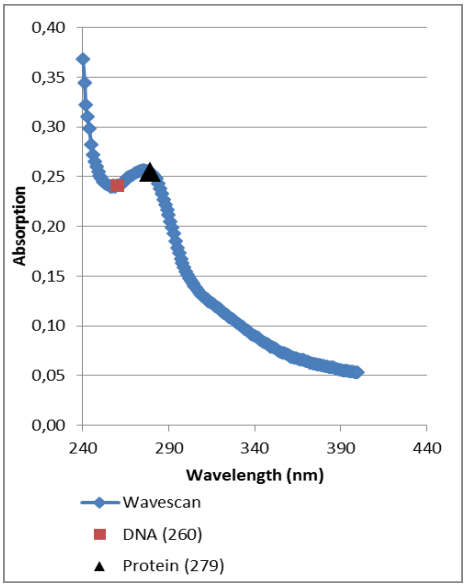


Figure 20: SDS-gel showing the protein in the stages from shock dilution and the eventual sample used for crystallization. The shock dilution is shown in lane 4, lane 2 shows the same sample 3x concentrated, lane 3 shows the sample prepared for crystallization by dialyzing it through a concentrator. The sample prepared through dialysis with a 10kDa membrane is shown in lane 5. For reference reasons, lane 6 and 7 show the denatured forms of p10 and p20, after expression through BL21(DE3)pRARE2 cells.*



DNA (260)	0,144
Protein (279)	0,167
Ratio Prot/DNA	1,16
C Protein (mg/mL)	4,453

Figure 21: UV-scan of 1mL Caspase-1 sample used for setting up crystals (1st batch)



DNA (260)	0,241
Protein (279)	0,255
Ratio Prot/DNA	1,06
C Protein (mg/mL)	6,80

Figure 22: Uv-scan of a 1mL Caspase-1 sample used for setting up crystals (2nd batch)

As the NDSB-201 signal is now significantly lower than that from the protein and given the fact that NDSB-201 has a higher absorption coefficient, we can conclude that the NDSB-201 concentration has been significantly reduced. The stability of the protein in low NDSB-201 concentrations is a further indication that we have achieved a soluble form.

Figure 22 shows that we have successfully reduced the NDSB-201 concentration to a point where the estimated NDSB-201 concentration is lower than that of the protein samples. This indicates we have purified a soluble protein ready for crystallization.

Circular Dichroism

To confirm correct folding of our samples we performed circular dichorism experiments to assess the secondary structural content of the sample.

In order to demonstrate correct folding of the sample, circular dichroism (CD) experiments were performed buy our collaborator Dr. Giovanni Ricercatori (University of Napoli). CD experiments are highly sensitive to the presence of alpha-helices and beta-sheets. Thus, a strongl signal in these regions is highly indicative of a correctly folded samples. The spectrum measured shows the protein has a secondary structure that strongly implies that the Caspase-1 has been folded correctly.

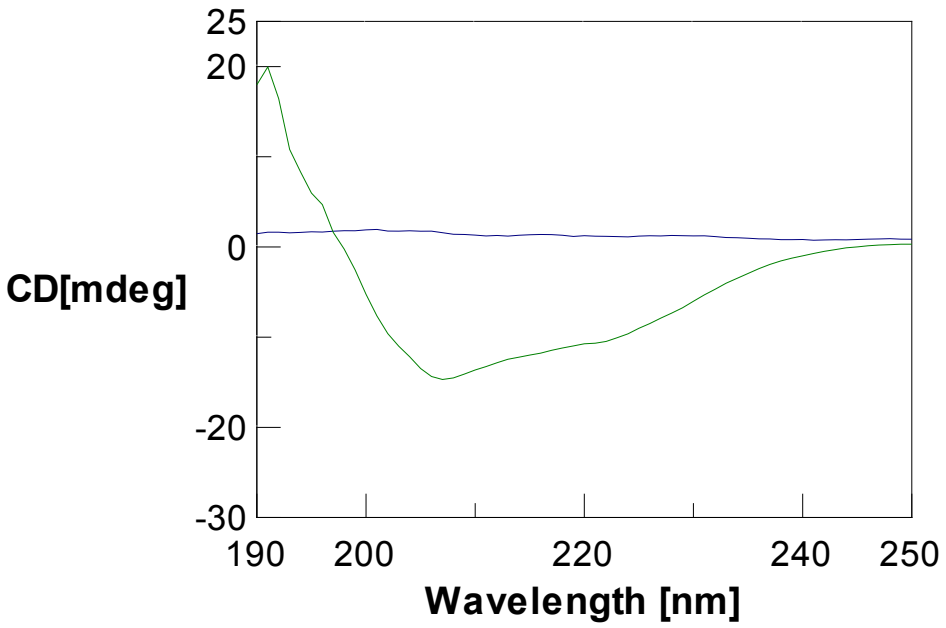


Figure 23: Circular Dichroism spectra of Caspase-1 at 12°C. The blue line represents the buffer signal (10mM phosphate buffer pH7.4). The green line represents the signal of a 0.5 mg/mL Caspase-1

Figure 23 shows that our sample contains α-helices and β-sheets. This is indicatinve that our sample is correctly folded and suitable for crystallization

Crystallization

The ultimate test of the overall structure is whether the sample will crystallize and show the expected overall fold. We also performed these experiments to look if the protein is able to co-crystallize with possible lead compounds.

Crystals have been observed of Caspase-1 alone and in the presence of ALC129, ALC150 and VX765. All crystals appeared after 3 days of vapor diffusion. Crystals in the presence of AD4 haven't yet been observed. All conditions in which the crystals have been observed can be found in Table 3. All crystals observed had a round transparent shape (Figure 24). The crystals have been analyzed on synchrotron beamline P11, PETRAIII, DESY, Hamburg. In Appendix 19 the data analyzed by XDS are shown of a Caspase-1 crystal with no compound attached to it. A correlation coefficient >95% is used as cutoff value, in this case down to a resolution of 3.19Å. Between 7.49 and 3.19 Å, where 79.5-85% of all possible reflections have been observed. The R-factors where between 6.1% and 26.3%.

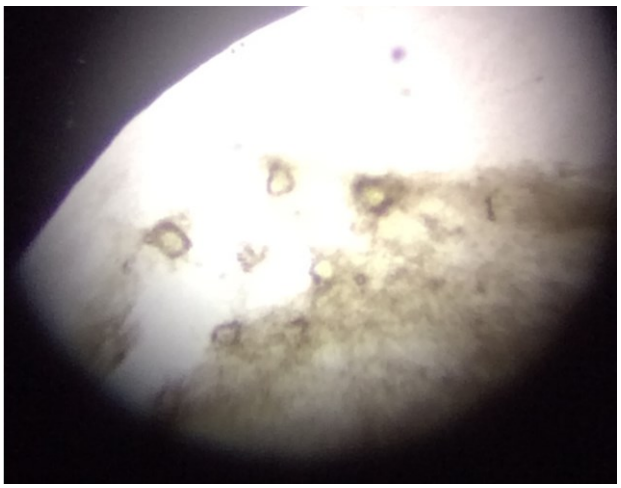


Figure 24: Picture of a Caspase-1 co-crystallized with ALC129 in 1.8M ammonium sulphate at pH=7.5

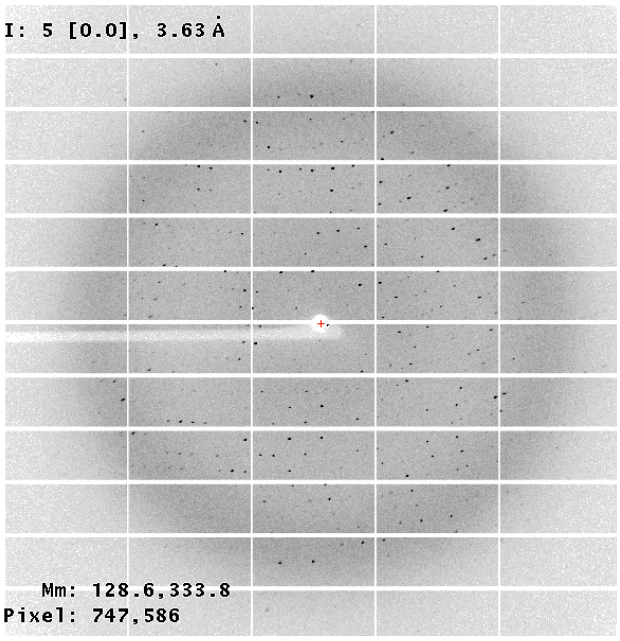


Figure 25: Snapshot of crystallization diffraction of a caspase-1 crystal

Table 3: conditions in which crystals were observed

compound	A.S.	pH
Casp-1 only	1,8	6,5
	2	6,5
	1,8	7,5
	2	7,5
ALC129	1,4	7
	1,8	7,5
	2	7,5
ALC150	2	8
VX765	1,6	7
	1,8	2

In order to acquire crystals of Casp-1 with AD4, some optimization might be needed. Suggestions are: Longer vapor diffusion time (>3 days), different conditions (pH), or different crystallization buffer (PEG, sodium malonate).

Figure 24 and Figure 25 show that we managed to crystallize the protein and acquired diffraction signals. This indicates that we have successfully expressed, purified, refolded and crystallized Caspase-1

Conclusion

While procedures applied in different articles[12][13][14] could not be reproduced in our lab we did manage to express and refold Caspase-1 properly and produce samples containing 6-9mg/mL. These samples were successfully crystallized. Caspase-1 was also successfully co-crystallized with ALC150, ALC129 and VX765.

Expression

The expression with BL21*(DE3)pRARE2 cells in 1L LB-media gave a yield of 167mg of p10 and 288 mg of p20 subunits. Concentrations in 8M urea can go at least as high as 17mg/mL for p10 and 29mg/mL for p20. In a 1:1 molar ratio 1.2mL of sample contained 30mg of protein used in 100mL shock dilution.

Refolding

The advised buffer solution used for shock dilution contains 1M NDSB-201 (Appendix 11). And the protein stays stable at room temperature. The dialysis method acquired from 2 different journals[12][13][14] did not work out because the protein precipitated during dialysis. The solution to prevent precipitation was to make a dialysis buffer containing 10mM HEPES and pH=8.0 (Appendix 12). After optimization of the dialysis procedure another problem showed up: The protein got digested. This problem was tackled by adding EDTA to the dialysis buffer which inhibited the protease responsible for the digestion. After dialysis and concentration the yield of Caspase-1 was 6-9 mg in 1 mL which is 20-28% of the initial protein added to the shock dilution.

A faster and more efficient process of dialysis is concentrating the sample with an Amicon stirred cell model 8050 (Millipore) 4050 concentrator instead of a classic membrane. The protein yield was similar, but the timespan of the procedure got reduced from 4 days to 1 day.

Circular Dichroism

In order to verify the protein got refolded correctly a sample was sent to the University of Naples, where G. Ricercatori made a Circular Dichroism spectrum, which indicated that the protein has a secondary structure which strongly implies that the Caspase-1 has been folded correctly.

Crystallization

Crystals of Caspase-1 co-crystallized with ALC129, ALC150 and VX765 were found in several conditions in the range between pH 6.5-8.0 and 1.4-2.2 ammoniumsulfate (Table 3). Crystals of Caspase-1 with AD4 haven't been observed (yet). Out of a Casapse-1 crystal diffraction data have been observed of particles between between 7.49 Å and 3.19 Å with a correlation coefficient >95%. Crystals of Casp-1 with AD4 haven't been observed, optimization with other conditions or longer vaporization time might be needed.

Overall

Acquiring correctly refolded Caspase-1 can effectively be achieved by expressing both subunits of the protein separately and refold them together through shock dilution. In this experiment the samples were pure and we were able to crystallize the protein itself, and together with possible lead compounds. However there is still room for optimization, one compound (AD4) did not crystallize in the used conditions. Maybe crystals will show up at a slower pace (>3 days), under different conditions (pH, temperature), or by using a different crystallization buffer (PEG, sodium malonate).

Future Perspectives:

We have now established a refolding mechanism for Caspase-1 as well as crystallization conditions and now can be used for screening on possible lead compounds. The SDS-PAGE, circular dichroism and the solubility of the sample all indicate that the protein has been refolded correctly, but a gel filtration has yet to be done to determine the purity of the protein. Besides optimizing the identification of the protein there is also room for optimization of the crystallization buffer.

Special thanks to M. Groves, S. Lunev, A. Ali and A. Chandgude for their patience, assistance and compounds during this project and G. Ricercatori for providing the Circular Dichroism spectra.

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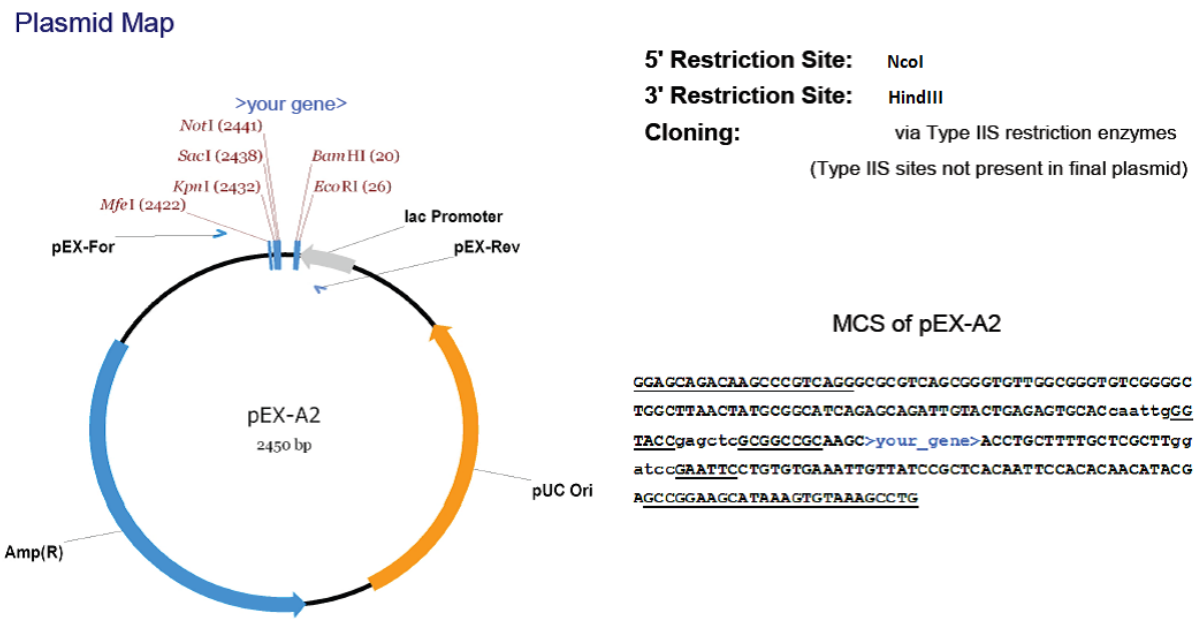
Appendix

Appendix 1: List of abbreviations

AIM2	absent in melanoma 2
ASC	apoptosis-associated speck-like protein containing a CARD
CARD	caspase activation and recruitment domain
DAMPs	damage-associated molecular patterns
PAMPs	pathogen-associated molecular patterns
NLR	NOD-like receptor
PRRs	pattern recognition receptors
IL1ß	Interleukin ß
BME	ß-Mercaptoethanol
SDS-PAGE	Sodiumdodecyl sulfate Polyacryl gel electrophorese
XDS	X-ray Detector Software

Appendix 2: Commercial plasmid map



Appendix 3: DNA extraction protocol

PURIFICATION PROTOCOL

Note

- Read IMPORTANT NOTES on p. 3 before starting.
- All purification steps should be carried out at **room temperature**.
- All centrifugations should be carried out in a table-top microcentrifuge at **>12000 × g** (10 000-14 000 rpm, depending on the rotor type).

Step	Procedure
1	Excise gel slice containing the DNA fragment using a clean scalpel or razor blade. Cut as close to the DNA as possible to minimize the gel volume. Place the gel slice into a pre-weighed 1.5 mL tube and weigh. Record the weight of the gel slice. **Note.** If the purified fragment will be used for cloning reactions, avoid damaging the DNA through UV light exposure. Minimize UV exposure to a few seconds or keep the gel slice on a glass or plastic plate during UV illumination.
2	Add **1:1 volume** of **Binding Buffer** to the gel slice (volume: weight) (e.g., add 100 µL of Binding Buffer for every 100 mg of agarose gel). **Note.** For gels with an agarose content greater than 2%, add 2:1 volumes of Binding Buffer to the gel slice.
3	Incubate the gel mixture at **50-60°C** for **10 min** or until the gel slice is completely dissolved. Mix the tube by inversion every few minutes to facilitate the melting process. Ensure that the gel is completely dissolved. Vortex the gel mixture briefly before loading on the column. Check the color of the solution. A yellow color indicates an optimal pH for DNA binding. If the color of the solution is orange or violet, add 10 µL of 3 M sodium acetate, pH 5.2 solution and mix. The color of the mix will become yellow.
4 for ≤500 bp and >10 kb DNA fragments	*Optional:* use this step only when DNA fragment is ≤500 bp or >10 kb long. • If the DNA fragment is ≤500 bp, add a 1:2 volume of 100% isopropanol to the solubilized gel solution (e.g. 100 µL of isopropanol should be added to 100 mg gel slice solubilized in 100 µL of Binding Buffer). Mix thoroughly. • If the DNA fragment is >10 kb, add a 1:2 volume of water to the solubilized gel solution (e.g. 100 µL of water should be added to 100 mg gel slice solubilized in 100 µL of Binding Buffer). Mix thoroughly.
5	Transfer up to 800 µL of the solubilized gel solution (from step 3 or 4) to the GeneJET purification column. Centrifuge for 1 min. Discard the flow-through and place the column back into the same collection tube. **Note.** • If the total volume exceeds 800 µL, the solution can be added to the column in stages. After each application, centrifuge the column for 30-60 s and discard the flow-through after each spin. Repeat until the entire volume has been applied to the column membrane. Do not exceed 1 g of total agarose gel per column. • Close the bag with GeneJET Purification Columns tightly after each use!
6	*Optional:* use this additional binding step only if the purified DNA will be used for sequencing. Add **100 µL** of **Binding Buffer** to the GeneJET purification column. Centrifuge for 1 min. Discard the flow-through and place the column back into the same collection tube.
7	Add **700 µL** of **Wash Buffer** (diluted with ethanol as described on p. 3) to the GeneJET purification column. Centrifuge for 1 min. Discard the flow-through and place the column back into the same collection tube.
8	Centrifuge the empty GeneJET purification column for an additional 1 min to completely remove residual wash buffer. **Note.** This step is essential to avoid residual ethanol in the purified DNA solution. The presence of ethanol in the DNA sample may inhibit downstream enzymatic reactions.
9	Transfer the GeneJET purification column into a clean 1.5 mL microcentrifuge tube (not included). Add **50 µL** of **Elution Buffer** to the center of the purification column membrane. Centrifuge for 1 min. **Note** • For low DNA amounts the elution volumes can be reduced to increase DNA concentration. An elution volume between 20-50 µL does not significantly reduce the DNA yield. However, elution volumes less than 10 µL are not recommended. • If DNA fragment is >10 kb, prewarm Elution Buffer to 65°C before applying to column. • If the elution volume is 10 µL and DNA amount is ≤5 µg, incubate column for 1 min at room temperature before centrifugation.
10	Discard the GeneJET purification column and store the purified DNA at -20°C.

Appendix 4: Genejet plasmid miniprep protocol

PURIFICATION PROTOCOL

Note

- Read IMPORTANT NOTES on p.3 before starting.
- All purification steps should be carried out at **room temperature**.
- All centrifugations should be carried out in a table-top microcentrifuge at **>12000 × g** (10 000-14 000 rpm, depending on the rotor type).

Use 1-5 mL of *E. coli* culture in LB media for purification of **high-copy** plasmids.

For **low-copy** plasmids use up to 10 mL of culture.

Step	Procedure
1	Resuspend the pelleted cells in **250 µL of the Resuspension Solution**. Transfer the cell suspension to a microcentrifuge tube. The bacteria should be resuspended completely by vortexing or pipetting up and down until no cell clumps remain. **Note.** Ensure RNase A has been added to the Resuspension Solution (as described on p.3)
2	Add **250 µL of the Lysis Solution** and mix thoroughly by inverting the tube 4-6 times until the solution becomes viscous and slightly clear. **Note.** Do not vortex to avoid shearing of chromosomal DNA. Do not incubate for more than 5 min to avoid denaturation of supercoiled plasmid DNA.
3	Add **350 µL of the Neutralization Solution** and mix immediately and thoroughly by inverting the tube 4-6 times. **Note.** It is important to mix thoroughly and gently after the addition of the Neutralization Solution to avoid localized precipitation of bacterial cell debris. The neutralized bacterial lysate should become cloudy.
4	Centrifuge for 5 min to pellet cell debris and chromosomal DNA.
5	Transfer the supernatant to the supplied GeneJET spin column by decanting or pipetting. Avoid disturbing or transferring the white precipitate. **Note. Close the bag with GeneJET Spin Columns tightly after each use!**
6	Centrifuge for 1 min. Discard the flow-through and place the column back into the same collection tube. **Note.** Do not add bleach to the flow-through, see p.7 for Safety information.
7	Add **500 µL of the Wash Solution** (diluted with ethanol prior to first use as described on p.3) to the GeneJET spin column. Centrifuge for 30-60 seconds and discard the flow-through. Place the column back into the same collection tube.
8	Repeat the wash procedure (step 7) using **500 µL of the Wash Solution**.
9	Discard the flow-through and centrifuge for an additional 1 min to remove residual Wash Solution. This step is essential to avoid residual ethanol in plasmid preps.
10	Transfer the GeneJET spin column into a fresh 1.5 mL microcentrifuge tube (not included). Add **50 µL of the Elution Buffer** to the center of GeneJET spin column membrane to elute the plasmid DNA. Take care not to contact the membrane with the pipette tip. Incubate for 2 min at room temperature and centrifuge for 2 min. **Note.** An additional elution step (optional) with Elution Buffer or water will recover residual DNA from the membrane and increase the overall yield by 10-20%. For elution of plasmids or cosmids >20 kb, prewarm Elution Buffer to 70°C before applying to silica membrane.
11	Discard the column and store the purified plasmid DNA at -20°C.

Appendix 5: 10x TAE buffer

compound	pH/conc.	mass/volume
Tris	0,3M	48,4g
Acetic acid (glacial)	0.189M	11.42g
EDTA	10mM	20mL (0.5M)
H_2O	solvent	1L

Appendix 6: LB-medium

compound	pH/conc.	mass/volume
LB broth	25mg/mL	25g
Kanamycin	35mg/L	35mg
Chloramphenicol	35mg/L	35mg
H_2O	solvent	1L

Appendix 7: Ni-NTA (Lysis) buffer recipe

compound	pH/conc.	mass/volume
Tris HCl	50mM	7,88g
NaCl	300mM	17,53g
Triton (optional)	5%	5mL
BME	5mM	210µL of 14.3M
pH	8	
H_2O	solvent	1L

Appendix 8: Urea 8M + BME recipe

compound	pH/conc.	mass/volume
Urea	8M	480 g
BME	20mM	1.4mL * 14.3M
H_2O	solvent	1L
pH	8	

Appendix 9: PBS shock dilution buffer

compound	pH/conc.	mass/volume
Na2HPO4	10mM	1,44g
KH2PO4	1,8mM	0,24g
NaCl	137mM	8g
KCl	2,7mM	0,2g
BME (optional)	5mM	210µL of 14.3M
pH	8	
H2O	solvent	1L

Appendix 10: TBS shock dilution buffer

compound	pH/conc.	mass/volume
Tris HCl	50mM	6,05g
NaCl	150mM	8,76g
BME (optional)	5mM	210µL of 14.3M
pH	8	
H2O	solvent	1L

Appendix 11: NDSB-201 Shock dilution buffer

For 100 mL:

compound	pH/conc.	mass/volume
HEPES	50mM	1,19g
NaCl	100mM	590mg
NDSB-201	1M	20,12g
BME	20mM	0,14mL * 14.3M
H_2O	solvent	1L
pH	8	

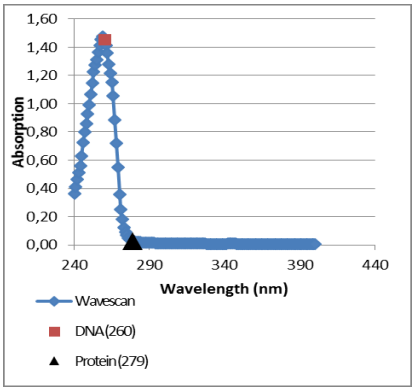
Appendix 12: Dialysis buffer

compound	pH/conc.	mass/volume
HEPES	10mM	2,4g
NaCl	50mM	2,9g
BME (optional)	20mM	1400µL of 14.3M
pH	8	
H2O	solvent	1L

Appendix 13: table of buffers used to find the variable causing precipitation

	buffer 1	buffer 2	buffer 3	buffer 4
compound	pH/conc.	pH/conc.	pH/conc.	pH/conc.
HEPES	50mM	50mM	50mM	50mM
NaCl	100mM	100mM	100mM	100mM
NDSB-201	0M	1M	1M	0M
BME	20mM	20mM	20mM	20mM
Urea	0,8M	0,8M	0M	0,8M
pH	8	5,9	8	5,9
result	solution	solution	solution	precipitation

Appendix 14: Biodrop Uv-Vis absorption spectrum of a sample containing 1M NDSB-201



DNA (260)	1,456
Protein (279)	0,028
Ratio Prot/DNA	0,02
C Protein (µg/mL)	746,67

Appendix 15: Crystallization buffers

compound	concentration	pH	volume
$NH_4(SO3)_2$	3,5M		50mL
MES	1M	6,5	10mL
HEPES	1M	7	10mL
HEPES	1M	7,5	10mL
HEPES	1M	8	10mL
DTT	1M		1mL
Triton x-100	1%		0,5mL

Appendix 16: Setup for crystallization plate

A.S. = ammonium sulphate, all added solutions are displayed in Appendix 15

row		1	2	3	4	5	6
column		1,4M A.S.	1,6M A.S.	1,8M A.S.	2,0M A.S.	2,2M A.S.	2,4M A.S.
A	A.S.	400µL	457 µL	514 µL	571 µL	628 µL	685 µL
	MES 6,5	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL
	DTT	25 µL	25 µL	25 µL	25 µL	25 µL	25 µL
	Triton	10 µL	10 µL	10 µL	10 µL	10 µL	10 µL
	H_2O	465 µL	408 µL	351 µL	294 µL	237 µL	180 µL
B	A.S.	400µL	457 µL	514 µL	571 µL	628 µL	685 µL
	HEPES 7	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL
	DTT	25 µL	25 µL	25 µL	25 µL	25 µL	25 µL
	Triton	10 µL	10 µL	10 µL	10 µL	10 µL	10 µL
	H_2O	465 µL	408 µL	351 µL	294 µL	237 µL	180 µL
C	A.S.	400µL	457 µL	514 µL	571 µL	628 µL	685 µL
	HEPES 7,5	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL
	DTT	25 µL	25 µL	25 µL	25 µL	25 µL	25 µL
	Triton	10 µL	10 µL	10 µL	10 µL	10 µL	10 µL
	H_2O	465 µL	408 µL	351 µL	294 µL	237 µL	180 µL
D	A.S.	400µL	457 µL	514 µL	571 µL	628 µL	685 µL
	HEPES 8	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL
	DTT	25 µL	25 µL	25 µL	25 µL	25 µL	25 µL
	Triton	10 µL	10 µL	10 µL	10 µL	10 µL	10 µL
	H_2O	465 µL	408 µL	351 µL	294 µL	237 µL	180 µL

Appendix 17: Chemical properties of p10 subunit

Number of amino acids		88
Molecular weight		10243,7
Theoretical pI		7,1
neg. residues (Asp + Glu)		11
pos. residues (Arg + Lys)		11
Atomic composition:		
Total number of atoms:		1415
Carbon	C	457
Hydrogen	H	696
Nitrogen	N	126
Oxygen	O	129
Sulfur	S	7

Ext. coefficient 8730
Abs 0.1% (=1 g/l) 0.852,
assuming all pairs of Cys residues form cystines

Ext. coefficient 8480
Abs 0.1% (=1 g/l) 0.828,
assuming all Cys residues are reduced

Aliphatic index: 62.05

Amino acid composition:			
Ala	(A)	5	5.7%
Arg	(R)	7	8.0%
Asn	(N)	1	1.1%
Asp	(D)	4	4.5%
Cys	(C)	4	4.5%
Gln	(Q)	3	3.4%
Glu	(E)	7	8.0%
Gly	(G)	4	4.5%
His	(H)	4	4.5%
Ile	(I)	6	6.8%
Leu	(L)	3	3.4%
Lys	(K)	4	4.5%
Met	(M)	3	3.4%
Phe	(F)	8	9.1%
Pro	(P)	5	5.7%
Ser	(S)	6	6.8%
Thr	(T)	6	6.8%
Trp	(W)	1	1.1%
Tyr	(Y)	2	2.3%
Val	(V)	5	5.7%
Pyl	(O)	0	0.0%
Sec	(U)	0	0.0%

Sequence

10	20	30	40	50	60
AIKKAHIEKD	FIAFCSSTPD	NVSWRHPTMG	SVFIGRLIEH	MQEYACSCDV	EEIFRKVRFS
70	80				
FEQPDGRAQM	PTTERVTLTR	CFYLFPGH			

Appendix 18: Chemical properties of p20 subunit

Number of amino acids		178
Molecular weight		19843,8
Theoretical pI		7,06
neg. residues (Asp + Glu)		22
pos. residues (Arg + Lys)		22
Atomic composition:		
Total number of atoms:		2785
Carbon	C	866
Hydrogen	H	1399
Nitrogen	N	241
Oxygen	O	267
Sulfur	S	12

Ext. coefficient 14230
Abs 0.1% (=1 g/l) 0.717, assuming all pairs of Cys residues form cystines

Ext. coefficient 13980
Abs 0.1% (=1 g/l) 0.705, assuming all Cys residues are reduced

Aliphatic index: 81.63

Amino acid composition:			
Ala	(A)	10	5.6%
Arg	(R)	8	4.5%
Asn	(N)	9	5.1%
Asp	(D)	10	5.6%
Cys	(C)	5	2.8%
Gln	(Q)	6	3.4%
Glu	(E)	12	6.7%
Gly	(G)	10	5.6%
His	(H)	4	2.2%
Ile	(I)	14	7.9%
Leu	(L)	14	7.9%
Lys	(K)	14	7.9%
Met	(M)	7	3.9%
Phe	(F)	6	3.4%
Pro	(P)	9	5.1%
Ser	(S)	16	9.0%
Thr	(T)	11	6.2%
Trp	(W)	2	1.1%
Tyr	(Y)	2	1.1%
Val	(V)	9	5.1%
Pyl	(O)	0	0.0%
Sec	(U)	0	0.0%

Sequence

10	20	30	40	50	60
NPAMPTSSGS	EGNVKLCSLE	EAQRIWKQKS	AEIYPIMDKS	SRTRLALIIC	NEEFDSIPRR
70	80	90	100	110	120
TGAEVDITGM	TMLLQNLGYS	VDVKKNLTAS	DMTTELEAFA	HRPEHKTSDS	TFLVFMSHGI
130	140	150	160	170	
REGICGKKHS	EQVPDILQLN	AIFNMLNTKN	CPSLKDKPKV	IIIQACRGDS	PGVVWFKD

Appendix 19: Crystallisation data from syntron beamline analyzed with XDS

SUBSET OF INTENSITY DATA WITH SIGNAL/NOISE >= -3.0 AS FUNCTION OF RESOLUTION

RESOLUTION	NUMBER OF REFLECTIONS			COMPLETENESS	R-FACTOR	R-FACTOR	COMPARED	I/SIGMA	R-meas	CC(1/2)	Anomal	SigAno	Nano
LIMIT	OBSERVED	UNIQUE	POSSIBLE	OF DATA	observed	expected					Corr		
10.59	1208	371	486	76.3%	6.1%	10.7%	1149	16.98	7.0%	99.4*	-33	0.413	141
7.49	2303	647	814	79.5%	6.2%	9.6%	2211	17.10	7.1%	99.4*	-31	0.498	316
6.12	2843	890	1077	82.6%	8.8%	10.3%	2695	13.69	10.4%	98.8*	-17	0.653	293
5.30	3621	1036	1247	83.1%	9.5%	10.9%	3480	12.88	11.0%	98.3*	-16	0.667	484
4.74	3980	1187	1424	83.4%	9.9%	10.4%	3803	13.41	11.6%	98.3*	-15	0.733	484
4.33	4097	1299	1538	84.5%	11.4%	10.5%	3874	12.62	13.5%	97.2*	-20	0.768	435
4.00	4774	1417	1692	83.7%	11.9%	10.9%	4552	11.53	14.0%	97.8*	-12	0.850	588
3.75	5250	1535	1808	84.9%	14.2%	12.2%	5015	10.44	16.7%	97.1*	-10	0.794	678
3.53	5127	1651	1948	84.8%	17.7%	14.8%	4836	8.46	21.1%	93.7*	-11	0.813	505
3.35	5739	1730	2018	85.7%	20.7%	18.5%	5484	6.92	24.4%	96.2*	-5	0.786	652
3.19	6302	1826	2132	85.6%	26.3%	26.5%	6046	5.53	30.8%	95.9*	-2	0.721	766
3.06	6919	1958	2260	86.6%	34.4%	37.0%	6666	4.26	40.1%	94.4*	1	0.707	875
2.94	6721	2005	2307	86.9%	41.2%	46.5%	6431	3.30	48.5%	93.9*	-1	0.655	758
2.83	6757	2113	2440	86.6%	55.1%	61.8%	6431	2.58	65.3%	90.9*	-1	0.619	680
2.74	7443	2204	2520	87.5%	62.2%	75.5%	7147	2.20	73.1%	90.4*	2	0.598	848
2.65	7607	2249	2566	87.6%	80.4%	100.6%	7305	1.76	94.2%	86.2*	-1	0.576	904
2.57	7421	2306	2637	87.4%	80.7%	110.4%	7066	1.42	95.1%	88.1*	-3	0.532	900
2.50	7084	2381	2764	86.1%	104.2%	146.2%	6679	1.03	124.4%	75.9*	-2	0.527	719
2.43	4615	1952	2798	69.8%	199.1%	280.2%	3981	0.68	244.2%	52.3*	0	0.460	335
2.37	1141	964	2911	33.1%	233.4%	365.6%	329	0.20	319.3%	-26.1	-52	0.150	5