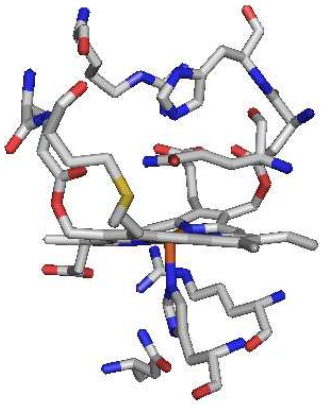
University of Natural Resources and Applied Life Sciences, Vienna
Department of Chemistry, Division of Biochemistry
Metalloprotein Research Group

# Characterization of the myeloperoxidase mutant Asn421Asp produced in CHO and HEK cells

Diploma thesis



submitted by

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# Index

# 1. Introduction

## *1.1. Recombinant expression of proteins*

The recombinant expression of proteins enables the production of relatively large amounts of proteins, which can be either obtained only by great effort and costs out of the natural resources or are not accessible at all. Furthermore, the recombinant expression allows rather easily the production of mutant variants of proteins like the MPO mutant Asn421Asp.

There is a great variety of expression system available. Therefore it is possible to choose one adequate for the complexity of the wanted protein. Other criteria like technical considerations, the ease of use, time and costs have to be taken into account as well.

The rather laborious cultivation of cells, which require complex media with special additives and the big effort necessary to ensure the sterility of the culture as well as the low production rate compared to the biomass are some clear disadvantages of animal cell culture. On the other hand, this expression system allows the expression of more elaborate proteins. They are able to perform posttranslational modifications like correct protein folding, glycosylation, phosphorylation, acetylation and formation of disulfide bridges. Furthermore, the purification of the proteins is facilitated by secretion into the media.

In contrast to human mature leucocytes MPO, recombinant produced MPO is a monomer with propeptide. However, glycosylation, heme incorporation and protein folding are very similar. Therefore, the spectral as well as the kinetic properties are comparable. (*1-3*)

### 1.1.1. Properties of CHO cells

Chinese hamster ovary (CHO) cells are a long established fibroblastic cell line and often used in the medical sector. There are a number of reasons for that. First, adherent as well as suspension cultivation is possible and second they are relatively stable against mechanical and chemical stress. Further, it is possible to produce serum- free complex

proteins and no human pathogens are known as contaminants. At last they provide high protein quality due to human-like post-translational modification.
In 1958 the CHO-K1 cell line was derived as a sub clone from the parental cell line by a biopsy of ovary of an adult Chinese hamster (*Cricetulus griseus*) by Puck. (*4*)

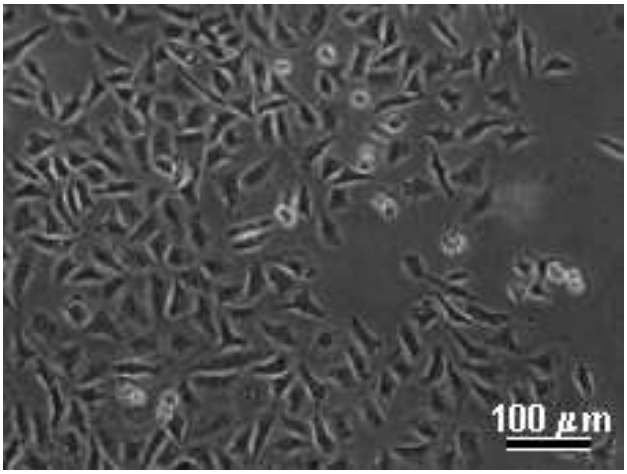



**Figure 1-1:** CHO-K1 cells with high density (IFO Animal Cell Bank, Japan)

### 1.1.2. Dihydrofolate reductase negative cells and gene amplification

Dihydrofolate reductase (DHFR) plays a central role in the synthesis of nucleic acid precursors. A dhfr$^-$ CHO cell line was developed by Urlaub and Chasin. (*5*) These mutants are not able to use the de novo synthesis way. However they are still viable if precursors for salvage synthesis are present in the media. This defect can be compensated by transfection with a *dhfr* gene on a vector containing the gene of interest. If the cells are cultivated in nucleotide free media, only transfected cells possessing the *dhfr* gene can survive. Methotrexate (MTX) is a competitive inhibitor of DHFR. Cells that are cultivated with a small amount of MTX increase their DHFR production by gene amplifying. Gene amplification involves a segment of 100 to 2 000 kb, thus the neighboring gene of interest is amplified too. (*6*)

### 1.1.3. Properties of HEK cells

The HEK 293 cell line is used extensively across many scientific disciplines and is considered as a first choice for recombinant expression of many proteins. The transformation of human embryonic kidney (HEK) cells after the exposure to sheared

fragments of human adenovirus type 5 (Ad5) DNA generated the widely used HEK 293 cell line. (*7*) The transformation was performed by Graham, Smiley, Russell and Nairn in 1977.


**Figure 1-2:** HEK 293 cell (*7*)

## *1.2. Myeloperoxidase (MPO)*

Myeloperoxidase (MPO, EC 1.11.1.7) is a heme containing enzyme and the most abundant protein in human neutrophils. It plays an important role in the antimicrobial armory of neutrophils, the major effector cell of the innate immune system. (*8*)

### 1.2.1. Heme peroxidases superfamilies

The majority of the currently known peroxidases belong to two superfamilies, which arose independently. They differ greatly in their primary and tertiary structure as well as in their prosthetic heme group. The non-animal peroxidase superfamily is mainly represented by catalase peroxidases, ascorbate peroxidases, cytochrome *c* peroxidases, manganese and lignin peroxidases and plant secretoric peroxidases. The other superfamily, the peroxidase-cyclooxygenase superfamily, includes myeloperoxidase (MPO), eosinophil peroxidase (EPO), lactoperoxidase (LPO) and thyroid peroxidase. A common feature of the non-animal peroxidase superfamily is to contain a ferriprotoporphyrin IX chromophore at their active site, which is not covalently linked. The peroxidase-cyclooxygenase superfamily possesses a ferriprotoporphyrin IX derivate covalently linked to the protein by autocatalytic formation of two ester bonds with highly conserved aspartate and glutamate residues. MPO is unique in possessing

an additional sulfonium ion linkage between the heme 2-vinyl group and a conserved methionine 243. (*9*)

MPO shows 70%, 51% and 44% homology in amino acidswith EPO, LPO and TPO. (*10-12*)

## 1.2.2. Structure

The name myeloperoxidase itself indicates its myeloid origin. It was formerly called verdoperoxidase due to its intensive green color. (*13*) With an isoelectric point of > 10, MPO is evidently a strongly basic protein (*14*). It is a glycosylated homodimer with a molecular mass of approx. 150 kDa. Each half consists of one heav*y* α-chain with a molecular weight of 58.5 kDa and one light β-chain with a molecular weight of 14.5 kDa. (*15*) Two identical monomers are linked together by a disulfide bond. (*16*)

Selective cleavage of this connecting disulfide bridge yields the monomers. The spectral and catalytic properties of MPO monomer are indistinguishable from those of the intact enzyme. (*17*) Additionally, there are six intrachain disulfide bonds. Five of them are found in the heavy chain, one in the light chain. (*18*)

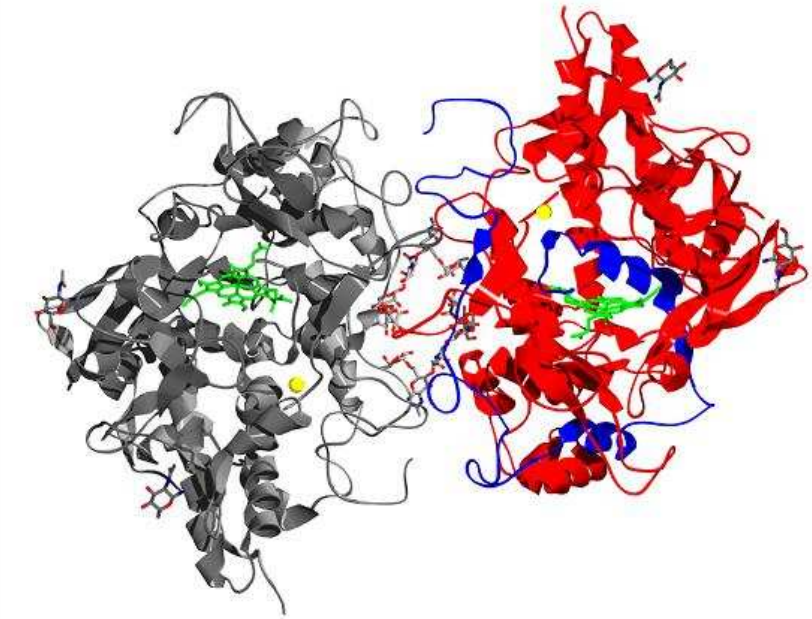


**Figure 1-3:** MPO homodimer, each monomer consisting of one heavy and one light chain and one heme

The MPO heavy chain sequence contains five potential asparagine glycosylation sites (Asn-X-Ser and Asn-X-Thr), four of them (Asn157, Asn189, Asn225, Asn317) had been

biochemically confirmed. (*9*) Moguilevsky et al. showed that the sugar content of the natural MPO is about 12%, whereas the sugar content of the recombinant produced MPO is 15% of the total mass. (*3*)

The heme is a derivative of protoporphyrin IX, in which the methyl groups on the pyrrole rings A and C have been modified. That is why an ester formation with the carboxyl groups of Glu242 and Asp94 (the numbering starts after the propetide) is possible. A third covalent link is a sulfonium ion linkage between the sulfur atom of Met243 and the terminal β-carbon of the vinyl group on pyrrole ring A.

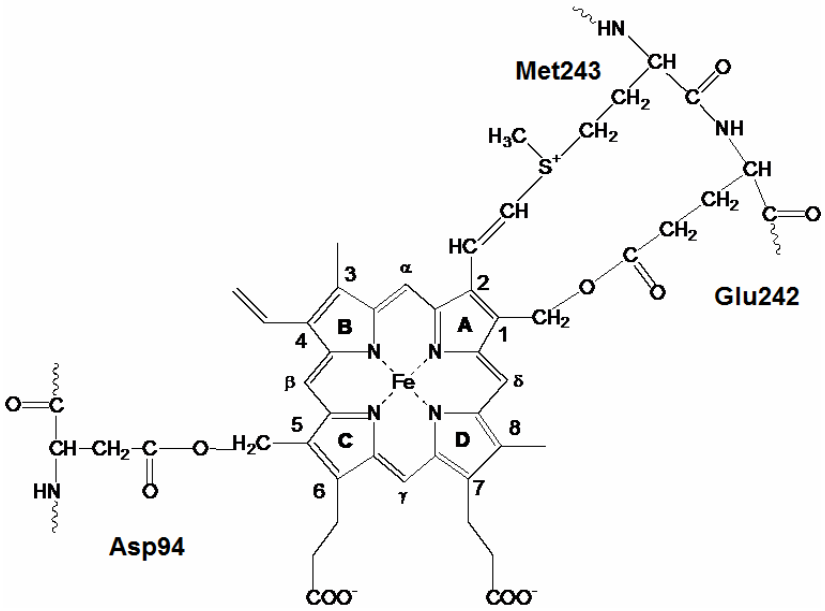



**Figure 1-4:** Heme structure with covalent links between the pyrrole rings and residues Asp94, Glu242 and Met243

This unique sulfonium linkage significantly affects the redox properties of MPO. It distorts the heme from planarity, resulting to a bow-shaped structure and withdraws electron density because of its positive charge. (*8, 19*) The absorption maximum of peroxidases with a covalently-linked heme is at 413 nm and already red shifted compared to other peroxidases with no covalently-linked heme group. The ring deformation of MPO leads to a Soret maximum at 430 nm, hence causes an even stronger red shift. (*20*)

His336 is the proximal ligand in MPO. It is conserved in all mammalian peroxidases as well as the neighboring Asn421 and it is of crucial importance for the maintenance of the redox properties of the heme iron.

The distal heme cavity is occupied by the side chains of Gln91, His95, Arg239 and five water molecules, which form hydrogen bonds with the residues, the propionate group on ring C as well as themselves. In the resting ferric, high-spin state of MPO, the iron atom is positioned slightly to the proximal side. The $N_\varepsilon$ of His95 is hydrogen bonded via a buried water molecule linked to Asp237, His250 as well as to a chain of four water molecules leading to the molecule surface. (*8, 19, 21*)

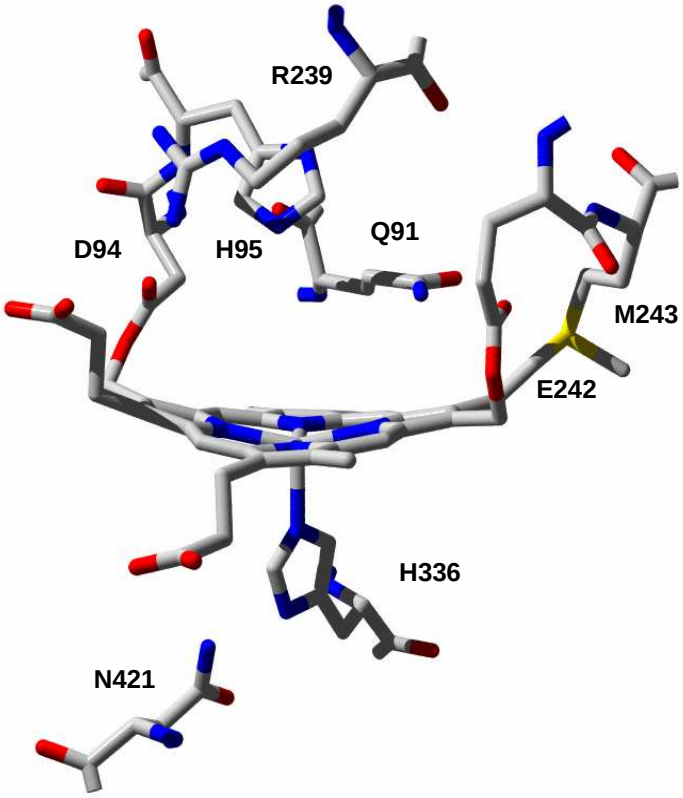



**Figure 1-5:** The non- planar porphyrin ring and its covalent attachment to the protein via two ester bonds and one sulfonium linkage (*21*)

Each half of dimeric MPO possesses a calcium. Its binding site has a typical pentagonal bipyramidal coordination. Most of the ligands belong to the large polypeptide chain, except Asp96, which is adjacent to distal His95 and is part of the small polypeptide chain. (*19*) As the removal of calcium leads to protein precipitation, the calcium ion is not

only important for the correct orientation of the distal histidine but also impacts the interaction between the small and the large polypeptides. (*21*)

## 1.2.3. Reaction mechanism

Change in oxidation- and spin-status of heme proteins allows the spectrophotometrical observation of redox intermediates as well as their interconversion.

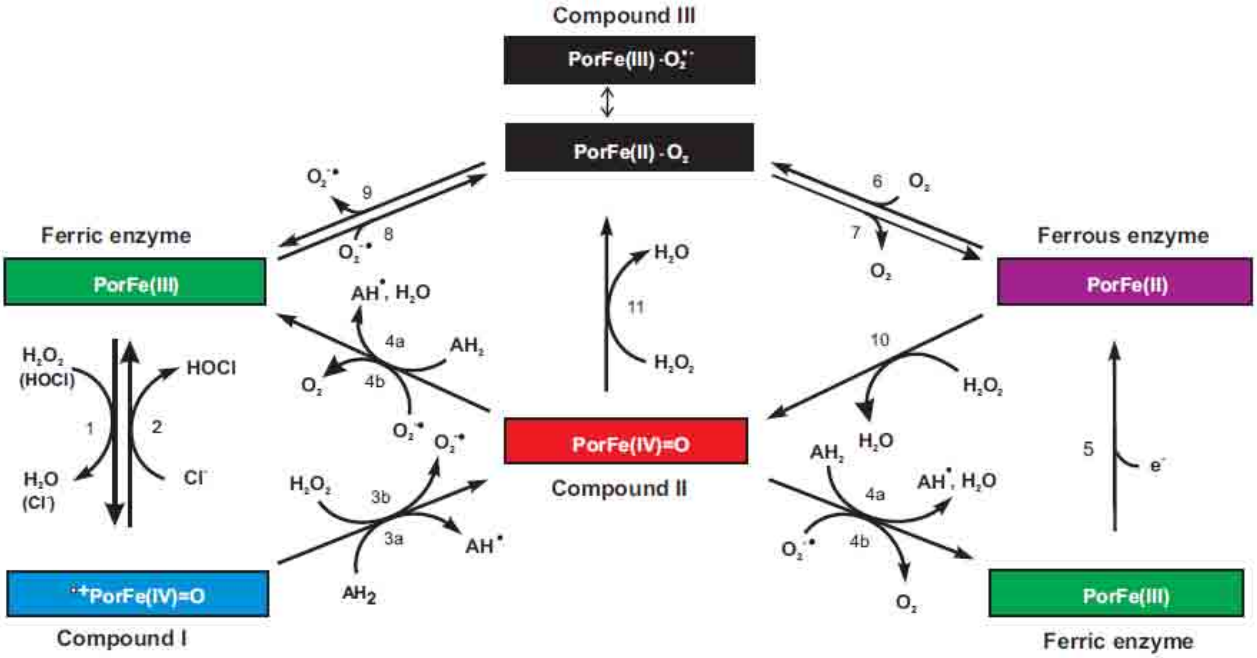



**Figure 1-6:** Redox pathways in myeloperoxidase (*22*)

The native enzyme is present in its ferric form with a $Fe^{III}$ ion and an absorption coefficient of $\varepsilon = 95\,000\ M^{-1}\ cm^{-1}$ at $\lambda = 430$ nm. The peroxidation cycle and the halogenation cycle are both started by reaction of ferric enzyme with hydrogen peroxide to form compound I. Thereby one oxidizing equivalent is stored as oxyferryl moiety and the other as a porphyrinyl radical cation, which can be transferred to the protein in the absence of exogneous electron donor(s). Its formation happens within milliseconds. The half life of compound I is about 100 ms. (*23*) Its Soret band is still at 430 nm, but the absorption coefficient is about 50% lower (Reaction 1 in Figure 1-6). (*21*)

$$Fe^{III} + ROOH \rightarrow \text{Compound I} + ROH \qquad E^{0'}(cI/Fe^{III}) = 1160\ mV \qquad (1)$$

Peroxidation cycle

As mentioned before the reaction starts with the formation of compound I coupled with the reduction of $H_2O_2$. Compound I is reduced via an one-electron step, forming compound II, an oxyferryl species (Reaction 3 in Figure 1-6). (*21*)

$$\text{Compound I} + AH \rightarrow \text{Compound II} + A\bullet \qquad E^{0'}(\text{cI/cII}) = 1\,350\ \text{mV} \qquad (3)$$

Another one electron oxidation reduces compound II back to the native enzyme (Reaction 4 in Figure 1-6).

$$\text{Compound II} + AH \rightarrow Fe^{III} + A\bullet + H_2O \qquad E^{0'}(\text{cII/Fe}^{III}) = 970\ \text{mV} \qquad (4)$$

The first electron transfer forming compound II is much faster than the second transfer that leads to back to the native enzyme form. Thus, the peroxidation cycle is dominated by compound II. The cycle can be summarized with the following equation:

$$ROOH + 2\,AH \rightarrow ROH + 2A^{\bullet} + H_2O$$

Physiological relevant organic peroxidase substrates for MPO include a range of endogenous compounds. MPO can oxidize a wide range of substrates due to the unusually large reduction potential of its compound I/compound II couple. (*8*) Especially MPO compound I is a very potent oxidant. (*21*)

Reduction to ferrous MPO

Human peroxidases are able to release molecular oxygen at low rates if one-electron donors are absent. Responsible for this (pseudo-) catalytic reaction is an alternative cycle involving ferrous peroxidase, compound II and compound III, (Reactions 7, 10 and 11 in Figure 1-6). Under strictly anaerobic conditions and in the presence of a suitable electron donor ferric MPO is reduced to its ferrous form.

$$\text{MPO}\,(Fe^{III}) + e^{-} \rightarrow \text{MPO}\,(Fe^{II}) \qquad E^{0'}(Fe^{III}/Fe^{II}) = 25\ \text{mV} \qquad (5)$$

Ferrous MPO can react with dioxygen to compound III, a ferrous-dioxy/ ferric-superoxide complex (Reaction 6 in Figure 1-6). $H_2O_2$ mediates transition of the ferrous MPO form to compound II (Reaction 10 in Figure 1-6) and transition of compound II to compound III (Reaction 11 in Figure 1-6). This last reaction is very slow and therefore it seems to be the rate-limiting factor in the (pseudo-) catalytic cycle of human peroxidases. (*21*)

Halogenation cycle

Compound I is a very reactive species, it can undergo a two-electron reaction with halides and pseudohalides:

$$\text{Compound I} + X^- \rightarrow Fe^{III} + HOX \qquad (2)$$

The reduction of compound I to ferric MPO is coupled with the oxidation of halides and pseudohalides to their corresponding hypohalous acids. All heme peroxidases are able to oxidize iodide, but due to its high reduction potential MPO is able to oxidize even chloride at a reasonable rate. The ease of halide ions oxidation is: $I^- > Br^- > Cl^-$.

Cyanide binding

In its native form the heme iron of MPO is in a high-spin configuration with a maximum of unpaired electrons ($S$ = 5/2). Cyanide binds very tightly and converts MPO into a low-spin configuration with a minimum of unpaired electrons ($S$ = 1/2). Another so called low-spin ligand is nitrite, binding directly to heme iron, in contrast to high-spin ligands like fluoride or chloride. These low-spin ligands are responsible for a significant red shift in the UV/VIS spectra.

## 1.2.4. Biosynthesis

Human MPO is encoded by a single gene, spanning 14 kb on the long arm of chromosome 17 and its expression is restricted to myeloid cells. A variety of transcription factors regulate normal MPO gene expression in tissue and differentiation specific manner. Myeloperoxidase synthesis terminates as myeloid progenitors enter the

myelocyte stage of differentiation. (*24*) The cDNA for MPO consists of a signal peptide sequence and several consensus sequences for asparagines linked glycosylation.

The primary translation product is a 80 kDa protein precursor (prepro MPO), which is released into the endoplasmatic reticulum (ER). The MPO precursor is co- translationally glycosylated with a bloc of $GlcNAc_2Man_9Glu_3$ residues. This step and the removal of the signal peptide lead to the formation of the enzymatically inactive apopro MPO. Glycosylation is of great importance for the association of the ER molecular chaperons calreticulin (CRT), calnexin (CLN) and ERp57. Insertion of heme into the peptide backbone of apopro MPO generates the already enzymatically active pro MPO. (*25*) The three covalent bonds connecting the protein with the heme are formed through an autocatalytical process. However, most mechanistic details of this process are still unknown.

After the acquisition of heme, which is crucial for subsequent processing, pro MPO undergoes a series of proteolytic events. A 74 kDa intermediate remains after the removal of the 125 amino acid propeptide. In a second proteolytic step the intermediate is cleaved into a heavy and a light subunit, linked via covalent bonds associated with the heme group. Two subunits are interacting to an approximately 150 kDa dimer, linked by a disulfide bond. Figure 1-7 A gives an overview of the maturation of MPO.

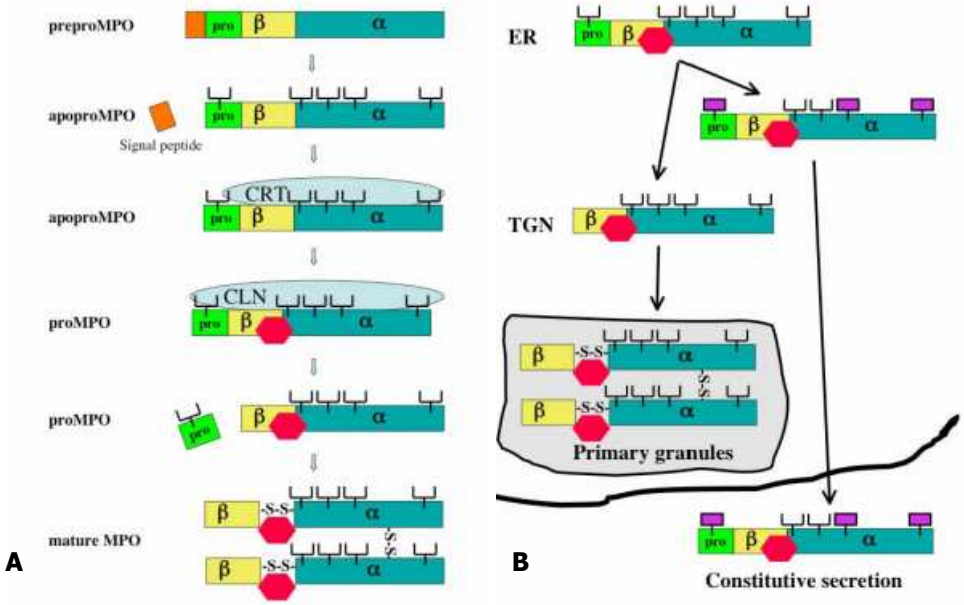



**Figure1-7: A -** MPO biosynthesis **B-** Storage in granules or secretion (*24*)

After passing through the ER and the complex trans-Golgi network (TNG), MPO precursors either reach their final intracellular destination in azurophilic granules or exit into the extracellular space as shown in Figure 1-7 B. (*24*)

## 1.2.5. Physiological role

Myeloperoxidase is the most abundant protein in neutrophils, it makes up to 5% to the dry weight of the cells. The primary function of neutrophils is the phagocytosis leading to the destruction of microorganism. (*26*)

During phagocytosis a microbe is ingested into a phagosome. Azurophilic granules become attached to the phagosome, their membranes fuse and the content of the granules including MPO and a number of cationic antimicrobial proteins are discharged into the cleft between the bacterial cell wall and the inner lining of the phagosome. A burst of oxygen consumption is associated with these events and most of the extra consumed oxygen, is converted into toxic oxygen metabolites. (*26*) The first product of this respiratory burst is the formation of the superoxide anion ($O_2^{\bullet}$) by the NADPH-oxidase. The superoxide anion dismutates to $H_2O_2$ and dioxygen. The toxic properties of $H_2O_2$ itself are not sufficient enough to kill and degrade the entity present in the phagosome. However the reactivity is increased by reaction with iron to form hydroxyl radicals ($OH^-$) or by reaction with peroxidases. As described before peroxidases catalyze the reaction with halides to form highly potent bactericides. (e.g. HOCl)

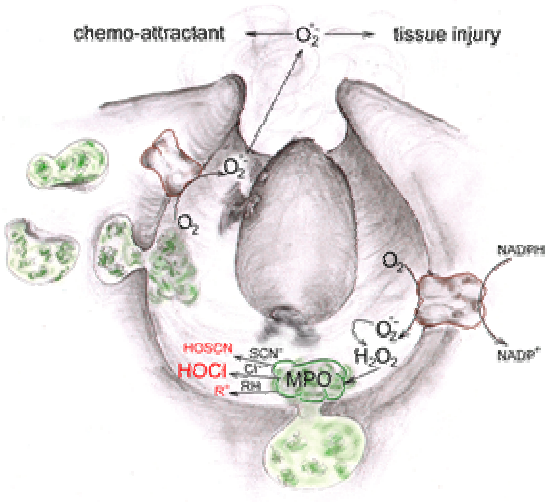



**Figure1-8:** Inside the phagosome: MPO is released and catalyzes the reaction of hydrogen peroxide and (pseudo) halides ions to potent bactericides.

The initial protein concentration is very high (30% - 40%) and diluted in course of time due to osmotic influx. An appropriate ion concentration inside the phagosome is necessary to compensate the consumption of protons. This is achieved by several ion pumps. (*27*)

The oxidants generated by peroxidases play a key role in microbial killing and viral inactivation, but excessive or misplaced generation of these reactive species has also been linked to tissue damage. (*8*) Hence, the peroxidases MPO, EPO and LPO are associated with disease like e.g. artherosclerosis, cystic fibrosis, asthma, neurodegenerative disease and carcinogenesis.

### 1.2.6. N421D mutant

For a better understanding of the interactions within the active center and its surroundings, specific mutants of myeloperoxidase were created. One of these mutants is the variant N421D in order to probe the role of the proximal asparagine in MPO catalysis.

In the peroxidase – cyclooxygenase superfamily the proximal histidine (His336) interacts with a fully conserved asparagine. So far it was assumed that the His336 interacts with the carbonyl oxygen of Asn421. The impact of this interaction was unknown. However this orientation, further referred as orientation OHis (shown in Figure 1-8), would lead to a very close contact between the amid group of the Asn421 and the positively charged guanidinium group of Arg333. Since it is very unlikely to find a neutral guanidium at this position, the hydrogen distance between these two groups would be less than 1 A. This would result into strong repulsive interactions. Flipping of these residues could result into an orientation OArg. In this orientation the carbonyl group of Asn421 points towards Arg333 (shown in Figure 1-9). However, if His336 is present in its neutral form, there would be again a very unfavorable hydrogen contact. Hence, the OArg orientation is only stable when His336 is present in its anionic form. (*9*)

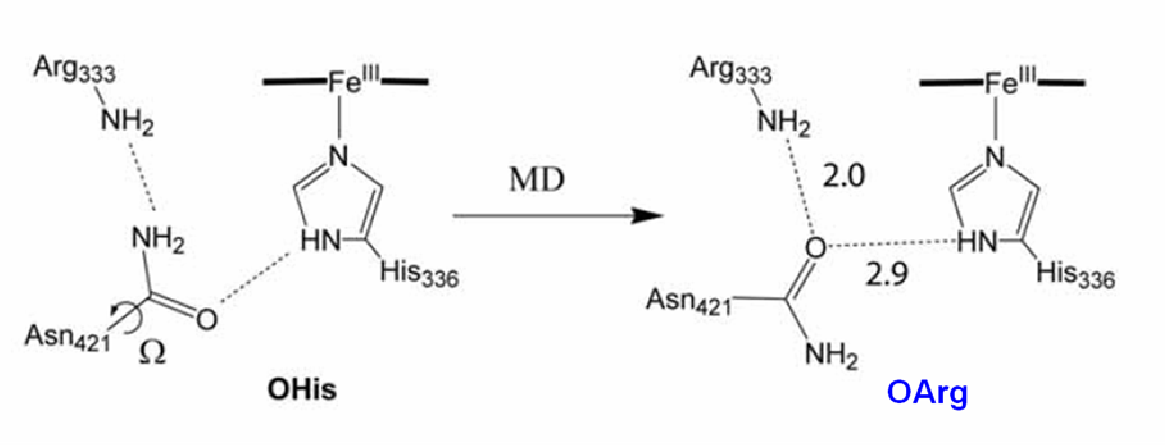



**Figure 1-8:** Molecular dynamics simulation. The starting situation is the OHis orientation and a neutral His 336. (*9*)

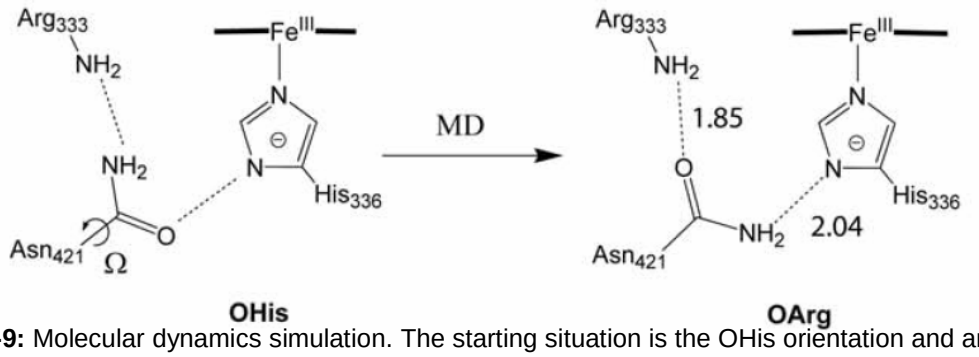



**Figure 1-9:** Molecular dynamics simulation. The starting situation is the OHis orientation and an anionic His 336 (*9*)

Analysis of the catalytic properties of this N421D mutant should underline the importance of the close His-Asn-Arg interaction for the maintenance of the heme cavity architecture.

# 2. Aims of investigation

## *2.1. Production of the mutant Asn241Asp in animal cell culture*

Expression of recombinant MPO and its mutants in CHO cells has been described by Moquilevski (*3*) and Kooter (*28*)

One of the main objectives of this work was the production of mutant protein at an adequate yield, but since the expression in CHO cells was insufficient, alternative strategies were explored. One approach was to increase productivity by gene amplifying with MTX in *dhfr*$^-$ CHO cells. Another was to establish the cultivation of HEK 293 cells as an expression system. Since the latter was the more successful approach endeavors have been made in order to optimize its productivity.

## *2.2. Characterization of the recombinant protein*

The spectrophotometrical properties and peroxidation as well as halogenation activity of the mutant were investigated and compared with wild-type MPO. Obtained biochemical data should be discussed with respect to the three-dimensional structure of MPO and recent molecular simulations. Finally, the role of the environment of proximal histidine in both heme peroxidase superfamilies (His-Asp versus His-Ans) should be outlined.

# 3. Materials and Methods

## *3.1. Cloning of the recombinant protein*

### 3.1.1. DNA preparations

#### 3.1.1.1. Vector for transfection into CHO cells

The vector pmCBm6 (see Figure 3.1.) was used as a cloning and expression vector in CHO dhfr$^-$ cells. It was provided by Lukas Mach, Department of Applied Genetics and Cell Biology at BOKU.

It possesses a multiple cloning site (BamHI restriction site), an ampicillin and neomycin resistance gene and a DHFR encoding site. However, the specific size and sequence of this vector is unknown.

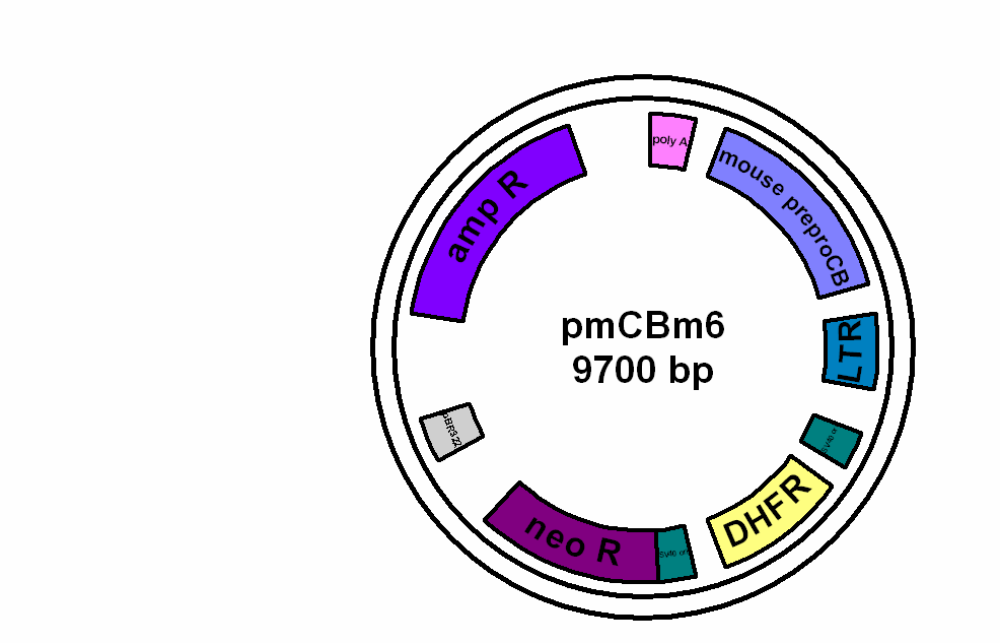



**Figure 3-1:** pmCBm6 vector containing the mouse prepro CB

#### 3.1.1.2. Vector for transfection into HEK 293 cells

The cloning vector pcDNA3.1 (see Figure 3.2.) was purchased from Invitrogen.

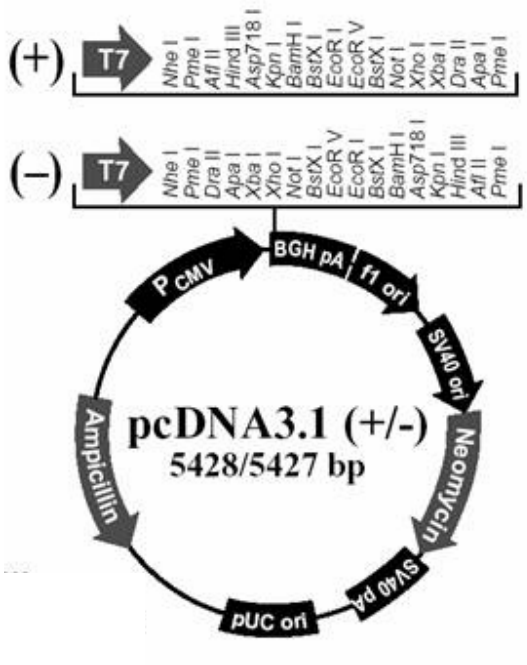



**Figure 3-2:** pcDNA3.1 vector

The vector containing the MPO N421D mutant cDNA insert was already available in the beginning of this work. Hence, the following DNA preparations were performed in order to clone the insert of the pcDNA3.1 vector into the pmCBm6 vector.

### 3.1.1.3. Insert DNA amplification by PCR

Since the pmCBm6 vector only possess a BamHI restriction site the straightforward way of cutting the insert out of the pcDNA3.1 vector and then directly ligate it into the vector with the *dhfr* gene was not possible. An alternative approach had to be explored.

In order to amplify the insert DNA for further cloning into pmCBm6 a polymerase chain reaction (PCR) has to be performed. A miniprep (see chapter 3.3.1.) of the vector pcDNA3.1 containing the MPO insert was used as template.

During PCR, the template DNA is denatured and subsequently specific primers are able to anneal to the single stranded DNA. Afterwards a DNA polymerase synthesizes a new DNA strand complementary to the template. This procedure is repeated various times, so eventually there will be sufficient insert DNA for cloning.

Procedure:

Chart 3-1 shows the components of the PCR probe for the insert DNA preparation, which were pipetted into a PCR tube and Chart 3-2 shows the PCR program used for the amplification. A pcDNA3.1/N421D Miniprep was used as a template and to start the reaction 1 µL Pfu Polymerase was added.

**Chart 3-1:** PCR Mastermix

| | |
|---|---|
| Template | 1µL |
| Primer T7 | 2 µL |
| Primer NDBamH I_2 | 2 µL |
| dNTP's | 1 µL |
| 10* Buffer + $MGSO_4$ | 10 µL |
| AD | 260 µL |

**Chart 3-2:** PCR program

| | | |
|---|---|---|
| 94℃ | 2 min | |
| 94℃ | 30 sec | |
| 52℃ | 30 sec | 28* |
| 72℃ | 5 min | |
| 72℃ | 10 min | |
| 10℃ | hold | |

Material and Equipment

| | | |
|---|---|---|
| Primer NDBamH I_2 | 10* Buffer + $MGSO_4$ | Fermentas |
| Primer T7 | Pfu Polymerase | Fermentas |
| dNTP's | Thermocycler | Techne |

### 3.1.1.4. Restriction enzyme digest

A restriction endonuclease is a bacterial enzyme that is able to recognize and cleave at a certain nucleotide sequence. Originally it is a bacterial protection mechanism against invading virus DNA but nowadays restriction enzymes are also an important tool for recombinant DNA technology.

Procedure

In order to perform a restriction enzyme digest the double stranded DNA was incubated with a suitable restriction enzyme and corresponding buffer recommended by the supplier. The temperature and the duration of the incubation vary depending on the restriction enzyme. In order to prepare the vector and the insert for the ligation both were incubated with the restriction enzyme BamH I for 120 min at 37℃. The Charts 3-3 and 3-4 show the components of these digests. To prevent the reconnection of the linearized

vector 1 µL alkaline phosphatase was added to the digests after 60 min and after 90 min.

**Chart 3-3:** Restriction enzyme digest of vector

| pmCBm6 vector digest with BamH I | |
|---|---|
| pmCBm6 vector 4x mini | 90 µl |
| BamH I (10 units/µL) | 10 µL |
| Buffer BamH I | 4 µL |

**Chart 3-4:** Restriction enzyme digest of insert

| Insert N421D multiplied by PCR | |
|---|---|
| Insert | 90 µl |
| BamH I (10 units/µL) | 10 µL |
| Buffer BamH I | 4 µL |

After the restriction a purification step is necessary to remove the remaining restriction enzymes in order to prevent DNA damage. Therefore the plasmid miniprep kit was used with some modifications. The digest was transferred to a column already containing 500 µL neutralization buffer and centrifuged for 1 min at 12 000 rpm. The following steps were performed like described in chapter 3.3.1.

**Chart 3-5:** Used restriction enzymes

| Enzyme | Sequence | Incubation temperature | Concentration | Buffer | Producer |
|---|---|---|---|---|---|
| BamHI | 5' G/GATCC 3' | 37℃ | 10 units/µL | Buffer BamH I | Fermentas |

Material and Equipment

| | | | |
|---|---|---|---|
| Plasmid to be digested | pmCBm6 | Plasmid DNA GeneJET™ | |
| 10 x restriction buffer | see table alkaline | Plasmid Miniprep Kit | Fermentas |
| Incubator | Memmert BE400 | Restriction enzyme | see table |
| Alkaline phosphatase | Fermentas | | |

### 3.1.1.5. Purification of DNA fragments from agarose gel

As a further purification step an agarose gel electrophoresis was performed with the linearized vector and the insert DNA followed by cutting-out of the specific DNA bands and extraction of the DNA from the gel slices. Therefore, a DNA

extraction kit (Fermentas) was used. The kit utilizes chaotropic salts to promote the binding of the DNA to the specifically prepared glass particles. After capturing of the DNA impurities can be removed. Finally, DNA can be eluted either in TE buffer or water.

Procedure

The DNA containing gel slice was excised by a razor blade and transferred into a pre-weighed Eppendorf tube. For each volume of gel, three volumes of binding solution had to be added, followed by incubation at 55℃ for 5 min in order to dissolve the agarose. Then 5 µL of silica powder suspension were added and again the probe was incubated for 5 min at 55℃. Afterwards the silica powder was spinned down to form a pellet and the supernatant was removed. 500 µL ice cold washing buffer was added. This step was repeated three times. Eventually the DNA was eluted from the silica into 50 µL water and the supernatant carefully transferred into a new Eppendorf tube.

Material and Equipment

| | | | |
|---|---|---|---|
| DNA Extraction Kit | Fermentas | Vortex | Janke Kunkel |
| Microcentrifuge | Sigma | | IKA VF2 |
| 1.5 mL tubes | Eppendorf | Thermosmixer compact | Eppendorf |

### 3.1.1.6. Ligation

The connection between the linearized vector and the prepared insert is catalyzed by a DNA ligase. Thereby the sticky ends are joined together through the formation of covalent phosphodiester bonds between the 5'-phosphate ends of one nucleotide with the 3'-hydroxyl end of another. The ratio between vector and insert should be approximately 1 : 6. Therefore, it is necessary to estimate the amount of vector DNA and insert DNA. On that account equal amount of those solutions were applied and separated by agarose gel electrophoresis.

Procedure

The appropriate amount of vector and insert DNA are mixed together in an Eppendorf tube and incubated for two hours at room temperature. Afterwards the probe was purified in order to remove the remaining ligase.

Material and Equipment

| | | | |
|---|---|---|---|
| Linearized vector and insert | | T4 DNA ligase (10x) | BioLabs |
| T4 DNA ligase | BioLabs | E- gel | Invitrogen |
| E-gel runner | Invitrogen | | |

## 3.1.2. Transformation of bacteria

### 3.1.2.1. Cultivation of bacterial strains

The *E. coli* strain JM107 was used for all cloning experiments. The vector pmCBm6 carries an ampicillin resistance gene. Therefore bacterial cells containing a plasmid can be selected by the addition of ampicillin to the LB-medium.

Procedure

LB-medium was inoculated with the *E. coli* strain JM107 and incubated at 37℃ under agitation.

Material and Equipment

| | | | |
|---|---|---|---|
| LB- medium: | 5 g NaCl<br>5 g Peptone<br>2.5 g Yeast extract<br>HQ- $H_2O$ added to 500mL | Ampicillin stock solution: | 100 mg sodium ampicillin / 1mL $H_2O$<br>Sterile filtrated and stored at –20℃ |
| $LB\text{-}medium_{AMP}$: | LB- medium and addition of 100 µg / mL Ampicillin | | |
| Shaker | INFORS HT Ecotron | | |

### 3.1.2.2. Preparation of electrocompetent *E. coli* JM107 cells

In order to prepare *E. coli* cells for electroporation they need to be transferred into a medium of low conductivity.

Procedure

10 mL $LB\text{-}medium_{AMP}$ were inoculated with cells and grown overnight at 37° and 180 rpm. The next day the overnight culture was used to inoculate 500 mL $LB\ medium_{AMP}$ that was then incubated at 37° and 180 rpm until the $OD_{600}$ reached 0.6. Then the culture was incubated for 30 min on ice and afterwards the cells were

harvested by centrifugation for 8 min at 4 000 rpm and 4℃. The supernatant was discarded and the pellet resuspended in 250 mL chilled 1 mM Hepes. The cells were again harvested by centrifugation for 10 min at 4 000 rpm and 4℃ and once again the supernatant was discarded and the pellet resuspended in 125 mL chilled 1 mM Hepes. After centrifugation and decanting under the conditions described above the cells were resuspended in 50 mL chilled 1 mM Hepes and likewise centrifuged. The pellet was then dissolved in 15 mL cooled 10% glycerol, followed by a last centrifugation step for 10 min at 5 000 rpm and 4℃. The pellet was finally resuspended in 2 mL cooled 10% glycerol and aliquots of 60 µL respectively 120 µL were stored at -80℃.

Material and Equipment

| | | | |
|---|---|---|---|
| LB medium$_{AMP}$ | | *E. coli* JM107 | |
| 1 mM Hepes: | 238.3 mg Hepes | Shaker | INFORS HT Ecotron |
| | HQ- $H_2O$ added to 1000 mL | Spectrophotometer | Hitachi U- 1100 |
| 10% Glycerol: | 5.05 mL 99% Glycerin | Centrifuge | Sorvall RC5C |
| | HQ- $H_2O$ added to 50 mL | Centrifuge beaker | |

## 3.1.2.3. Electroporation of bacteria

Due to a strong but short electric puls, the bacterial cell wall becomes permeable to larger molecules like plasmid DNA which can then enter and transform the bacteria cell.

Procedure

At first 1 mL SOC- medium had to be pre-warmed for each transformation sample and the frozen competent cells were slowly thawed on ice. The samples had to be prepared as shown in Chart 3-6 whereupon dd$H_2O$ was used as negative control and an uncut vector as positive control.

**Chart 3-6:** Transformation sample

| | |
|---|---|
| pmCBm6 / MPO N421D | 15 µL ligation sample |
| pmCBm6 | 15 µL vector control |
| positive control (pcDNA3.1/MPO N421D) | 10 µL dd $H_2O$ + 1 µL |
| negative control (dd $H_2O$) | 10 µL dd $H_2O$ |

The cells were put into a cooled, UV- cross linked electroporation cuvette together with the transformation sample and the electroporation was performed under the following parameters:

1000 Ohm
25 µF
2.5 kV

After electroporation the cells were transferred into prewarmed SOC-medium and after 30 min incubation at 37℃ plated onto selective LB agar and grown overnight at 37℃.

Material and Equipment

SOC- medium: 20 g Peptone
5 g Yeast extract
7.5 g Bacto-agar
2.46 g $MgSO_4.7H_2O$
3.6 g Glucose
0.58 g NaCl
0.19 g KCl
2.03 g $MgCl_2.6H_2O$
HQ- $H_2O$ added to 1000 mL

LB- $agar_{AMP}$: LB-medium (500 mL)
100 µg / mL Ampicillin
Electroporation cuvette Biorad
Gene Pulser Biorad
UV- cross linker

## 3.1.3. Screening of transformed bacterial cells

### 3.1.3.1. Miniprep

Miniprep refers to a small scale preparation of highly pure plasmid DNA from bacterial cell suspensions. The procedure is based on alkaline lysis, which denatures nucleic acid as well as proteins. Furthermore it takes advantage of the fact that plasmid DNA is relative small and super-coiled whereas bacterial chromosomal DNA is longer and less super-coiled. Therefore, after addition of a neutralization solution chromosomal DNA and proteins precipitated whereas plasmid DNA can renature and stay in solution.

Procedure

1.5 mL of saturated bacterial suspension was transferred into a 1.5 mL tube and the cells were precipitated by centrifugation at 10 000 rpm for 2 min. All steps were performed at room temperature. The supernatant was discarded and 250 µL

resuspension solution was added. After vortexing and resuspending the pellet 250 µL lysis buffer was added and mixed by inverting 4 – 6 times. Finally, 350 µL neutralization solution was added and mixed through inverting 4 – 6 times then the chromosomal DNA and cell debris were precipitated by centrifugation at 10 000 rpm for 5 min. Afterwards the supernatant was transferred to a GeneJET™ Spin Column and centrifuged at 12 000 rpm for 1 min. The throughput was collected in a collection tube and eventually discarded. Subsequently a washing step was performed where 500 µL of washing buffer were added to the column and centrifuged for 30 sec at 12 000 rpm. This step was repeated and followed by an additional centrifugation for 1 min to remove the residual washing solution. Then the column was placed into a new 1.5 mL tube and 50 µL of $ddH_2O$ was added. After an incubation period of 2 min the plasmid DNA was eluted by centrifugation for 2 min at 12 000 rpm.

Material and Equipment

| | | | |
|---|---|---|---|
| GeneJET™ Plasmid Miniprep Kit | Fermentas | Microcentrifuge | Sigma 1-15 |
| | | Vortex | Janke Kunkel IKA VF2 |
| Components: | Resuspension Solution | 1.5 mL tubes | Eppendorf |
| | Lysis Solutions | Saturated bacterial culture containing plasmid DNA | |
| | Neutralization Solution | | |
| | RNase A | | |
| | Wash Solution | | |
| | Elution Buffer | | |
| | Collection Tubes (2 mL) | | |
| | GeneJET™ Spin Column | | |

### 3.1.3.2. PCR screening

A PCR Screening is performed with specific primers to amplify the insert DNA in order to proof the integration into the bacterial genome. The DNA fragments can then be separated by agarose gel electrophoresis and positive clones can be determined.

Procedure

The overnight colonies grown on selective ampicillin agar were picked and each dissolved in 50 µL $ddH_2O$. These probes were used for the PCR Screening as well as

for the cultivation of a master plate. Chart 3-7 shows the mastermix components for 20 probes and Chart 3-8 the specific PCR program.

**Chart 3-7:** PCR Mastermix

| | |
|---|---|
| 5'Primer | 12 µL |
| 3'Primer | 12 µL |
| dNTP's (25 mM) | 1,2 µL |
| 10*Taq buffer + $ASO_4$ | 60 µL |
| $MgCl_2$ | 48 µL |
| AD | 260 µL |

**Chart 3-8:** PCR program

| | | |
|---|---|---|
| 95℃ | 4 min | |
| 94℃ | 40 sec | |
| 51℃ | 30 sec | 28* |
| 72℃ | 1 min 30 sec | |
| 72℃ | 10 min | |
| 10℃ | hold | |

After vortexing 6 µl of Taq Polymerase were added.

Material and Equipment

| | | | |
|---|---|---|---|
| PCR tubes | Biozym | 3'Primer | |
| Thermocycler | Techne | MgCl2 | Fermentas |
| dNTP's | | 10*Taq buffer + ASO4 | Fermenats |
| 5'Primer | | Taq Polymerase | Fermentas |

## 3.1.3.3. Restriction screening

In order to confirm not only the integration of the insert DNA itself but also to proof the correct orientation and size a restriction screening was performed. Therefore, it is necessary to choose restriction enzymes, which produce a specific band pattern in either case. Chart 3-9 enlists the restriction enzymes used.

**Chart 3-9:** Restriction Enzyms

| Enzyme | Sequence | Incubation temperature | Buffer | Producer |
|---|---|---|---|---|
| BamH I | 5' G/GATCC 3' | 37℃ | BamH I buffer | Fermentas |
| Bgl II | 5' A/GATCT 3' | 37℃ | Buffer 3 | Bio Labs |

Procedure

An overnight culture of already positive clones confirmed by PCR screening was grown at 37℃ followed by a miniprep preparation the next day. Subsequently two small restriction digests were performed with the restriction enzymes BamHI I and Bgl II. The components of the digest were pipetted together in an Eppendorf reaction tube as

shown in the Charts 3-10 and 3-11 and incubated for 90 min at 37℃. Before the probes could run on an agarose gel a further purification step was necessary. Therefore, the same procedure as mentioned in chapter (3.1.1.4.) was used.

**Chart 3-10:** Digest with BamHI I

| Digest with BamH I | |
|---|---|
| Miniprep | 17 µl |
| BamH I (10 units/µL) | 1.7 µL |
| Buffer BamH I | 0.5 µL |

**Chart 3-11**: Digest with Bgl II

| Digest with Bgl II | |
|---|---|
| Miniprep | 17 µl |
| Bgl II (10 units/µL) | 1.7 µL |
| Buffer 3 | 0.5 µL |

Material and Equipment

| | | | |
|---|---|---|---|
| restriction enzyme | see table | Plasmid Miniprep Kit | Fermentas |
| 10 x restriction buffer | see table | Plasmid DNA Purification Kit | Fermentas |
| Incubator | Memmert BE400 | Microcentrifuge | Sigma 1-15 |

## 3.1.3.4. DNA sequencing

To verify that the final DNA construct contains the correctly amplified insert DNA sequencing is necessary. The comparison of the obtained sequence with the expected sequence can confirm the correct integration of the desired insert into the vector.

Procedure

In an Eppendorf tube 10 µL of the plasmid DNA of one selected positive clone (80 ng/µL) are mixed together with 4 µL of the specific primer solution (5 pmol/µL). Chart 3-11 lists the selected primers for the DNA sequencing.

The probes were sent to the company AGOWA (Berlin, Germany).

**Chart 3-12:** Primer for DNA sequencing

| primer name | binding site |
|---|---|
| endesign_rv | ← 179 |
| 1720_rv | ← 807 |
| 1291_fw | 1464 → |
| 4667_fw | 709 → |
| N421D_fw | 1797 → |

Material and Equipment

| | | | |
|---|---|---|---|
| Plasmid DNA GeneJET™ | | Primers | see table |
| Plasmid Miniprep Kit | Fermentas | Microcentrifuge | Sigma 1-15 |

### 3.1.3.5. Cryocultures of sequenced clones

To store positive clones over a longer period cryocultures were made.

Procedure

900 µL of an overnight culture and 100 µL of 30% glycerol were pipetted into a cryotube. The tube was stored at –80°C.

Material and Equipment

| | | | |
|---|---|---|---|
| 30% Glycerol: | 15.15 mL 99% Glycerin | $LB\text{-}medium_{AMP}$ | |
| | $H\text{-}H_2O$ added to 50 mL | Shaker | INFORS HT Ecotron |

### 3.1.3.6. Endotoxin free midiprep

Endotoxins are lipopolysaccharids and therefore elements of the outer membrane of gram-negative bacteria. During the normal plasmid DNA isolation endotoxins are set free and remain in the miniprep. However, to protect the cells after the transfection it is necessary to remove these endotoxins. Hence a special “Endofree- Kit” was used that contains an additional buffer for the removal of endotoxins. Furthermore, it is advisable to use endotoxin- and pyrogen-free material for the isolation.

Procedure

At first a starter culture is grown for 8h at 37°C from which 100 µL are used to grow 100 mL overnight culture. The next day the overnight culture was centrifuged at 4°C and 6 000 g for 15 min. Afterwards the supernatant was discarded and the pellet resuspended in 10 mL buffer P1 with a RNase A end concentration of 100 µg/mL. Additionally 10 mL of buffer P2 were added, inverted 4 - 6 times and incubated at room temperature for 5 min. After the incubation 10 mL chilled buffer P3 were added, mixed by inverting 4 - 6 times and finally the lysat was poured into QIAfilter Cartridge. After another incubation at room temperature for 10 min the lysat was filtered and 2.5 mL ER buffer were added and mixed by inverting. While the filtrate was incubated on ice for 30 min, a QIAGEN- tip was equilibrated. Then the filtrate was applied to the column and

washed 2 times with 30 mL QC buffer. Finally, the DNA was eluted with 15 mL QN buffer and precipitated by the addition of 10.5 mL isopropanol. The solution was centrifuged for 30 min at 4℃ with 15 000 g and the DNA pellet washed with 70% ethanol for 10 min at 15 000 g. Eventually the pellet was air dried and for 5 – 10 min and dissolved in 100 µL TE-buffer.

Material and Equipment

| | | | |
|---|---|---|---|
| LB-medium$_{AMP}$ | | Centrifuge | Sorvall RC5C |
| Shaker | INFORS HT Ecotron | EndoFree Plasmid | |
| Microcentrifuge | Sigma 1-15 | Purification Maxi Kit | QIAGEN |

### 3.1.3.7. Quantification of the isolated DNA

To estimate the concentration of the DNA ultraviolet (UV) spectrophotometry is most commonly used. DNA has a maximum absorption at 260 nm, therefore the DNA solution is measured at this wavelenght. An absorption of 1 equals 50 µg/mL double stranded DNA.

As the maximal absorption of proteins is at 280 nm, the ratio between the absorption at 260 nm and the absorption at 280 nm is a measure for the purity of the isolated DNA and should be around 1.8. Measurements at 230 nm and 320 nm are further indicators for a contamination. At 320 nm neither proteins nor nucleic acids absorb therefore the absorption should be at 0.0. The ratio between the absorption at 230 nm and the absorption at 260 nm should be less than 0.6 and is a measure for the contamination with phenols, peptides, carbohydrates and aromatic hydrocarbons.

Procedure

First the DNA sample was diluted in TE buffer (usually 1 : 50). The spectrophotometer was calibrated with TE buffer too and finally the DNA was measured and the concentration as well as the ratios of purity were calculated.

Material and Equipment

| | | | |
|---|---|---|---|
| DNA sample | | EndoFree Plasmid | |
| Spectrophotometer | Zeiss | Purification Maxi Kit | QIAGEN |

## *3.2. Protein production*

### 3.2.1. Cell culture

#### 3.2.1.1. Sterile technique

Every treatment of the cells or solutions in contact with the cells was performed in the laminar flow hood. The fan and the UV lamp were turned on at least 15 min before working. Furthermore the working place, every vessel and every other material inserted into the laminar was cleaned with 70% ethanol. After finishing work in the laminar, the bench was cleaned again with 70% ethanol and fan and UV lamp was turned off after 30 min.

Reusable material like centrifuge tubes, supply bottles with cap and pasteur pipettes were autoclaved before use and after contamination and cleaned. Cell waste and contaminated disposable material were decontaminated and afterwards discarded.

Material and Equipment

| | | | |
|---|---|---|---|
| Laminar flow hood | Heraeus Instruments | $CO_2$ incubator | Heraeus |
| 70% Ethanol | Merck | Incubator BD 240 | Binder |
| UV lamp | Osram HNS 15 watt | | |

#### 3.2.1.2. Stock solution preparation

The powder chemicals were weighed, dissolved in dd$H_2O$ except MTX, hemin and hematin which were dissolved in 0.1 N NaOH and filtered sterilely in the laminar. They were stored in suitable aliquots at –20℃ or 4℃. T he supplements hemin and hematin, added to the media for the improvement of heme incorporation, had to be prepared freshly before use. The liquid supplements FBS and L-glutamine were aliquoted respectively and stored at –20℃.

**Chart 3-13:** Stock solutions

| | |
|---|---|
| Penicillin (10 000 U/mL) | G418 (40 000 U/mL) |
| Streptomycin (10 mg/mL) | Hemin (1 mg/mL) |
| MTX (10 µM) | Hematin (1 mg/mL) |

Material and Equipment

| | | | |
|---|---|---|---|
| Penicillin G (sodium salt) | Sigma | L-glutamine 200 mM (10x) | Gibco |
| Streptomycin (sulfate salt) | Sigma | Syringe (20 mL) | Braun |
| Methotrexate (MTX) | Sigma | Syringe Filter | Roth |
| Geneticin sulfate (G418) | Gibco | Hemin | Sigma |
| Fetal bovine serum (FBS) | Gibco | Hematin | Sigma |
| FBS, dialyzed | Gibco | | |

### 3.2.1.3. Media preparation

Medium supplies the cells with amino acids, vitamins, salts and an energy source. Moreover do cells need a pH around 7.4 to grow and therefore $NaHCO_3$ is added. Together with the $CO_2$ enriched atmosphere in the incubator it can provide pH adjustment with a $pK_a$ of 6.3. However to fulfill its purpose the flasks need to be left slightly opened. Addition of phenol red, which is red at pH 7.4, indicates acidic conditions below pH 6.5 by turning yellow and alkaline conditions above pH 7.8 with a color change to purple. The supplement FBS is complex and contains among other growth factors, hormones, adhesion factors, carrier molecules and a trypsin inhibitor. To avoid contamination antibiotics are added. In this case penicillin was added against gram-positive bacteria and streptomycin against gram-positive as well as gram-negative bacteria.

Procedure

Powdered MEM-α-medium and 2.2 g $NaHCO_3$ per liter were dissolved in HQ-$H_2O$. The pH was adjusted to approximately 6.9, because due to the following filtration the pH increases for 0.2 – 0.3 units. Finally the medium was filtrated sterile with bottle top filters in batches of 2 L into autoclaved bottles with cap and then stored at 4℃.

Powdered DMEM medium was prepared similar but only the amount of added $NaHCO_3$ was changed to 3.7 g/L. Before use all media, trypsin and supplements were tempered at 37℃ in the water bath.

Material and Equipment

| | | | |
|---|---|---|---|
| Water bath | Julabo | pH- Meter | Radiometer Copenhagen |
| Bottle top filters | | $NaHCO_3$ | Merck |
| (PES membrane, 0.2 µm) | Nalgene | | |

### 3.2.1.4. Cultivation of cells

Human Embryo Kidney (HEK) cells were grown in DMEM + Glutamax® supplemented with penicillin/streptomycin (100 µg/mL) and 10 % fetal bovine serum (FBS). The cells were maintained in a humidified atmosphere containing 5% $CO_2$ at 37°C.

Chinese Hamster Ovary (CHO) cells were cultivated in MEM - α- medium supplemented with penicillin / streptomycin (100 µg/mL), 10% FBS and 2% L-glutamine. To cultivate $dhfr^-$ mutants MEM-α-medium (+) and non-dialyzed FBS was used whereas for $dhfr^+$ mutants MEM-α-medium (-) and dialyzed FBS was used and MTX for gene amplification was added. The cells were grown in a humidified atmosphere containing 5% $CO_2$ at 37°C.

In order to avoid frequent tempering, batches of 500 mL complete medium were prepared and stored at 4°C.

### 3.2.1.5. Cell trypsinization

Trypsin is a pancreatic serine protease and is used for detaching cells from the surface of the culture flask. It is necessary to remove FBS by washing as it contains a trypsin inhibitor as well as $Ca^{2+}$ and $Mg^{2+}$ ions which support cell attachment. To avoid irreversible damage the process has to be stopped by the addition of FBS containing medium.

Procedure

The medium was discarded and the surface of the cell culture vessel washed with PBS:

| | |
|---|---|
| CCF T25 | 5 mL |
| CCF T75 | 15 mL |
| CCF T175 | 25 mL |

The cells were then covered with the appropriate amount of trypsin-EDTA:

| | |
|---|---|
| CCF T25 | 500 µL |
| CCF T75 | 1 000 µL |
| CCF T175 | 2 000 µL |

Afterwards the flask was put in the incubator for a few minutes until the cells had detached and showed a round shape, which was observed in the microscope. The cells were knocked off and the trypsinization process was stopped by addition of media. The remaining adhering cells were flushed by repeated pipetting steps and the cell suspension was eventually transferred into a new flask and incubated with no further manipulation for 24 hours.

Material and Equipment

| | | |
|---|---|---|
| Trypsin (0.05%) | Invitrogen | Phosphate buffered saline (PBS) |
| cell culture vessel | Greiner | without $Ca^{2+}$, $Mg^{2+}$, pH 7 |
| sterile pipettes | TRP | 1.54 mM $KH_2PO_4$ |
| | | 154 mM NaCl |
| | | 2.71 mM $Na_2HPO_4.7H_2O$ |

### 3.2.1.6. Media change

Depending on the metabolism and the growth rate of the cells the nutrients of the medium are metabolized and toxic metabolites can be accumulated. Furthermore, certain components like penicillin and L-glutamine are not stable for more than five days at 37°C, therefore it is necessary to passage the cells or to change the media every 3 - 4 days. Supplements like hemin or hematin, which are added for better heme incorporation, are prepared freshly before use. They are added to fresh media directly before media change.

Procedure

The old medium was poured off and stored at –4°C. If necessary the surface was washed with PBS, then new medium was added optionally together with media supplements and the flasks were incubated again at 37°C.

### 3.2.1.7. Subcultivation

Monolayer cell cultures stop growing when the surface is fully occupied due to contact inhibition. Therefore the cells should be transferred to a larger culture vessel when they have reached 80 – 90% confluency. Depending on the growth behavior of the cells the appropriate dilution factor needs to be estimated. For this calculation the surface ratio of

the cell culture vessels needs to be considered and the fact that at low dilution rates the cells have to be passaged more frequently.

**Chart 3-14:** used culture vessels and its surface ratio

| Cell culture vessels | Surface ratio ($cm^2$) |
|---|---|
| 96 well | 0.28 |
| 12 well | 3.9 |
| 6 well | 10 |
| Petri dish ∅60 | 20 |
| T25 | 25 |
| Petri dish ∅90 | 58 |
| T75 | 75 |
| T125 | 125 |
| T175 | 175 |
| Triple Flask | ~500 |
| Cell factory | 6 320 |

Procedure

The cells were trypsinated and transferred into a new cell culture vessel according to the desired dilution.

### 3.2.1.8. Thawing of the cells

To preserve cells over a long period they are stored at –80℃ and in liquid nitrogen at –196℃. In order to prevent any cell damage to the cells rapid but gentle thawing is necessary. The cryocultures of CHO cells and already transfected HEK cells were very stable and did easily attach to the surface of the culture vessel. Therefore it was not necessary to remove DMSO whereas HEK cells which have not been transfected did not adhere to the surface if the DMSO remained in the media.

Procedure

For CHO cells and transfected HEK cells:

6 – 10 mL tempered medium were propounded in a T25 flask. The vial with the cells was thawed in the hand without shaking. The cells were pipetted carefully with a single-use

pipette into the medium. The cells were incubated for the following 24h without any manipulation or movement in order to assist cell attachment.

For HEK cells:

The thawed cells were suspended carefully in 6 mL cooled media and centrifuged for 10 min at 1 700 U/min. Then the supernatant was removed and the cells were resuspended in 6 mL temperated media before the transfer into a T25 flask.

Material and Equipment

| | |
|---|---|
| single use pipette | Roth |

### 3.2.1.9. Freezing of the cells

The cells used for cryogenic storage should be healthy and capable of proliferation; therefore a T75 flask with about 80% confluency is used. As a cryogenic vial should contain 2 – 5 x $10^6$ cells per 1 mL suspension it is possible to obtain three vials from one T75- flask. The freezing medium consists of complete growth medium and a cryogenic protectant like DMSO.

Procedure

The supernatant was removed; the cells were washed with PBS and afterwards detached by trypsinization. The process was stopped by the addition of media containing FBS and the media with the suspended cells was centrifuged for 10 min at 170 g. The supernatant was discharged and the pellet resuspended thoroughly in the cooled freezing media consisting of normal growth medium with 10% DMSO and portionated in cooled cryogenic vials. The vials were pre-frozen at -80℃. After 24h some of them were transferred to the liquid nitrogen tank for long time storage.

Material and Equipment

| | | | |
|---|---|---|---|
| Cryogenic vials | Nalgene | Centrifuge | Hettich |
| DMSO | Sigma | | |

### 3.2.1.10. Transfection

Two different transfection methods were used for CHO and HEK cells. CHO cells were transfected by lipofection whereas for the HEK cells $Ca^{2+}$ precipitation was used.

Lipofection:

Lipofectin interacts spontaneously with DNA to form a lipid DNA complex, which fuses with the cell membrane of the cultured cells resulting in the DNA uptake.

Procedure

The recommended confluency for lipofection is 50 – 70%. As the transfection was performed in a 12 well plate, for each transfection 2 µg DNA was diluted in 200 µL serum free media and after the addition of 4 µL TurboFect to the diluted DNA the solution was incubated for 15 - 20 min at room temperature. Finally, the solution was added dropwise to each well and incubated at 37℃. After 24h the media were changed.

Material and Equipment

Turbofect Fermentas

$Ca^{2+}$ precipitation:

DNA can enter cultured cells by endocytosis as a copreciptate with calcium phosphate. Some of these coprecipitates are able to escape from endosomes or lysomes and reach the cytoplasm from where they are transferred into the nucleus.

Procedure

The transfection was performed in a 12 well plate with a confluency of 40 – 50%. Before passaging the cells into the 12 well plate, they were pretreated with 25 µg/mL polyethylenimine (PEI) as the HEK cells don't adhere so well on the surface.

Four hours before transfection the cells were incubated with 25 µM chloroquine. 1 µg DNA was diluted in 35.5 µL 0.25 M $CaCl_2$ before 35.5 µL HEPES-buffered saline pH 7.05 was added during vortexing. Finally the solution was pipetted carefully to the cells and incubated. After 24h the media were changed.

Material and Equipment

Polyethylenimine Chloroquine

### 3.2.1.11. Selection and singularization of positive cells clones

Twenty-four hours after transfection the cells were subcultured in various dilution steps and finally 48h after transfection they were subcultured in selective media containing

geneticin. So only positively transfected clones, which possess the neomycin resistance gene can survive and proliferate.

For singularization of effectively transfected cells it is possible to either pick single cells forming little islands generated from one geneticin resistant clone and transfer it to a 96 well plate or to singularize cells by serial dilution in 96 well plates.

Procedure

The selective media containing geneticin were used for cultivation 48h after transfection and was renewed every 2 – 3 days. After one week the dead cells were washed away from the plate and only positive clones were left on the plate.

It is possible to transfer single clones grown apart from each other to 96 well plates by manual picking of the cells. Therefore, the clones were marked under the microscope, subsequently detached from the plate with a pipette tip and eventually resuspended in a well of a 96 well plate.

On the other hand it might be necessary to singularize cells by serial dilution if the cell colonies have grown too close to allow manual picking. Thereby 100 µL of media was pipetted into each well of a 96 well plate besides the well A1 in which 200 µL of the trypsinated cell suspension ($5 \times 10^3$ - $1 \times 10^4$ cells) was transferred. From this cell suspension 100 µL were transferred into the well B1 and carefully mixed. This dilution step was repeated all over the whole first column and the last 100 µL were discarded. With a multichannel pipette 100 µL from each column were transferred to the following column whereas the final 8 x 100 µL were again discarded. At last 100 µL media were added to each well and the cells were incubated at 37°C.

Material and Equipment

Multichannel pipette Gibon

### 3.2.1.12. Screening of the transfected cell clones

For the selection of clones which express the protein in an active form and a reasonable yield two screening methods, ABTS screening and immune detection were performed.

Immune Detection:

Already little amounts of protein can be successfully detected with specific antibodies in an immune detection assay. Thereby the specific antibody is captured by a secondary antibody connected with a phosphatase that reacts with 5-brom-4-chlor-3-indolylphosphate (BCIP) and nitroblue tetrazolium (NBT) to a colourful compound indicating the presence of the protein.

Procedure

The harvested supernatant is dotted on a nitrocellulose membrane and after drying of the dots, the membrane was incubated on a shaker for 1h in 10 mL blocking solution at room temperature. Then the dot blot was incubated for 2h in 10 mL binding solution, containing 2 µL of the primary antibody again at room temperature and agitation. After a washing step with binding buffer, the membrane was transferred into a 10 mL binding solution containing 0.5 µL of the secondary antibody and was shaken at room temperature for another 2h.

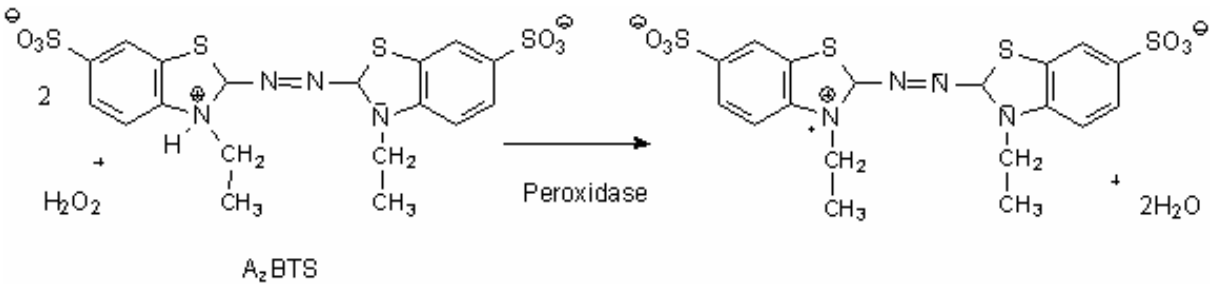
Finally the blot was washed again with binding buffer to remove the unbound secondary antibody and for the formation of the colorful compound 5 mL of AP-buffer were added to the membrane as well as 33 µL NBT and 16.5 µL BCIP.

Material and Equipment

| | | | |
|---|---|---|---|
| Nitrocellulose membrane | Amersham Biosciences | Blocking buffer: | 0.2 g BSA<br>10 mL PBS |
| AP- buffer: | 100 mM Tris- HCl<br>pH 9.5<br>100 mM NaCl<br>5 mM $MgCl_2$ | Binding buffer: | 2g BSA<br>100 mL PBS<br>2 µL Tween 20 |
| | | NBT | Promega |
| Shaker | INFORS HAT Ecotron | BCIP | Promega |

ABTS Screening:

Peroxidase catalyzed reduction of hydrogen peroxide to water is connected with oxidation of ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)) to a green product leading to a color change of the tested media.




Procedure

The assay was performed directly in the harvested media of the 96 well plate. In each well 2 µL 10 mM $H_2O_2$, 10 µL 200 mM phosphate buffer pH 5.0 and 10 µL 50 mM ABTS were added. Furthermore, a negative and positive control for easier comparison of the color change of the media was performed.

**Figure 3-3:** ABTS Screening in 96 well plate

Material and Equipment

| | | | |
|---|---|---|---|
| ABTS | Sigma | Phosphate buffer 200 mm pH 5.0 | |
| $H_2O_2$ | Sigma | Microtiter plate | Nunc |

## 3.2.1.13. Cell factory

In order to produce recombinant protein at a higher yield, cell factories are used for large scale production. A cell factory is a closed multilayer system with two outlets. One is used for media exchange and the other one for gas exchange via a sterile filter. The multilayer system provides enough surface area for monolayer cell growth and the handling itself is relatively simple. Media exchange is performed two times a week and harvested media are filtrated via bottle top filter.

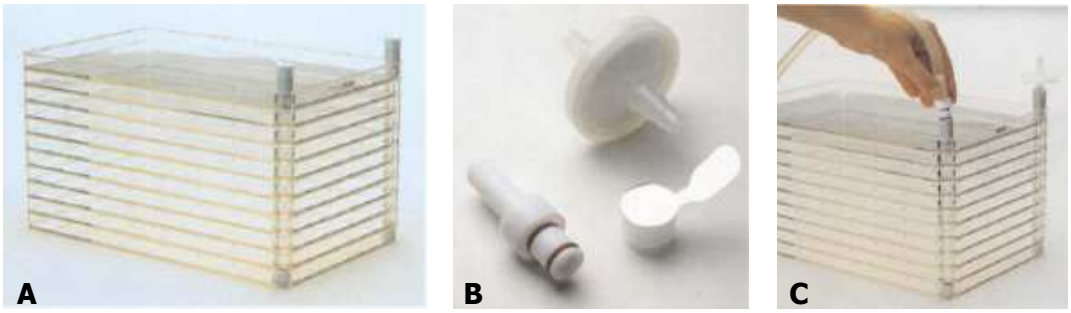



**Figure 3-4:** A – Cell factory, B – Starter kit, C – Connecting with hose

Procedure

After unpacking and the attachment of a sterile air filter, the cell factory was filled with 2 L media in which the trypsinated cells of six T175 flasks were suspended. For filling of the cell factory an additional sterile funnel with attached tube was connected and the cell factory needed to be stored sideways so that in the 10 chambers the media was distributed equally. The media were changed every 3 – 4 days and the supernatant was filtrated with a bottle top filter before storage at 4℃.

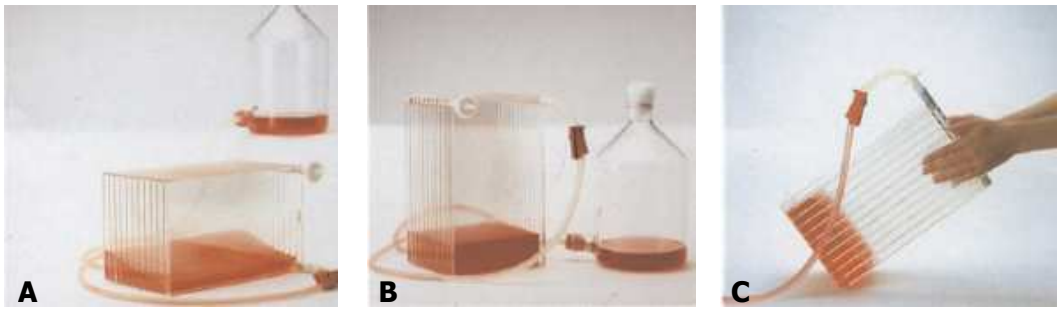



**Figure 3-5:** A – Filling, B – Checking even distribution, C – Setting in incubation position

Material and Equipment

| | | | |
|---|---|---|---|
| Cell factory | Nunc | Vacuum pump CVC 2 | Vacuumbrand |
| Sterile air filter | Nunc | Bottle top filter | Sarsted Inc. |
| Sterile funnel | Nunc | | |

## *3.3. Protein purification*

The protein is purified from the harvested media by a cation-exchange-chromatography (Carboxylmethyl-Sepharose CM). The eluated fractions are pooled and concentrated before the next purification step, the gel filtration. Finally, the purified enzyme is pooled

again, concentrated and desalted. For long time storage the protein is portioned, lyophilized and kept at –80°C.

### 3.3.1. Cation-exchange-chromatography

Ion-exchange-chromatography separates proteins due to their charge. The main principle of the purification is the reversible interaction between charged molecules and the oppositely charged matrix. The bound proteins can be eluted either by increasing salt concentration or pH change.

CM- Sepharose Fast Flow is a weak cation exchanger with good flow properties and the negatively charged carboxymethyl group maintains a good capacity at a pH range of 6 – 13, so that cationic proteins like the MPO mutant N421D can bind to the gel.

Procedure

The packed gel has been stored in 20% ethanol and was washed with RO water and then equilibrated with 20 mM phosphate buffer pH 7.0. Before the cell culture supernantant was loaded on the column it was filtrated and diluted with the same amount of RO water in order to prevent column overload due to the media components. Afterwards the gel was washed with the equilibration buffer until no detectable absorbance at 280 nm was found. For the elution of the protein a linear gradient of 0 – 1 M NaCl in 20 mM phosphate buffer pH 7.0 was used. The eluate was collected in fractions and analyzed spectrophotometrically in order to estimate the protein content at 280 and 410 nm before storage at 4°C. For storage the column was first washed with 200 mM NaCl in 20 mM phosphate buffer pH 7.0, then with RO water and eventually with 20% ethanol.

Material and Equipment

Phosphate buffer 20 mM pH 7.0 + 1M NaCl
Phosphate buffer 20 mM pH 7.0 + 2M NaCl
Matrix CM Sepharose “Fast Flow” Pharmacia Biotech
Pump Pharmacia Peristaltic
Fraction Collector Frac 920 Amersham Bioscience
Gradient former Model 395 BioRad
Phosphate buffer 20 mM pH7.0
Ultrasonic bath Sonorex Super RK510H Bandelin
Filter HVLPO4600, pore size 0.45 µm Millipore

## 3.3.2. Gel filtration

Molecules in solution can be separated according to their size as they pass through a column containing a gel matrix, in this case Superdex 200. Smaller molecules can diffuse into the pores of the gel matrix and are therefore delayed in their passage down the column whereas larger molecules will be prevented by their size from defusing into pores in the same extent. Hence are the larger molecules leaving the column before the smaller molecules.

Prepacked Superdex 200 HR 10/30 connected with the ÄKTA design system is used for this purification step. Superdex 200 is produced by covalent bonds between dextran and highly cross-linked porous agarose. It gives a good separation of proteins in the molecular range 10 000 - 600 000 Da and is stable over the pH range 3 - 12.

Procedure

The column is stored in 20% ethanol and therefore it is necessary to wash the column with RO water with a flow rate of 0.2 mL/min. Then the column was equilibrated with two column volume 20 mM phosphate buffer pH 7.0 + 0.100 M NaCl before the sample was injected. After the protein was eluated in fractions, the column was washed again with RO water and eventually stored in 20% ethanol.

Material and Equipment

| | | |
|---|---|---|
| Phosphate buffer 20 mM pH 7.0 | ÄKTA | Amersham pharmacia |
| Superdex 200 HR 10/30 | Amersham pharmacia | |

## 3.3. 3. Concentration, buffer exchange and vacuum-drying

To concentrate the purified enzyme and to lower the salt concentration ultrafiltration is performed with Centripreps YM-30. As centrifugation increases the hydrostatic pressure inside the tubes, substances with a molecular weight of less than 30 kDa are forced through the membrane into the filtrate collector, whereas substances with a higher molecular weight, thus the produced protein, remains inside the sample container.

By applying pressure to portioned liquid protein solution water is removed and the fully dried protein can be stored at -80℃ without loss o f activity or quality reduction.

Procedure

The pooled fractions were concentrated until the desired volume for the protein solution was reached, which was performed by centrifugation at 2 500 rpm for 20 min at 4℃. Afterwards 5 mM phosphate buffer pH 7.0 was added to the enzyme solution in the sample chamber for desalting. After centrifugation the filtrate was discarded and this step was repeated several times depending on the sample volume.

The final solution was analyzed spectrophotometrically and then divided in portions, vacuum dried and eventually stored at –80℃.

Material and Equipment

| | | | |
|---|---|---|---|
| Phosphate buffer 5mM pH 7.0 | | Centriprep | YM-30 (15 mL)Amicon |
| Centrifuge | Du Pont Instruments, Sorvall, RC-5C | Speed Vac | SVC-100 H SAVANT Instruments |

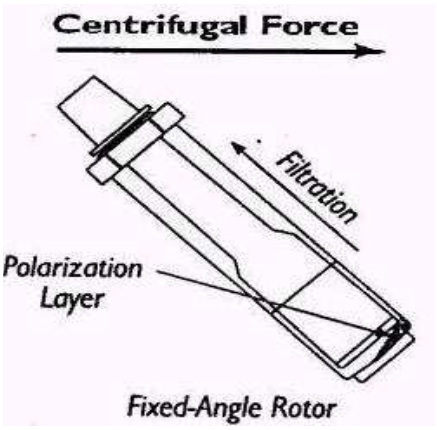



**Figure 3-6:** Filtration during centrifugation

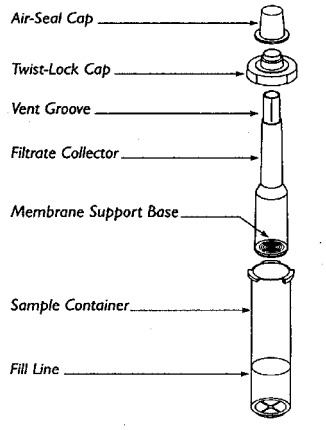



**Figure 3-7:** Centriprep

## *3.4. Protein characterization*

### 3.4.1. Spectral properties

The absorption spectra of MPO and respectively the mutant N421D show besides the protein peak at 280 nm also a Soret peak and its position is depending on heme coordination and covalent linkage. The ratio between the Soret peak and protein peak

describes the purity number of the protein solution. The enzyme concentration can be calculated with Lambert- Beer's law:

$$A = \varepsilon \times c \times d$$

| | |
|---|---|
| A | Absorbance |
| ε | molar extinction coefficient [$M^{-1}$ $cm^{-1}$] |
| c | concentration [mol/L] |
| d | cuvette diameter [cm] |

$$RZ = A_{heme} / A_{280}$$

| | |
|---|---|
| RZ | purity number |
| $A_{heme}$ | heme peak absorption |
| $A_{280}$ | protein peak absorption |

Procedure

The spectra were measured with RO water as reference. In order to reduce background noise the enzyme solution was first centrifuged and than 0.3 – 1.0 mL were pipetted into the cuvette, set into the light path and a wavelength scan from 200 – 800 nm was performed.

Material and Equipment

Spectrophotometer Specord UV/VIS S10 Quartz cuvette (light path 1 cm)
Diodenarray Zeiss Instruments

## 3.4.2. SDS-Polyacrylamide-gel electrophoresis (SDS-PAGE)

In an electric field charged molecules are able to migrate, with their mobility depending on their charge, shape and size. Electrophoresis is carried out in an inert homogenous carrier like a polyacrylamide gel. The porosity can be determined by the concentration of the reagents acrylamid and methylenebisacrylamide. As the unpolymerized substances are toxic, protective gloves should be worn. Sodiumdodecylsulfate (SDS) is an anionic detergent with denaturing effect, binds on proteins and induces negative charge. β-Mercaptoethanol has a reducing effect and breaks disulfide bonds.

The SDS-PAGE is one of the most commonly used method to separate protein solutions and to determine the relative molecular mass (Mr) as the pace of the protein – SDS complex is directly proportional to its logarithm. However, the electrophoretic movement depends also on the ionic strength, viscosity and temperature of the medium. Therefore,

it is suitable to determine the molecular weight by comparing the protein band with a marker.

In order to visualize the separated protein bands, the gel is stained with sensitive methods like the fast and easy Coomassie-stain.

Procedure

The gels were pipetted together as shown in Chart 3-15.

**Chart 3-15:** Composition of separating and stacking gel

| | Separation gel (for 2 gels) Monomer conc. 10% | Stacking gel (for 2 gels) Monomer conc. 4% |
|---|---|---|
| HQ-$H_2O$ | 3.96 mL | 2.97 mL |
| 1.5 M Tris-HCl | 2.5 mL | - |
| 0.5 M Tris-HCl | - | 1.25 mL |
| Acrylamid/ Bis | 3.33 mL | 670 µL |
| 10% SDS | 100 µL | 50 µL |
| Degasing | | |
| 10% APS | 100 µL | 50 µL |
| TEMED | 10 µL | 10 µL |

The separation gel was cast and then covered with 2-butanol in order to achieve a plain surface. After polymerization the 2-butanol was removed and the surface rinsed with water. The stacking gel was cast and as last step before polymerization the comb was set in order to form the slots. The gels were connected with the electrode unit after polymerization and put into the electrophoresis chamber, which was filled with 1 x running buffer. The samples were diluted with the same amount of 2 x sample buffer, mixed and after removing the comb loaded into the slot with a syringe. The running conditions were 200 V and max. 70 mA and electrophoresis was stopped when the bromphenolblue-front reached the end of the gel. Eventually, the gel was rinsed with water before it was either dyed or blotted.

For staining with Coomassie-stain the gel was incubated in the staining solution on a shaker at room temperature and afterwards transferred into a destaining solution, incubated under the same conditions. The solution was exchanged several times until the bands were clearly visible.

Material and Equipment

| | |
|---|---|
| 5x Electrophoresis buffer: | 15 g/L Tris<br>72 g/L Glycine<br>5 g/L SDS<br>HQ- $H_2O$ added to 1000 mL |
| 1x Electrophoresis buffer: | 200 mL 5 x Electrophoresis Buffer HQ- $H_2O$ added to 1000 mL |
| 2x Sample buffer: | 900 µL SB- Stock<br>100 µL β-Mercaptoethanol |
| 2x Sample buffer Stock: | 2 mL 125mM Tris/HCl<br>1.6 mL Glycerin<br>3.2 mL 15% SDS<br>0.4 mL 0.5% Bromophenol Blue |
| Acrylamide/Bis: | 500 ml<br>46.0 g acrylamide<br>4.0 g Bis |
| Destaining solution: | 40% (v/v) methanol<br>10% (v/v) acetic acid<br>50% (v/v) RO water |
| | filtrated, stored in the dark at 4℃ |
| 1.5 M Tris-HCl, pH 8.8: | 300 ml<br>54.45 g Tris Base<br>pH adjustment with 6 M HCl |
| 10% (w/v) SDS: | 1.0 g SDS<br>10 mL RO water |
| 0.5 M Tris-HCl, pH 6.8: | 100 ml<br>6.0 g Tris Base<br>pH adjustment with 6 M HCl |
| 10% (w/v) APS: | 50 mg ammonium persulfate<br>500 µL RO water |
| N,N,N',N'-Tetramethyle-ethylenediamine (TEMED) | |
| Power supply | BioRad |
| Electrophoresis Equipment | BioRad |
| Shaker | IKA- VIBRAX VXR |
| Staining solution | 0.1% (w/v)Coomassie Blue R-250<br>40% (v/v) methanol<br>10% (v/v) acetic acid<br>50% (v/v) RO water |

## 3.4.3. Western blot

A very sensitive method to detect small amounts of proteins specifically is a Western blot. First, the already via SDS-PAGE separated proteins are transferred from the gel to a nitrocellulose membrane. Therefore the gel, the membrane and filter papers are placed together between two electrodes like a sandwich. The methanol in the transfer buffer removes the SDS and enhances the binding of proteins to the membrane.

For specific detection of proteins immundetection is performed. (see chapter 3.4.12.)

Procedure

The filter papers as well as the nitrocellulose membrane were cut into the appropriate size and equilibrated together with the gel and two filter pads in chilled 1 x transfer buffer. The blot was assembled in the cassette without bubbles as shown in Figure 3-5 and then transferred into the electrode bracket with the membrane at the side of the anode. Next the buffer chamber was filled with 1 x transfer buffer and also a magnetic stirrer was added. The running conditions were set to 100 V at 4℃ for 55 min.

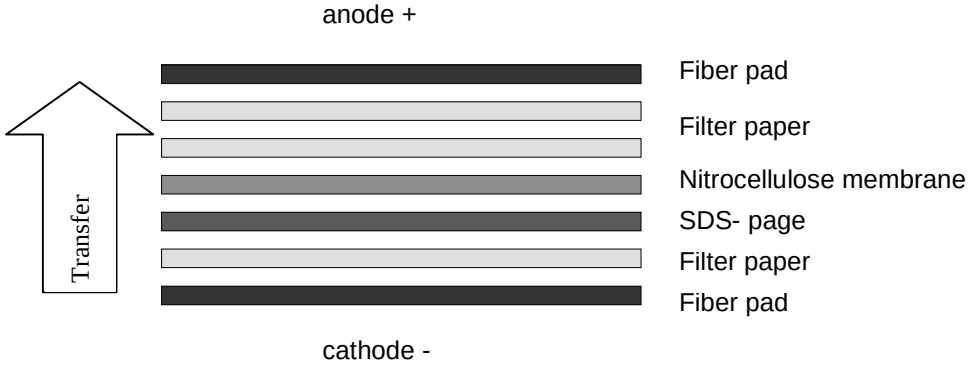



**Figure 3-8**: Assembly of a Western blot

Material and Equipment

5x transfer buffer pH 8.3: 1000 ml
- 15.15 g Tris Base
- 72.0 g glycine

Nitrocellulose membrane: BA 85; 0.45 µm
- Schleicher & Schuell

Power supply BioRad

Filter paper Whatman 3 MM

1x transfer buffer pH 8.3: 1000 ml
- 200 mL 5x transfer buffer pH 8
- 700 mL RO water
- 100 mL methanol

Mini Trans-Blot Electrophoretic Transfer Cell
- BioRad

## 3.4.4. Enzyme activity

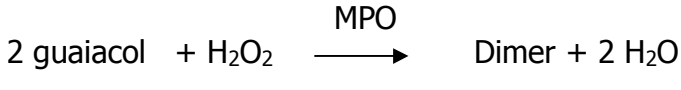
### 3.4.4.1. Peroxidase activity

Guaiacol Assay:

One way to determine the peroxidase activity of MPO and its mutant is the guaiacol peroxidase assay. Thereby guaiacol is used as a substrate for the peroxidatic activity

and the increase of the guaiacol oxidation product is measured as an increase in absorbance at 470 nm at pH 7.0 at 25℃.

$\varepsilon_{470}$ = 26 600 $M^{-1}$ $cm^{-1}$



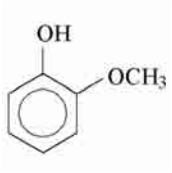




**Figure 3-9:** structure of guaiacol

Procedure

The following solutions except $H_2O_2$ were mixed together in a cuvette with a stirring bar, which was then set into a spectrophotometer. The reaction was started by the addition of $H_2O_2$.

**Chart 3-16**: Reaction mix for guaiacol assay (*29*)

| Stock solution | Volume | Final concentration |
|---|---|---|
| 200 mM phosphate buffer pH 7.0 | 500 µL | 100 mM |
| 1 mM guaiacol | 100 µL | 100 µM |
| Enzyme | 50 µL | |
| RO water | 340 µL | |
| 10 mM $H_2O_2$ (start) | 10 µL | 100 µM |
| Total | 1 000 µL | |

Material and Equipment

Guaiacol Sigma

Quartz cuvette

Spectrophotometer Specord UV/VIS S10

Diodenarray Zeiss Instruments

ABTS Assay:

The peroxidase catalyzed reduction of hydrogen peroxide to water is connected with oxidation of ABTS (see chapter ABTS screening). The increase of the absorption at 414 nm is measured at 25℃.

$\varepsilon_{414}$ = 36 000 $M^{-1}$ $cm^{-1}$

Procedure

The following solutions, except $H_2O_2$, were mixed together in a cuvette with a stirring bar and the cuvette was then set into a spectrophotometer. Again, the reaction was started by the addition of $H_2O_2$.

**Chart 3-17**: Reaction mix for ABTS assay

| Stock solution | Volume | Final concentration |
|---|---|---|
| 200 mM phosphate buffer pH 5.0 | 500 µL | 100 mM |
| 50 mM ABTS | 20 µL | 1 mM |
| Enzym | 50 µL | |
| RO water | 420 µL | |
| 10 mM $H_2O_2$ (start) | 10 µL | 100 µM |
| Total | 1 000 µL | |

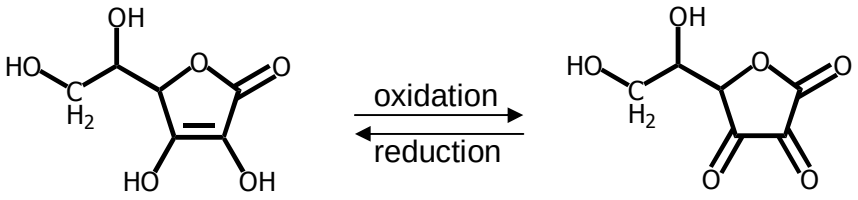
Material and Equipment

| | | | |
|---|---|---|---|
| ABTS | Sigma | Spectrophotometer | Specord UV/VIS S10 |
| Quartz cuvette | | Diodenarray | Zeiss Instruments |

Ascorbate assay:

In this assay ascorbate is used as a substrate for the peroxidatic activity. The decrease in absorbance at 290 nm is measured at 25℃.

$\varepsilon_{290}$= 2 800 $M^{-1}$ $cm^{-1}$




ascorbate dehydroascorbate

Procedure

During the preparation of the ascorbate stock solution, which had to be prepared freshly 100 µM EDTA was added in order to increase the stability. Then the solutions were pipetted into a quartz cuvette and mixed by a stirring bar in the cuvette. The probe was placed into a spectrophotometer and the reaction was started by adding $H_2O_2$.

**Chart 3-18**: Reaction mix for ascorbat assay (*30*)

| Stock solution | Volume | Final concentration |
|---|---|---|
| 67 mM phosphate buffer pH 7.0 | 700 µL | ~95 mM |
| 5 mM ABTS | 100 µL | 500 µM |
| Enzym | 100 µL | |
| 1 mM $H_2O_2$ (start) | 100 µL | 100 µM |
| Total | 1 000 µL | |

Material and Equipment

Ascorbate Merck

Quartz cuvette

Spectrophotometer Specord UV/VIS S10

Diodenarray Zeiss Instruments

## 3.4.4.2. MCD halogenation assay

Myeloperoxidase and its mutant are able to oxidize halids like chloride and bromide. The Monochloro Dimedon (MCD) assay is a very specific method in order to determine the halogenation activity of the enzymes.

$\varepsilon_{290} = 19\,900\ M^{-1}\ cm^{-1}$

$$Cl^- / Br^- + H_2O_2 + H^+ \xrightarrow{MPO} HOCl / HOBr + H_2O$$

HOCl / HOBr + Monochloro Dimedon ⟶ Dichloro Dimedon

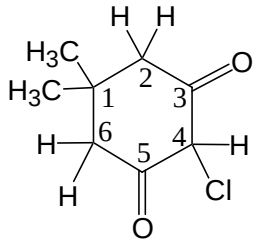



**Figure 3-10:** structure of MCD

Procedure

All of the following components, except $H_2O_2$ were mixed together in a cuvette with a stirring bar, which was then set into a spectrophotometer. The reaction was started with the addition of $H_2O_2$ to the probe and the decrease in absorbance at 290 nm at pH 5.0 or pH 7.0 at 25℃ was measured.

**Chart 3-18:** Reaction mix for MCD assay (*31*)

| Stock solution | Volume | Final concentration |
|---|---|---|
| 200 mM phosphate buffer pH 7.0 or pH 5.0 | 500 µL | 100 mM |
| 1 mM MCD | 100 µL | 100 µM |
| Enzym | 50 µL | |
| 1M NaCl or KBr | 100 µL | 100 mM |
| RO water | 340 µL | |
| 10 mM $H_2O_2$ (start) | 10 µL | 100 µM |
| Total | 1 000 µL | |

Material and Equipment

MCD Sigma

Quartz cuvette

Spectrophotometer Specord UV/VIS S10

Diodenarray Zeiss Instruments

## 3.3.4.3. Cyanide inhibition

Cyanide inhibition during the MCD assay:

The addition of cyanide ($CN^-$) to MCD assays stops the reaction as cyanide binds tightly to heme proteins in the ferric state, thus is leading to the inhibition of the halogenation.

Procedure

While the MCD assay was performed a small amount of higher concentrated cyanide solution was pipetted directly to the probe, which stopped the decrease of the absorbance at 290 nm.

Material and Equipment

| | | | |
|---|---|---|---|
| NaCN | Sigma | Spectrophotometer Specord UV/VIS S10 | |
| MCD | Sigma | Diodenarray | Zeiss Instruments |
| Quartz cuvette | | | |

Inhibition assay:

An inhibition assay is performed by adding an increasing concentration of cyanide solution to a probe of an enzyme activity assay like MCD halogenation assay. More precisely, a varying amount of a cyanide stock solution is added to the probe before starting the reaction. The decrease of activity is then plotted against the increasing cyanide concentration and the constant $K_{diss}$ is calculated.

Procedure

At first a suitable cyanide stock solution was prepared (1 mM or 100 mM). Then a MCD assay probe was mixed together like described in chapter 3.4.4.2. before adding a certain volume of the cyanide solution so that the probe contains the required cyanide end concentration. In order to obtain the same probe volume the difference was balanced with RO water. Eventually the reaction was started with $H_2O_2$.

Material and Equipment

| | | | |
|---|---|---|---|
| NaCN | Sigma | Quartz cuvette | |
| MCD | Sigma | Spectrophotometer Model U-3000 | Hitachi |

# 4. Results

## *4.1. Cloning of the human MPO mutant N421D*

### 4.1.1. Insert preparation

In order to obtain the N421D insert for cloning into the pmCBm6 vector a pcDNA3.1 / N421D miniprep was used as a template for PCR amplification.

Figure 4-1 shows the amplified N421D insert separated by agarose gel electrophoresis.

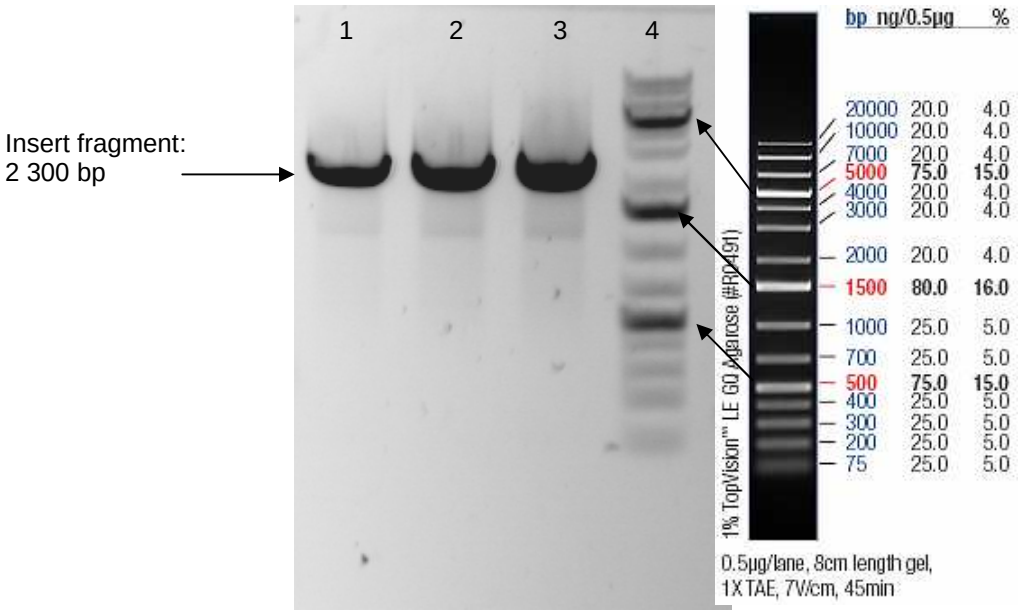



**Figure 4-1**: Amplification of insert DNA separated by agarose gel electrophoresis. Lane 1 - 3: ND insert; lane 4: marker

### 4.1.2. Restriction digest

Before ligation of N421D insert into the pmCBm6 vector a restriction digest had to be performed. Figure 4-2 shows the linearized vector with (v M6 +) and without (v M6 -) the mouse prepro insert separated by agarose gel electrophoresis. The restriction digest of the insert is not shown.

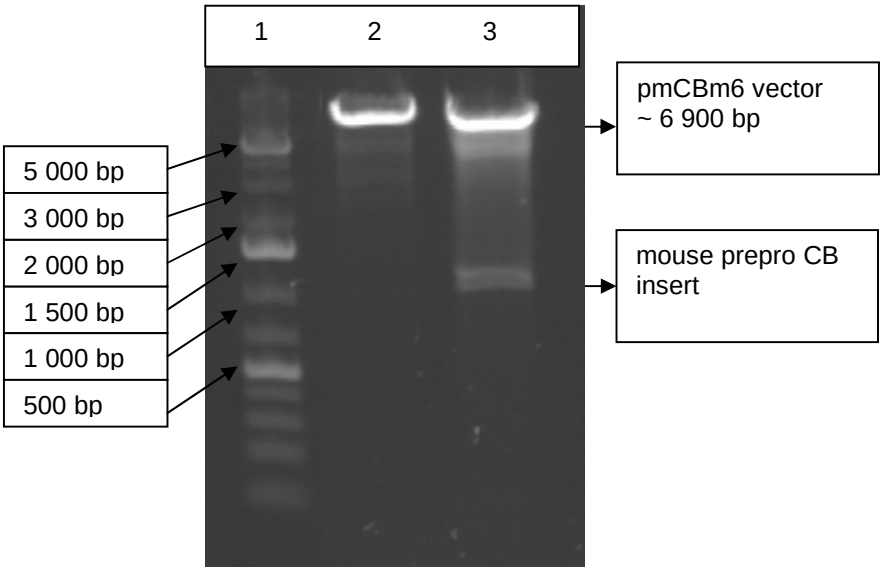



**Figure 4-2:** Linearized pmCBm6 vector with and without insert separated by agarose gel electrophroesis. Lane 1. Marker; lane 2: linearized vector without mouse prepro insert (v M6 -); lane 3: linearized vector with mouse prepro insert (v M6 +)

## 4.1.3. Ligation and transformation

On a ligation gel the same amount of vector and insert were applied in order to estimate the ratio of vector and insert needed for the ligation.

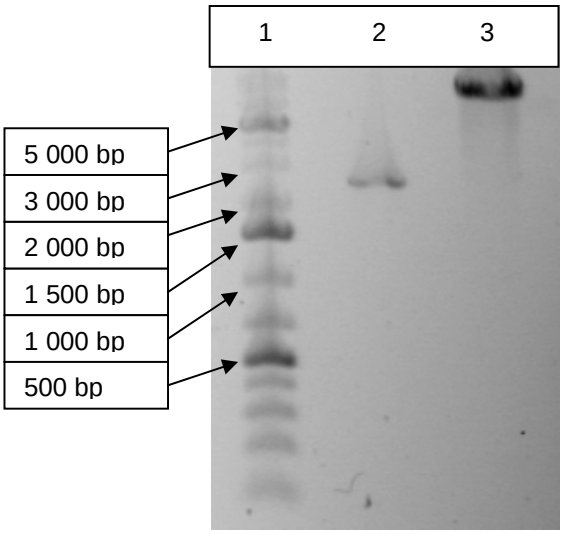



**Figure 4-3:** Ligation gel. Vector and insert were analyzed by agarose gel electorphoresis. Lane 1: marker; lane 2: insert; lane 3: vector

In this case 2 µL vector DNA and 15 µL insert DNA were used. The probe was mixed with water and ligase and incubated for 2h at room temperature.
After the transformation in *E. coli* JM107, the cells were plated on LB- $agar_{AMP}$. Positive clones were screened after incubation overnight at 37℃.

## 4.1.4. Screening

### PCR screening

Before a PCR screening was done, a master plate was prepared in order to ensure the further cultivation of the analyzed clones. The PCR screening was performed as described in chapter 3.3.2..
Figure 4-5 shows the PCR probes on an agarose gel. The marker did not run correctly, but as there is a positive control at the same height as the clone K5, it can be assumed that K5 contains the insert DNA.

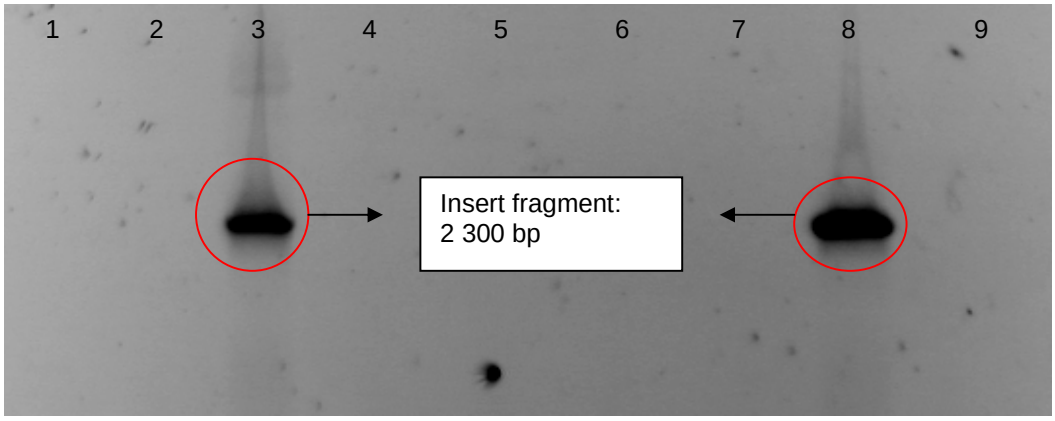



**Figure 4-5:** PCR screening of 6 different clones with a negative and a positive control, analyzed by agarose gel electrophoresis. Lane 1: marker (not visible); lane 2: negative control; lane 3: positive control; lane 4 – 7: K1 – K4, negative screened clones; lane 8: K5, positive screened clone; lane 9: K6, negative screened clone

### Restriction screening

In order to confirm correct integration a restriction screening was done. If the insert orientation is correct, a specific band pattern could be expected, which is shown in chart 4-1. BamH I was used once more to confirm the integration of the whole insert DNA whereas Bgl II cut in the vector and in the insert DNA. Depending on the orientation of the insert two differently sized fragments were possible. As the exact

vector sequence and size was unknown, the calculation for expected fragments was not accurate.

**Chart 4-1**: Expected band pattern for the restriction screening

| Restriction enzyme | Sequence | Expected fragment size |
|---|---|---|
| BamHI I | 5' G/GATCC 3' | ~2 300 bp |
| Bgl II | 5' A/GATCT 3' | ~2 500 bp<br>~4 000 bp<br>~5 000 bp |

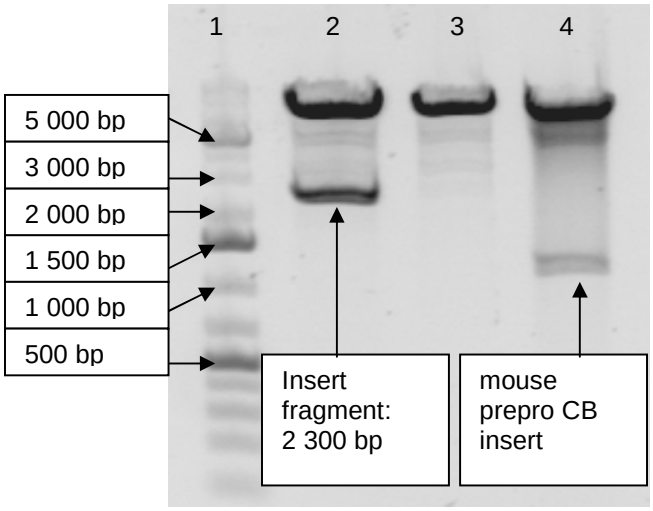



**Figure 4-6**: Restriction screening. The probes were digested with BamH I and separated by agarose gel electrophoresis. Lane 1: marker; lane 2: vector with ND insert; lane 3: empty vector; lane 4: vector with mouse prepro insert

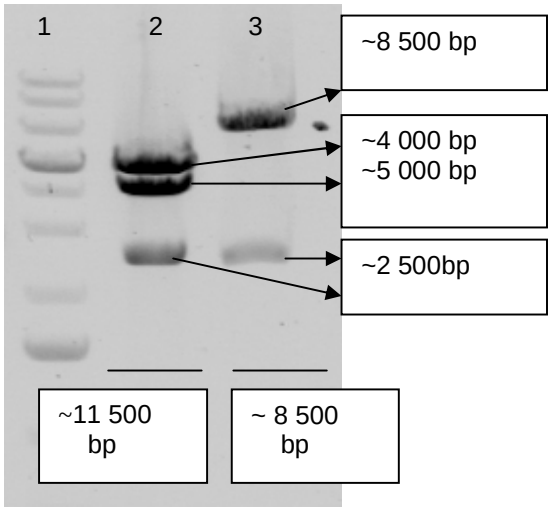



**Figure 4-7**: Restriction screening. The probes were digested with Bgl II and separated by agarose gel electrophoresis. Lane 1: marker; lane 2: vector with ND insert; lane 3: empty vector;

## 4.1.5. DNA sequencing

DNA sequencing is a tool to ensure that during PCR no unwanted DNA mutations did occur. Thereby the actual and theoretical sequences are compared. Figure 4-8 shows a small sequence section containing the targeted site of mutation (Asn421Asp).

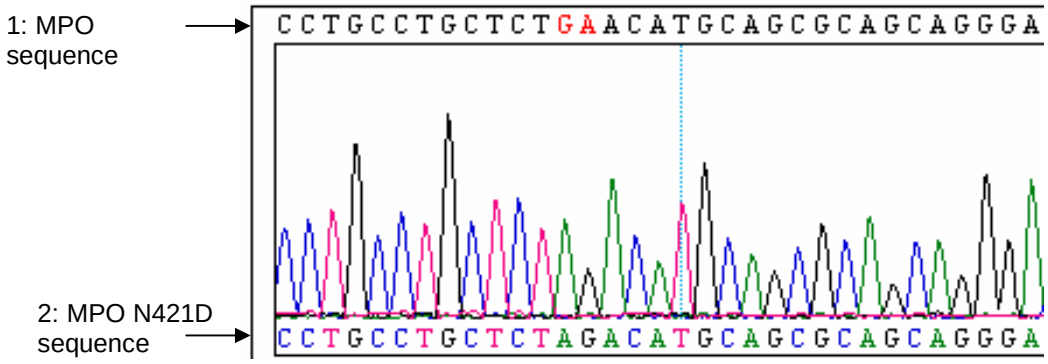



**Figure 4-8:** DNA sequencing, comparing the wt DNA and the DNA containing the mutation. Lane 1: wt MPO sequence; lane 2: MPO N421D sequence

## 4.1.6. Midiprep and purification of DNA

Chart 4-2 depicts absorption values of the isolated DNA at different wavelengths as well as various OD ratios.

**Chart 4-2**: Properties of endotoxin free DNA used for transfection

| | Wavelength (nm) | Absorption | Theoretical OD ratio | Actual OD ratio |
|---|---|---|---|---|
| pmCBm6/ N421D | 230<br>260<br>280<br>320 | 0.1990<br>0.4223<br>0.2185<br>0.0023 | $OD_{230/260} < 0.6$<br>$OD_{260/280} \sim 1.8$<br>$OD_{320} < 0.00$ | $OD_{230/260} < 0.47$<br>$OD_{260/280} \sim 1.9$<br>$OD_{320} < 0.0023$ |

The DNA solution matched the requirements and concentration could be estimated by measurement of the absorption at 260 nm. An $OD_{260}$ = 1 correlates with 50 µg/mL double stranded DNA, so the DNA concentration can be easily calculated as shown in Chart 4-3.

**Chart 4-3**: Determination of DNA concentration

| | $OD_{260}$ | Dilution factor | Concentration (mg/mL) |
|---|---|---|---|
| pmCBm6/N421D | 0.4223 | 1 : 50 | 1.05 |

## *4.2. Production of the MPO mutant N421D*

### 4.2.1. Cultivation of CHO cells

CHO K1 cells

PcDNA3.1 vector containing the insert DNA was used for transfection of CHO K1 cells. They were cultivated in Mem-$\alpha^+$-media supplemented with 20% FBS, 1% L-glut, 1% P/S and 500 µM G418. For production the media was renewed with 10% FBS, 2% L-Glut and 1% P/S.

In order to increase the yield 1 g/L glucose was added to the media and the produced amount protein was compared with the one without additional glucose. Another approach was to reduce the cultivation temperature from 37℃ to 33℃.

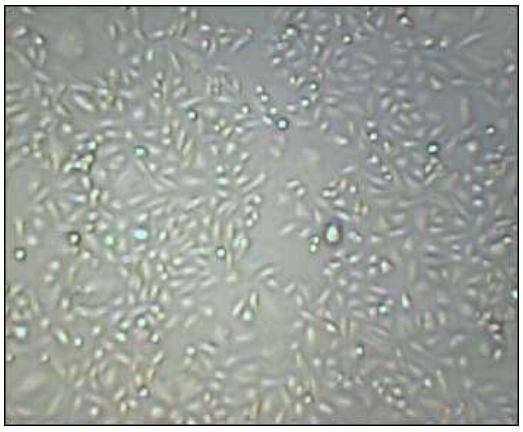


**Figure 4-9**: CHO K1 cells upbringing. Picture was taken 24h after thawing. (24/6/2008).

CHO dhfr$^-$ cells

CHO dhfr$^-$ cells have been used for transfection with the pmCBm6 vector.

For upbringing cells were grown in Mem-$\alpha^+$-media containing 1% P/S, 1% L-glutamine and 5% FBS. During the selection 500 µg/mL geneticin (G418) were added as a selection marker until stable clones have been established. To induce gene amplification the cells were cultivated in Mem-$\alpha^-$-media containing 25 nm methotrexate (MTX) and for production the FBS concentration was reduced to 2.5%.

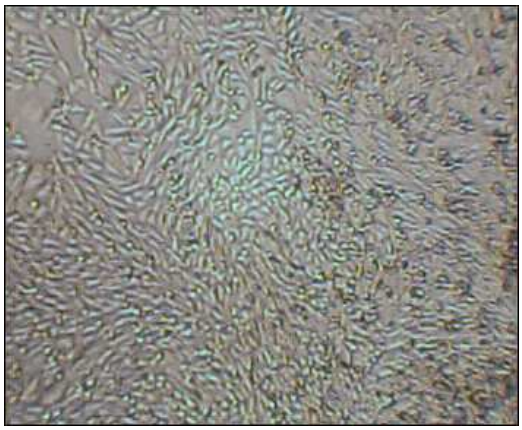


**Figure 4-10:** Transfected CHO cells (clone 9 C) treated with MTX for gene amplification. (16/9/2008)

Though it was possible to establish stable and protein producing clones, the production rate and purity number was still not satisfying.

As a consequence, recombinant protein production was carried out only in HEK 293 cells.

## 4.2.2. Cultivation and protein production in HEK cells

HEK cells have been transfected with a pcDNA3.1 vector containing the cDNA of the hMPO/N421D insert. They are grown in DMEM media containing 10% FBS, 1% P/S and 900 µg/mL G418 as a selection marker.

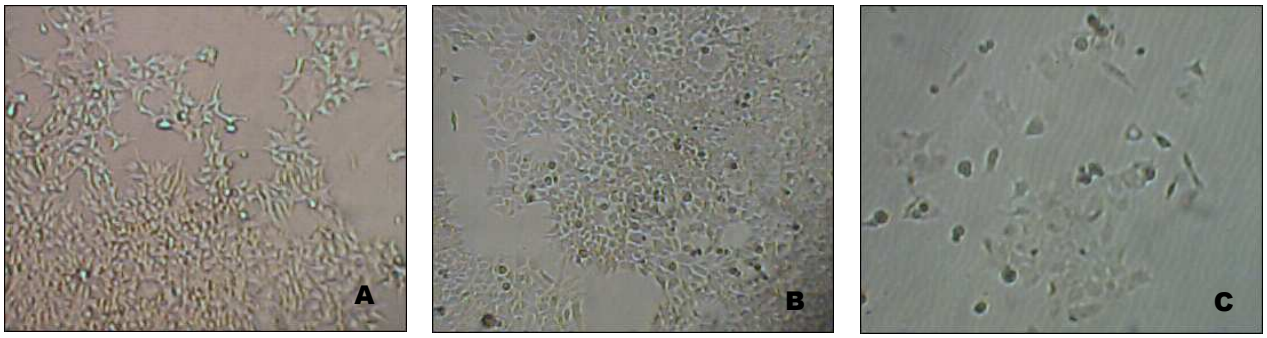



**Figure 4-11:** Transfected HEK cells. Morphological differences between clones can be observed. A: clone A1; B: clone B3, C: clone C2 (20/11/2009)

### Production in cell factories

The cells were passaged into a cell factory and grown in static culture. The attempt to adapt Mem-$\alpha^+$-media so that it can be a substitute for DMEM media led to a significant

decrease in protein production, therefore the further cultivation was continued with DMEM. Chart 4-3 shows the characteristics of a cell factory without any additives.

**Chart 4-3:** Cell factory characteristics without additives. Harvested media were pooled and purified (P1–5)

| | P1 | P 2 | P 3 | P4 | P5 |
|---|---|---|---|---|---|
| Number of harvests pooled | 2 | 3 | 3 | 3 | 2 |
| Yield (mg/L) | 0.18 | 0.40 | 0.74 | 0.10 | 0.06 |
| Purity number ($A_{410/280}$) | 0.12 | | | 0.10 | |

Additives

In order to test if the yield of recombinant protein can be enhanced by changing the FBS concentration (5 versus 10%), the activities of the produced protein was measured. Figure 4-11 depicts clearly that low FBS concentration led to lower yield. Therefore, the HEK cells were cultivated in media supplied with 10% FBS.

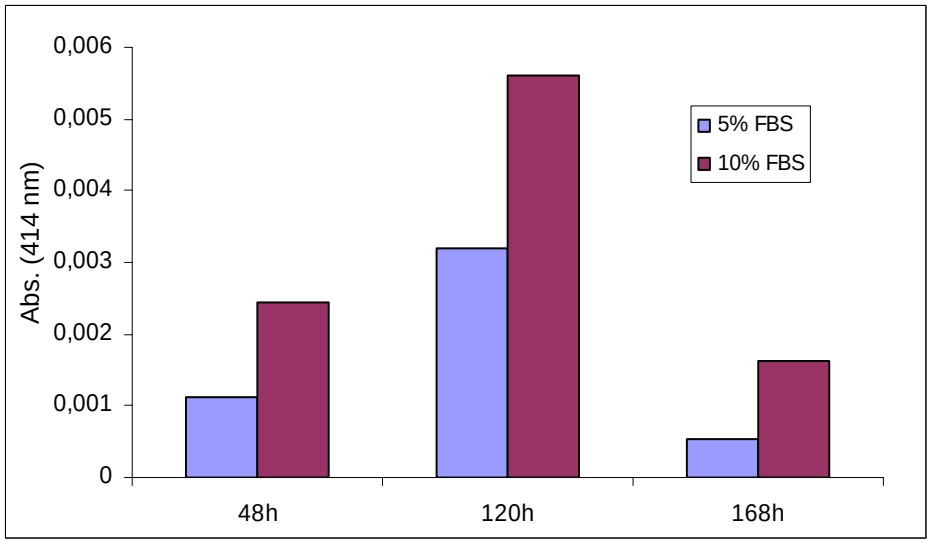



**Figure 4-12:** Comparison of the peroxidase activity (ABTS) in harvested media supplemented with 5% (blue) and 10% (red) FBS.

For better heme insertion, several supplements in the media have been tested like hemin, hematin, δ-aminolevulinic acid and sodium butyrate. The last two did not have

any positive effect on recombinant protein production, whereas hemin and hematin addition led to both a better protein yield and higher purity number.

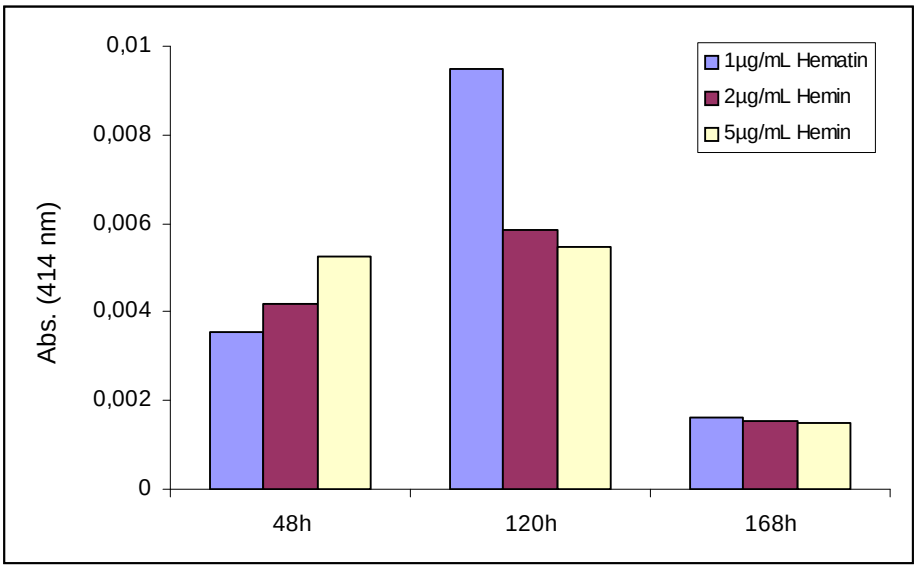



**Figure 4-13:** Comparison of the peroxidase activity (ABTS) of Asn421Asp in harvested media containing 1 µg/mL hematin (blue), 2 µg/mL hemin (red) and 5 µg/mL hemin (yellow)

Since cells treated with hematin might have a reduced life span, it was tested whether a lower hematin concentration allows longer cell cultivation. Chart 4-4 shows that lowering the hematin concentration did not improve cell survival.

**Chart 4-4:** Effect of addition of hematin on production of Asn421Asp and on cellular life span

| | 2 µg/mL hematin | 4 µg/mL hematin |
|---|---|---|
| Media volume | 500 mL | 500 mL |
| Protein produced | 0.32 mg | 0.44 mg |
| Purity number ($A_{410/280}$) | 0.24 | 0.30 |
| Cell survival in hematin containing media | 10 days | 10 days |

Consequently, the second cell factory was cultivated with 5 µg/mL hematin. Chart 4-5 lists properties of various protein harvests during cultivation.

**Chart 4-5:** Yield and purity number of As421Asp harvested, pooled and purified with 5µg/mL hematin. (Purification 1 – 6: P1 – 6)

| | P1 | P2 | P3 | P4 | P5 | P6 |
|---|---|---|---|---|---|---|
| Number of harvests pooled | 1 | 2 | 2 | 3 | 3 | 1 |
| Yield (mg/L) | 0.08 | 1.33 | 2.08 | 0.54 | 0.26 | 0.20 |
| Purity number ($A_{410/280}$) | 0.09 | pool1: 0.18<br>pool 2: 0.25 | pool1: 0.23<br>pool2: 0.40 | pool1: 0.14<br>pool2: 0.25<br>pool 3: 0.27 | pool1: 0.14 | pool1: 0.26 |

## *4.3. Protein purification*

Harvested media were pooled and 1 : 2 diluted with water before purification by cation chromatography. Concentration and desalting led to a small red-shift (1 – 2 nm) of the Soret band as depicts in Figure 4-14.

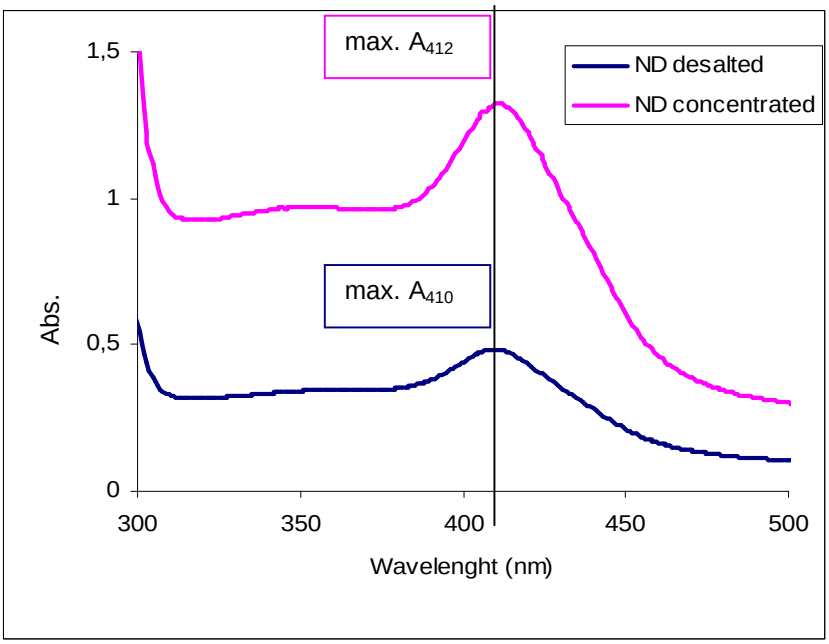



**Figure 4-14:** Shift of the Soret maximum of purified Asn421Asp after buffer exchange and desalting.

Principally, the various purification steps caused loss of protein, especially gel filtration. Depending on the following biochemical investigations, gel filtration was an optional step in the purification protocol.

## *4.4. Protein characterization*

### 4.4.1. Spectral properties

The average purity number of Asn421Asp produced in CHO cells was ~0.1, whereas production in HEK cells significantly enhanced the protein purity mainly by improved heme incorporation. Depending on supplementation of media by hematin, the corresponding purity numbers were calculated to be 0.25 – 0.4.

Generally, the Soret band of Asn421Asp was around 410 nm with a slight shoulder at 428 nm, but the extent of this shoulder varied depending on different batches and maybe also on the time of harvest.

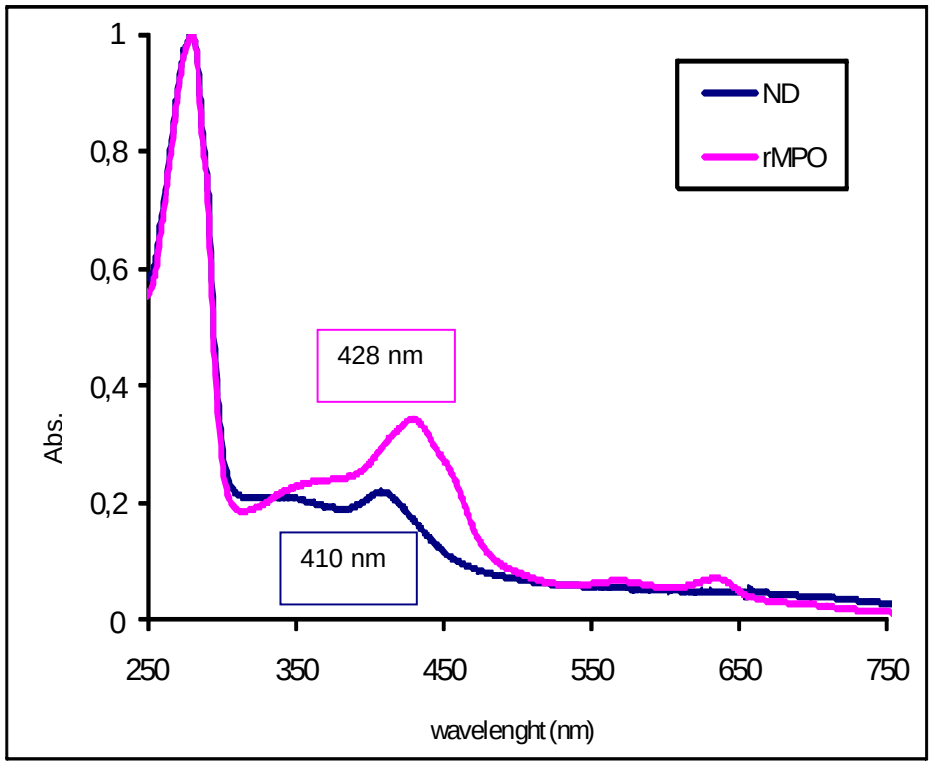



**Figure 4-15:** Comparison of the spectral properties of recombinant, monomeric wild-type MPO and the variant Asn421Asp

### 4.4.2. SDS-PAGE and Western blot

Purified recombinant N421D was analyzed by SDS-PAGE and Western blot using polyclonal antibodies raised against leucocyte myeloperoxidase.

Figure 4-16 show prominent bands around 73 kDA independent of the host cell line. These cells derive from the target protein as clearly demonstrated by Western blot analysis (Figure 4-17). Both Figure 4-16 and 4-17 suggest also a higher production rate for N421D of HEK ND1. As a consequence this colony was used for further production.

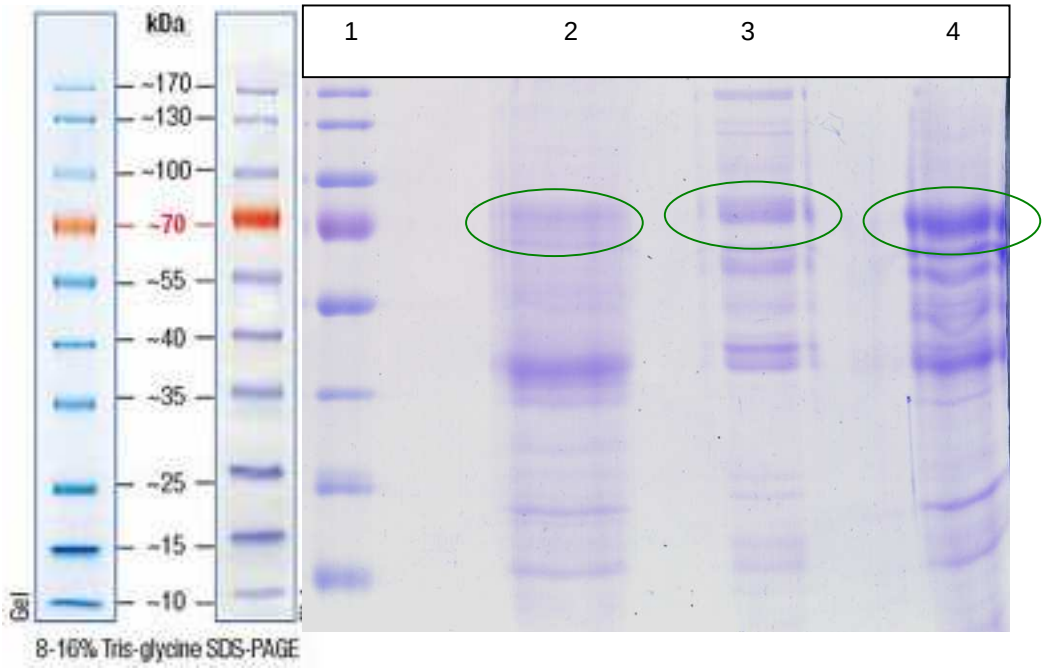



**Figure 4-16:** SDS- Page of N421D recombinantly expressed in CHO and HEK cells. Supernatants of cell cultures were purified by CM-chromatography and fractions with target protein have been pooled. Lane 1: Marker; lane 2: N421D produced in CHO cells; lane 3: clone ND2, N421D produced in HEK cells; lane 4: clone ND1, N421D produced in HEK cells

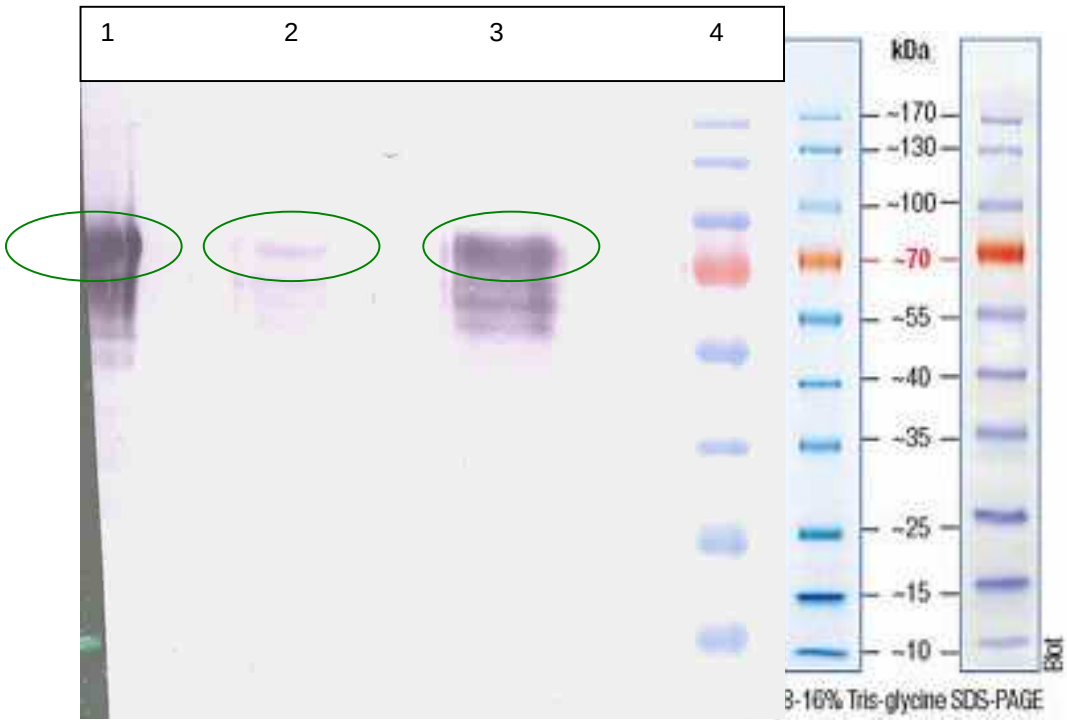



**Figure 4-17:** Western Blot of N421D produced in CHO recombinantly expressed in CHO and HEK cells. Supernatants of cell cultures were purified by CM-chromatography and fractions with target protein have been pooled. Lane 1: clone ND1, N421D produced in HEK cells; lane 2: clone ND2, N421D produced in HEK cells; lane 3: N421D produced in CHO cells; lane 4: Marker

## 4.4.3. CD spectra

It is necessary to ensure that the overall protein structure of the mutant is similar to that of the wild-type protein. Figure 4-18 indicates that the mutant is also mainly α-helical with a typical minimum at 208 nm and a shoulder around 222 nm. Compared to the wild-type protein the ellipticity at 208 nm was less pronounced in N421D.

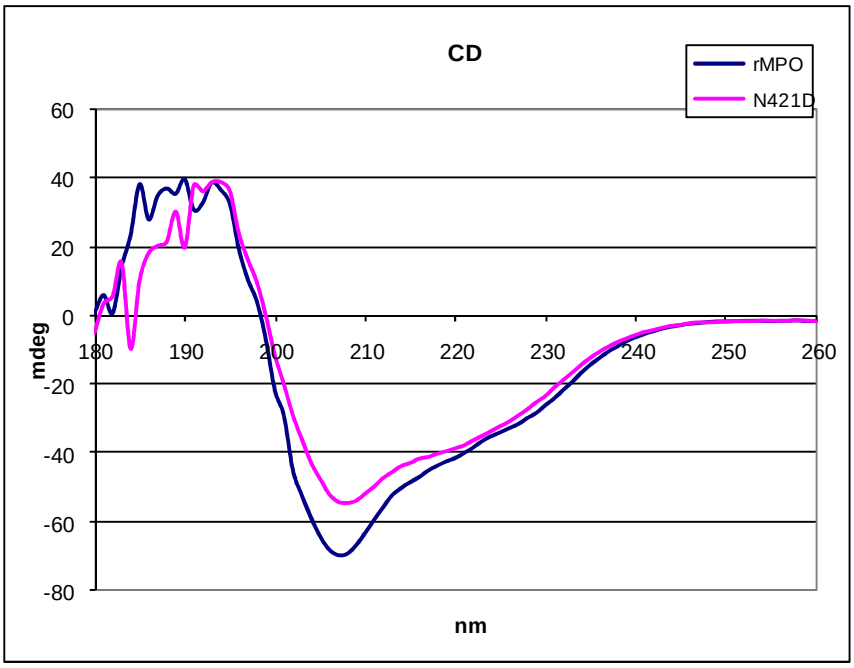



**Figure 4-18:** Overlay of far UV CD spectra of recombinant wild-type MPO and N412D produced in HEK ND1 clone in 10 mM phosphate buffer, pH 7.0, brought to identical absorbance at 280 nm.

## 4.4.4. Enzymatic activity

In order to investigate the enzymatic activity of the N421D mutant several activity assays were performed.

### 4.4.4.1. Peroxidase activity

Guaiacol assay

The guaiacol assay measures the peroxidase activity by MPO-mediated formation of a dimer that absorbs at 470 nm.

Chart 4-6 and Figure 4-19 summarizes activity measurements performed with 100 µM guaiacol, 100 µM hydrogen peroxide and 50 µL enzyme (mutant and wtMPO) in 100 mM phosphate buffer at pH 7.0.

Exchange of Asn421 by Asp has significantly reduced the oxidation capacity using guaiacol as one-electron donor. It is important to mention that the specific activity of mutant and wild-type protein was calculated on the basis of identical heme content.

**Chart 4-6:** Specific activity with guaiacol of N421D compared with recombinant wtMPO

| Guaiacol | wtMPO | ND |
|---|---|---|
| slope/min | 0.246 | 0.08174 |
| slope/min | 0.2484 | 0.07934 |
| slope/min | 0.2134 | 0.0731 |
| Average [min-1] | 0.2359 | 0.0781 |
| conc. [g/L] | 0.0063 | 0.0145 |
| U=µmol /mg enzym min | 37.3 | 5.4 |
| € =19 900 $M^{-1}$ $cm^{-1}$ | 1.88 | 0.27 |
| | **100.00%** | **14.46%** |

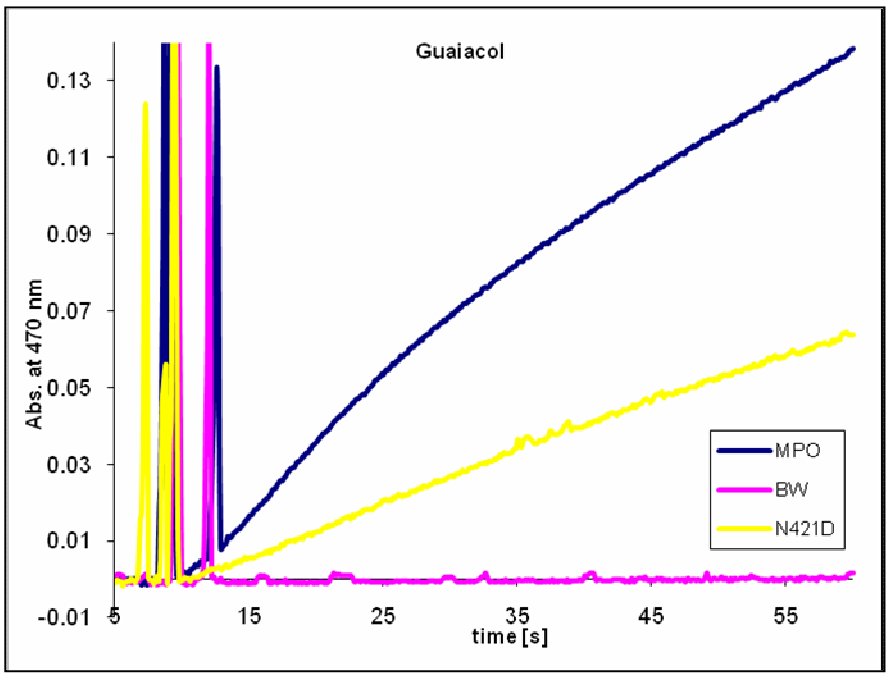



**Figure 4-19:** Guaiacol assay at pH 7.0, N421D activity compared with recombinant wtMPO activity. Typical time traces are depicted.

ABTS assay

Furthermore, ABTS was used as alternative one-electron donor and the reaction was followed at 414 nm.

Typically, the probe contained 1 mM ABTS, 100 µM hydrogen peroxide and 50 µL of the enzyme in 100 mM phosphate buffer at pH 5.0.

As shown in Chart 4-7 and Figure 4-20 the specific ABTS activity of N421D was even more diminished than with guaiacol as substrat.

**Chart 4-7:** Specific activity with ABTS of N421D compared with recombinant wtMPO

| ABTS | wtMPO | ND |
|---|---|---|
| slope/min | 4.798 | 1.034 |
| slope/min | 5.656 | 0.8838 |
| slope/min | 5.807 | 0.9789 |
| Average [min-1] | 4.798 | 1.034 |
| conc. [g/L] | 0.0025 | 0.0072 |
| U=µmol /mg enzym min | 2144.1 | 133.6 |
| € =19 900 $M^{-1}$ $cm^{-1}$ | 107.74 | 6.71 |
| | **100.00%** | **6.00%** |

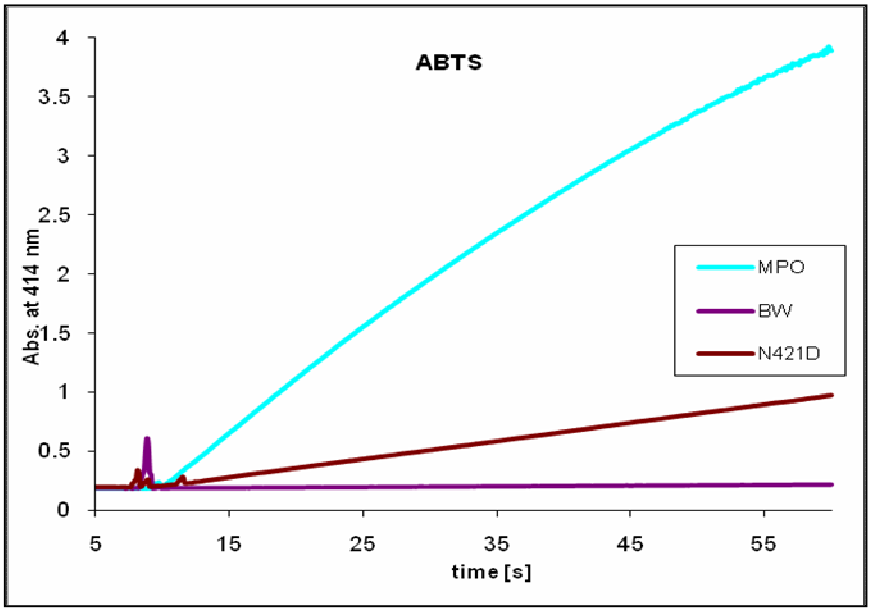



**Figure 4-20:** ABTS assay at pH 5.0, N421D activity compared with recombinant wtMPO activity. Typical time traces are shown. For reaction conditions see above.

Ascorbate assay

Ascorbate was used because it represents a hydrophilic one-electron donor. Its oxidation was followed at 280 nm. Typical assays contained 500 µM ascorbate, 100 µM hydrogen peroxide and 100 µL enzyme in 95 mM phosphate buffer pH 7.0.

Again a decreased peroxidase activity was observed as shown in Chart 4-8 and Figure 4-21. The specific ascorbate activity was about 19.5% of that of the wild type enzyme.

**Chart 4-8:** Specific activity with ascorbate of N421D compared with recombinant wtMPO

| Ascorbat | wtMPO | ND |
|---|---|---|
| Average [min-1] | 0.001675 | 0.00024 |
| conc. [g/L] | 0.015 | 0.0111 |
| U=µmol /mg enzym min | 0.1116 | 0.0217 |
| € =19 900 $M^{-1}$ $cm^{-1}$ | 0.0056 | 0.0011 |
| | **100.00%** | **19.45%** |

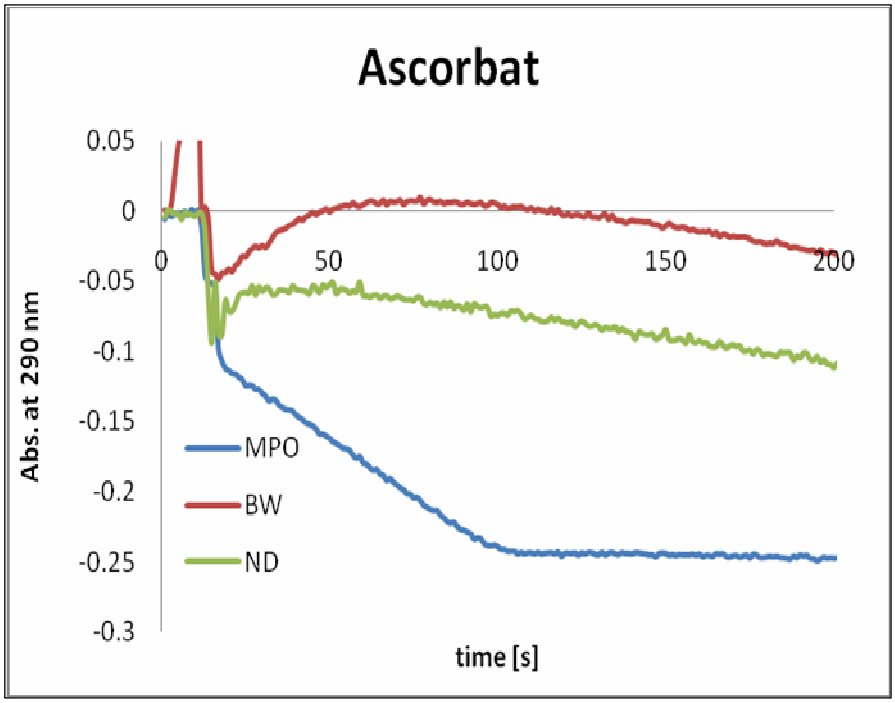



**Figure 4-21:** Ascorbate assay at pH 7.0, N421D activity compared with recombinant wtMPO activity. Typical time traces are shown. For reaction conditions see above.

### 4.4.4.2. MCD halogenation

The MCD (monochlorodimedone) assay measures indirectly the oxidation of halogenides like chloride and bromide by reaction of the products HOCl and HOBr with MCD. Activity measurements were performed with 100 µM MCD, 100 mM KBr or NaCl 100 µM hydrogen peroxide and 50 µL enzyme (mutant and wtMPO) in 100 mM phosphate buffer at pH 5.0.

The bromination activity of the mutant N421D was compared with that of the wild-type enzyme (Chart 4-9 and Figure 4-22). Obtained results unequivocally demonstrate a drastic decrease in bromination activity compared to wild-type MPO.

**Chart 4-9:** Bromination activity of N421D compared with recombinant wtMPO

| MCD KBr | wtMPO | ND |
|---|---|---|
| Average [min-1] | 8.682 | 0.5403 |
| conc. [g/L] | 0.0063 | 0.0158 |
| U=µmol /mg enzym min | 1373.7 | 34.2 |
| € =19 900 $M^{-1}$ $cm^{-1}$ | 69.03 | 1.72 |
| | **100.00%** | **2.49%** |

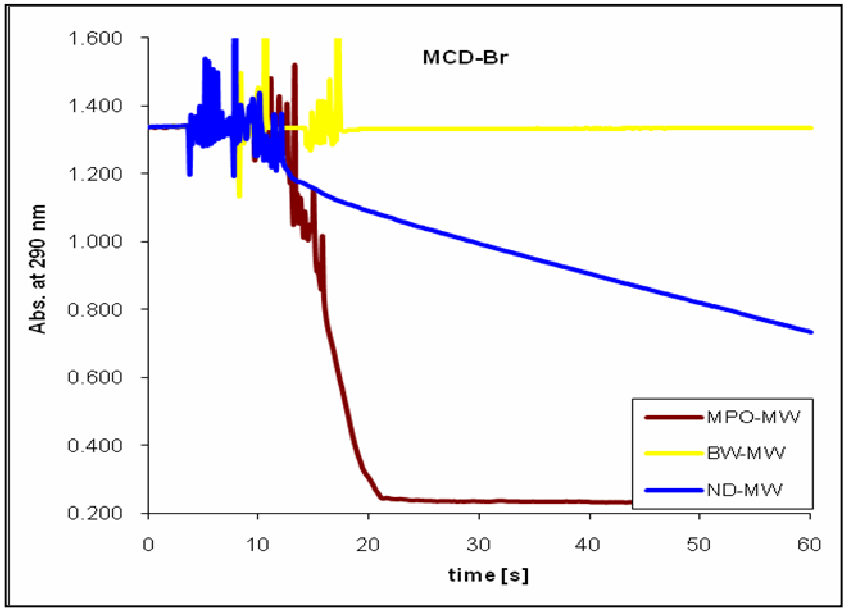



**Figure 4-22:** Bromination activity at pH5.0 of N421D compared with recombinant wtMPO activity. Typical time traces are shown.

The chlorination activity of the mutant is almost nonexistent as can be seen in Chart 4-10 and Figure 4-23.

**Chart 4-10:** Chlorination activity of N421D compared with recombinant wtMPO

| MCD NaCl | wtMPO | ND |
|---|---|---|
| Average [min-1] | 1.633 | 0.0412 |
| conc. [g/L] | 0.0063 | 0.0158 |
| U=µmol /mg enzym min | 258.4 | 2.6 |
| € =19 900 $M^{-1}$ $cm^{-1}$ | 12.98 | 0.13 |
| | **100.00%** | **1.01%** |

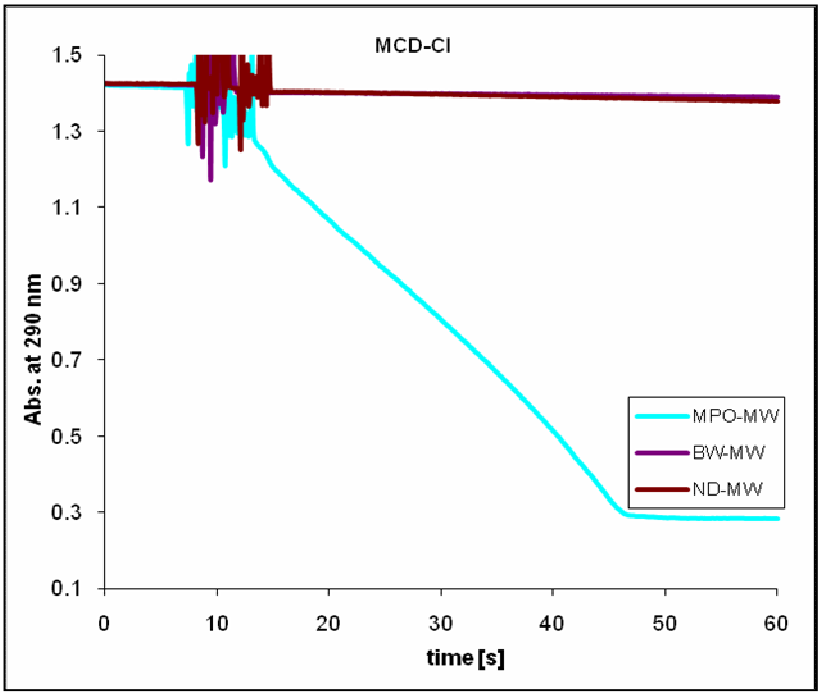



**Figure 4-23:** Chlorination activity at pH5.0 of N421D compared with recombinant wtMPO activity. Typical time traces are shown.

### 4.4.4.3. Cyanide inhibition

Figure 4-24 shows the impact of increasing cyanide concentration on the bromide oxidation mediated by N421D.

Reaction was followed at 290 nm (bromination of MCD).

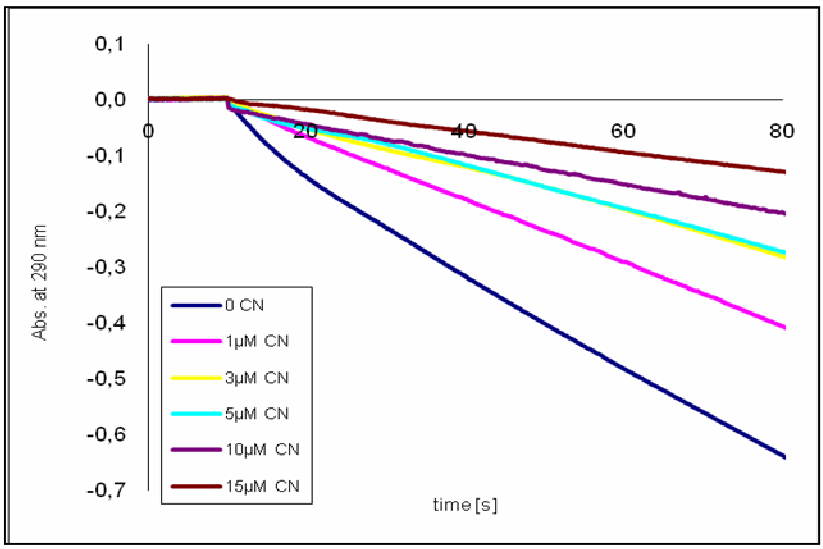



**Figure 4-24:** MCD bromination assay with increasing cyanide condition. Typical time traces are shown. Assay conditions see (chapter 4.4.4.2.)

Inhibition blot

Figure 4-25 shows an inhibition blot with increasing CN- concentration added to MCD bromination assay. A decrease in activity in relation to the $CN^-$ concentration can be observed. A concentration of 0.75 µM cyanide in the assay led to 50% inhibition of the specific activity of N421D.

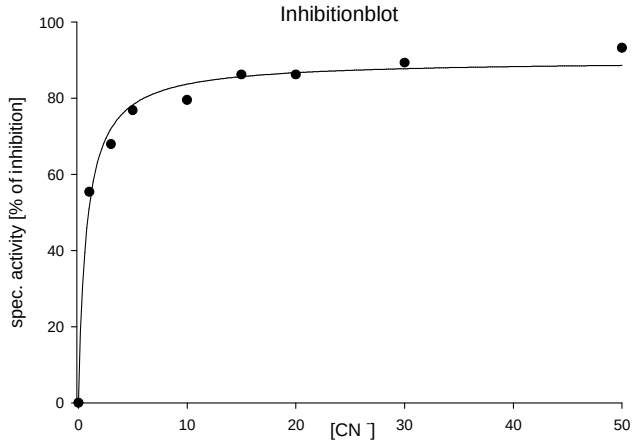



**Figure 4-25:** Inhibition blot with increasing cyanide concentration added to MCD bromination assay. Assay conditions see above.

# 5. Discussion

## *5.1. Cloning*

### 5.1.1. Cloning of the N421D insert into the pmCBm6 vector

The pcDNA3.1 vector, containing the insert for the MPO mutant N421D, was already available. The normal procedure would have been to cut the insert out of the vector and then ligate it directly into the multiple cloning site of a vector which possesses also the desired dhfr gene. However, the multiple cloning site of the pmCBm6 vector contains only one restriction site (BamH I). Therefore, it was necessary to amplify the insert by PCR amplification with a suitable primer, so a BamH I site is found at both ends. After a restriction digest, the insert was ligated into the vector.

Furthermore, the exact sequence of the vector was unknown. Thus, besides PCR screening and restriction screening, DNA sequencing was necessary in order to confirm the correct integration of the insert. Since primers were used which exceeded the insert, the obtained sequence was comparable with the sequence of the neighboring genes, confirming the correct orientation.

## *5.2. Protein production*

### 5.2.1. Protein production in CHO K1 cells

The production system of CHO K1 cells transfected with a pcDNA3.1 / N421D vector was already established at the beginning of this work. However the yield as well as the purity number of the protein produced was not satisfying. In order to enhance the quality and quantity of the protein produced several strategies have been explored.

At first, efforts have been made in order to improve the already existing production system. The addition of glucose (1 g/L) to the media increased the production slightly. However, as glucose is metabolized to lactate, the pH is decreased and worsens therefore the cultivation conditions for the cells resulting in a short production period.

Another approach was to reduce the cultivation temperature from 37℃ to 33℃. On one hand it leads to an increase of secondary metabolites production but on the other hand down-regulation of the cell growth is the consequence. Hence, this step could only be carried out at the end of a cultivation period.
Both strategies did have a slightly positive effect, but the overall protein production was still not sufficient enough for further investigations. Therefore, alternative expression systems like CHO dhfr$^-$ cells and HEK 293 cells were explored.

## 5.2.2. Protein production in CHO dhfr$^-$ cells

Gen amplification in dhfr$^-$ cells is a well known and widely used method. The specific procedure especially the amount of MTX supplied to the media varies extremely in the literature. The approach used in this work was adding MTX to all stable and already singularized cell clones. The MTX concentration in the media was 25 nM.
As a result clones were screened positive by ABTS screening, which have not been producing protein before. Cryocultures of the most promising clones have been established and cultivated in small scale in order to compare their productivity.
This has been done at the same time as the HEK 293 clones have been scanned for their productivity and the difference in yield and protein purity was enormous. So although the protein production in CHO dhfr$^-$ cells was better than in CHO K1 cells it could not keep up with protein production in HEK cells. Therefore, all further protein production was carried out in HEK 293 cells.

## 5.2.3. Protein production in HEK 293 cells

HEK 293 cells were provided by Lukas Mach, Department of Applied Genetics and Cell Biology. The cells have been transfected with the pcDNA3.1 / N412D vector by $Ca^{2+}$ precipitation. Handling of the HEK cells is relatively similar to CHO cells. Singularization of the cell clones is similar except the media composition and the amount of geneticin added for selection (900 µg/mL instead of 500 µg/mL for CHO cells). However HEK cells are more sensitive to additives and mechanical stress than CHO cells.

Two clones (HEK ND1 and HEK ND2) were chosen to be subcultivated in a small scale and their productivity was compared. HEK ND1 was clearly the better producer, thus having been selected for large scale production.
The protein yield of the first harvest of the cell factory always stayed behind the following harvests. So, it can be assumed that the protein production at a reasonable rate takes place only when the cells are confluent. The protein yield drastically decreased upon changing medium from DMEM to Mem $\alpha^+$ despite supplementation with extra glutamine.

Additives:
For further optimization of protein production several additives have been tested and are summarized here:

1) At 5% FBS concentration the protein yield was significantly decreased compared to 10% FBS.

2) Neither addition of δ-aminolevulinic acid nor of sodium butyrate led improvement of heme insertion into the target protein. However, addition of hemin or hematin increased both the purity number as well as the protein yield. Hematin had a more positive effect than hemin. Since cells seemed to have a reduced life span in the presence of added hematin, the effect of the actual hematin concentration on the cellular life span was tested. The optimum hematin concentration was shown to be 5 µg/mL. At lower concentration heme insertion was down-graded whereas the life span remained unchanged.

3) Interestingly, the used culture vessel had a significant influence on cell survival. Petri dishes with 90 cm diameter allowed cultivation for maximal one week, whereas triple flasks can be cultivated for nearly two weeks and in a cell factory cells survived for about one month. This is almost the same time period of a cell factory cultivated without hematin. The specific manufacturing of cell factory is probably responsible for that. Further, it is of advantage to wait with hematin supplement until cells are completely confluent.

## *5.3. Protein purification*

The purification protocol for N421D MPO mutant was similar to that developed for recombinant MPO. In any case, the high isoelectric point of the protein enabled a rather simple purification via CM chromtography.

Gel filtration as a second purification step enhances the purity number but since the protein loss is relatively high, the use of this method probably needs to be reconsidered.

The protein loss during concentrating and desalting in centripreps is also relatively high. Using a PD 10 column as an alternative desalting method seems to be promising but further comparison need to be done.

Another promising approach might be the protein treatment with $H_20_2$ . This should result into better and more stable heme integration into the protein due to autocatalytic formation of heme to protein linkages. Desalted protein solution was vacuum dried and stored at -80℃ until use. Storage had no impact on protein quality as demonstrated by UV-Vis spectroscopy and analysis of enzymatic activity.

## *5.4. Protein characterization*

### 5.4.1. Spectral characterization

The spectral properties of the MPO mutant N421D are different of those of the wild type enzyme. The mutant has its Soret band at 410 – 412 nm depending on the salt concentration of the protein solution whereas the native enzyme has the Soret maximum at 428 nm and additional bands at 498, 570, 622 and 690 nm, which are not visible in the mutant spectra. This blue shifted Soret band of the mutant enzyme suggests the absence of the typical third link of MPO; i.e. the sulfonium ion linkage. This is underlined by spectral analysis of lactoperoxidase as well as eosinophil peroxidase, which both lack the sulfonium link and have their Soret band at a similar wavelength range as the MPO variant N421D.

The N421D mutant further exhibits a slight shoulder at 428 nm, however the exact specificity varies from harvest to harvest. Hence, it seems likely that the condition of the cells during the time of the protein harvest is responsible for these variations.

Additionally, it has to be mentioned that the spectral properties of the enzyme were the same, regardless if they have been produced in CHO or HEK cells.

To rule out that no major structural rearrangements have occurred upon exchange of Asn 421 the far-UV CD spectrum was recorded. Since the overall structure of mutant and wild type enzyme were similar any change of the spectral and kinetic properties of the N421D mutant did result from the exchange of the amino acid and not from incorrect protein folding.

### 5.4.3. Enzymatic activity

The peroxidation activity of the N421D mutant was drastically reduced. The specific activity of guaiacol was only about 14.5%, the specific ascorbate activity about 19.5% and the specific ABTS activity merely 6% compared with the wild-type protein.

Halogenation activity was significantly decreased, as the bromination activity of the mutant was reduced to 2.5% of the wild type enzyme bromination activity and the chlorination activity was completely lost. In an inhibition blot 0.75 µM cyanide in the assay led to 50% inhibition of the specific activity of N421D whereas 8 µM cyanide was necessary for 50% inhibition of the native enzyme.

### 5.4.4. Impact of Asn421 exchange

Recent structural investigations have shown that the carbonyl group of Asn421 is oriented towards the positively charged guanidinium group of Arg333, while its amide group interacts with the unprotonated $N_\delta$ of the proximal His (see chapter 1.2.6.). (*9*)

Exchanging the Asn421 with an Asp underlines the importance of the close His-Asn-Arg interaction for maintenance of heme cavity architecture and catalysis. The resulting MPO mutant showed besides a lowered heme occupancy also altered modes of heme to protein linkages, indicated by the blue shifted Soret band, the calculated $A_{Soret}/A_{280}$ ratio as well as the before mentioned altered enzymatic activity.

Complete loss of the chlorination activity suggests that the oxidation capacity of compound I of N421D was significantly lower than in the wild type protein. As LPO and MPO with exchanged Met243 lack chlorination activity too, this and the blue shifted Soret band indicates the loss of the sulfonium ion linkage in the Asn421 mutant.

Hence, the interaction of the proximal His and Asn421 might have an impact in the autocatalytic formation of the heme to protein linkages.

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