Universität für Bodenkultur, Wien
Department für Chemie, Institut für Biochemie
Metalloprotein Research Group

Characterization of the myeloperoxidase mutants Q91T and R333A/K

Masterarbeit

Zur Erlangung des akademischen Grades Diplom Ingenieur

eingereicht von

Clemens Gruber

Betreuer: ao. Prof. Mag. Dr. Christian Obinger
Mitbetreuer: ao. Prof. DI Dr. Paul Georg Furtmüller

Wien, Februar 2011

Table of contents

Abstract

Myeloperoxidase (MPO) is the only chlorating enzyme in mammals. The prosthetic group, a modified heme *b* is covalently bound to the protein by three covalent bonds, which causes unique redox, chemical and spectral properties.

The physiological role of MPO is to catalyze the reaction of hydrogen peroxide and chloride to yield the hypochlorous acid, which is a strong oxidant and a antimicrobial agent. MPO is found in high concentration in the azurophilic granules of phagocytosing neutrophils and monocytes.

The aim of this investigation was to characterize two mutants of MPO. Site directed mutagenesis was performed to specifically exchange amino acid residues located in the distal and proximal heme cavity (glutamine 91 and arginine 333). Glu91 plays a major role in binding of small inorganic electron donors and Arg333 directly interacts with heme propionate and the H-bonding partner of proximal histidine (i.e. Asn 421). The corresponding variants were recombinantly produced in CHO and HEK 293 cell cultures.

Blue-shifted Soret maxima and decreased purity numbers demonstrated a significant impact of the mutations on heme modification (posttranslational modification) and autocatalytic formation of heme to protein bonds. In both Arg333 mutants the heme content was low and the activity negligible. Exchange of Gln91 decreased the peroxidase activity and impaired the halogenation activity.

For further investigations the protein yield and heme occupancy (despite addition of heme precursor) must be increased.

Zusammenfassung

Die Myeloperoxidase (MPO) ist das einzige chlorierende Enzym in Säugetieren. Die prosthetische Gruppe ist ein modifiziertes Häm *b,* das mit drei kovalenten Bindungen zum Protein gebunden ist, welche einzigartige redox, chemische und spektrale Eigenschaften verursachen.

Die physiologische Rolle der MPO ist es die Reaktion von Wasserstoffperoxid mit Chlorid zu katalysieren um Hypochlorsäure zu erzeugen. Diese ist ein starkes Oxidationsmittel und antimikrobiell. Die MPO wird in hohen Konzentrationen in den azurophilen Granulas von phagozytierenden Neutrophilen und Monocyten gefunden.

Das Ziel der Arbeit war zwei verschiedene Mutationen der MPO zu charakterisieren. Zuerst wurden durch Punktmutationen die gewünschte Aminosäuren ausgetauscht, eine an der distalen und die andere an der proximalen Häm Seite (Glutamin 91 und Arginin 333). Glu 91 spielt eine wichtige Rolle beim Binden von kleinen anorganischen Elektronen Donoren und Arg 333 beeinflusst direkt eine Seitenkette des Häm und das Wasserstoffbrücken Netzwerk des proximalen Histidin. Die verschieden Variationen der MPO wurden in CHO und HEK293 Zellen produziert.

Das veränderte Maximum der Soretbande und die schlechten Reinheitszahlen haben den großen Einfluss der Mutationen auf den korrekten Hämeinbau gezeigt. In beiden Argininmutanten war die Hämkonzentration sehr gering und die Aktivität vernachlässigbar. Der Austausch des Glutamins hat die Peroxidaseaktivität verkleinert und die Halogenierungsaktivität verhindert. Für weitere Untersuchungen müsste man die Proteinausbeute und den Hämeinbau verbessern.

1 Introduction

1.1 General Introduction

1.1.1 Recombinant expression of proteins

An easy way to produce high amounts of desired protein in high purity is to use well-established expression systems. Recombinant expression is applied to many biomolecules which can either be obtained only with great effort or costs out of natural sources or are not accessible at all. Therefore, it is often a good choice to produce recombinant proteins. Furthermore, the production of mutated variants of recombinant proteins is also quit easy to manage.

Prokaryotic expression systems are widely used for a great variety of proteins. For instance *E. coli,* a gram negative rod-shaped bacterium, which is well characterized, grows very quickly and target protein can be produced in high yields. Many mammalian proteins are quite complex and the simple machinery of *E. coli* cannot synthesize and fold the protein properly or is not able to perform many important posttranslational modifications. Therefore other, more complex systems are needed. In many cases the best choice is to use animal cells. They are able to perform posttranslational modifications like glycosylation, proteolytic trimming, phosphorylation, acetylation, formation of disulfide bridges and correct folding of the protein. Besides, they are able to secrete the recombinant expressed proteins into the media, which facilitates the purification. Compared to prokaryotic systems, animal cell culture has some disadvantages, namely slow growing rates and high costs.

Many different expression systems were tried for recombinant expression of myeloperoxidase (MPO), but the best results were achieved with animal cell culture. Bacterial systems formed insoluble inclusion bodies, yeast cells were not able to incorporate the heme and insect cells had a very low protein yield.

1.1.2 Protein expression in animal cells

Chinese hamster ovary (CHO) cells are commonly used for recombinant protein expression. They were introduced in the 1960s by Puck *(1)* and grow as cultured monolayer as well as in suspension. CHOs are fibroblastic cells, perform glycosylation similar to humans, but do not produce human pathogens. They are

easy in handling and quite stable in production. Hence they are often used in biological and medical research and commercially in the production of therapeutic proteins. *(2)* For the production of the MPO variant Gln 91 Thr (Q91T), CHO cells were used.

HEK293 cells are similar to CHO cells, easy in handling and stable in production. They were introduced in the 1970s by Graham *(3)* and are widely used for recombinant protein expression. Human embryonic kidney (HEK) cells were used for the production of the MPO variants Arg 333 Ala (R333A) and Arg 333 Lys (R333K).

1.2 Myeloperoxidase

Myeloperoxidase (EC 1.11.1.7) is the only chlorinating enzyme in mammals. It is the major protein of neurophilic leukocytes and found in the azurophilic granules. MPO is a major player of the innate immune system and contains a modified heme *b* covalently linked to the protein. *(4, 5)*

1.2.1 Physiological role

The major function of neutrophilic cells is to phagocytose pathogens and destroy them (figure 1-1). MPO plays a major role in destroying pathogens. *(6)*

During phagocytosis the granules fuse with the phagosome. MPO is released and produces potent antimicrobial agents (mainly hypochlorous acid out of chloride). Hydrogen peroxide is needed for the oxidation of chloride by the MPO, which is provided by the NADPH oxidase. The NADPH oxidase is a membrane bound protein of the phagosome which produces superoxide anion. Superoxide immediately dismutates to form hydrogen peroxide and molecular oxygen. *(7)*

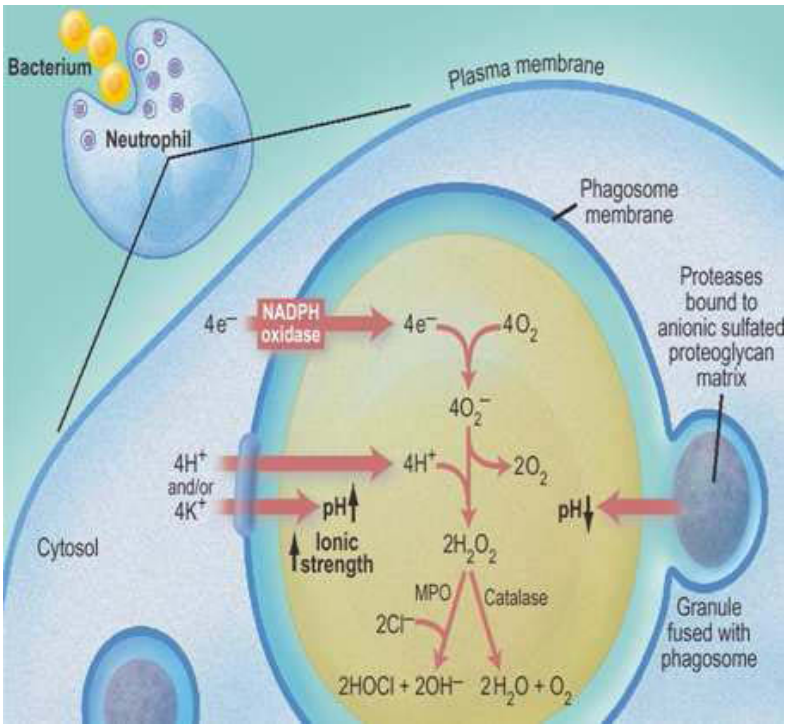


Figure 1-1: phagocytosis of a pathogen by a neurophil; Inside the phagosome: MPO is released and catalyzes the reaction of hydrogen peroxide and chloride to hypochlorous acid

Hypochlorous acid is a strong chlorinating and oxidizing molecule. Preferred it attacks thiols and thioethers and is able to convert amines to chloramines. Furthermore it chlorinates phenols and unsaturated carbohydrates, oxidizes iron centers and crosslinks proteins. In its protonated form hypochlorous acid is membrane permeable. *(7)* In figure 1-2 the effect of hypochlorous acid on bacteria is shown. At higher concentrations of hypochlorous acid less viable bacteria are detected and the amount of chlorotyrosines increased.

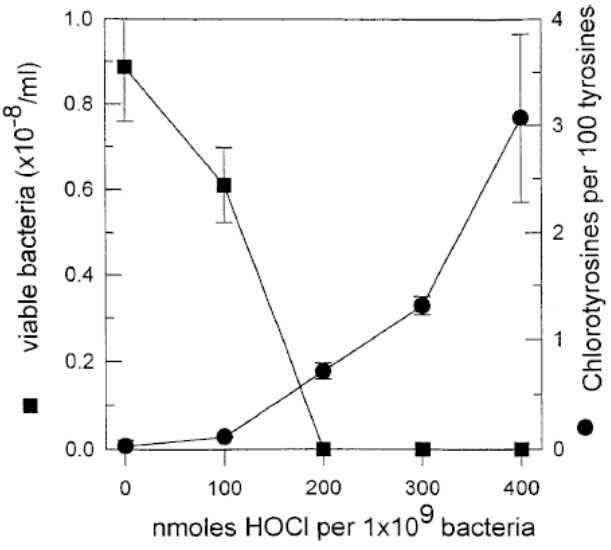


Figure 1-2: Bacteria were treated with a range of concentrations of HOCl and then analyzed for tyrosine and chlorotyrosine content and the number of remaining viable colony forming units. *(7)*

MPO not only plays a major role in microbial killing, but is also involved in many diseases. Excessive or misplaced production of reactive oxidants has been linked to tissue damage and might be involved in the pathogenesis of atherosclerosis, cystic fibrosis, asthma, neurodegenerative disease and carcinogenesis. *(4)*

1.2.2 Heme peroxidase superfamily

Today we distinguish between two heme peroxidase superfamilies. Plant, fungal and (archae)bacterial peroxidases comprise a superfamily, which has arisen by gene duplication of an ancestral gene and has been categorized into three classes, based on sequence alignment and biological origin. Myeloperoxidase (MPO) and other mammalian peroxidases constitute a separate peroxidase superfamily. Their primary and tertiary structures (based on MPO) as well as the nature of the prosthetic group differ greatly from those of plant or fungal peroxidases. The denomination mammalian peroxidase superfamily derived from the fact that its members MPO, eosinophil peroxidase (EPO), lactoperoxidase (LPO) and thyroid peroxidase (TPO) were originally identified in mammals. However, genes or ORF products showing significant similarity in amino acid sequence to mammalian peroxidases which have been identified from various invertebrates, such as arthropods, mollusks, as well as crayfish, *Caenorhabditis elegans* and *Drosophila*. Therefore, Taurog (1999) proposed calling it superfamily of “animal peroxidases”. Animal peroxidases arose independently from the plant/fungal/bacterial peroxidase superfamily and may represent an example of convergent evolution, i.e. they may be derived from a different ancestral gene, originally coding for a protein with some other function but later modified to include peroxidase activity *(4, 8, 9)*

1.2.3 Structure of MPO

Among the mammalian peroxidases only the 3-D structure of MPO is known. The overall protein fold of MPO was first revealed by a 3 Å resolution crystal structure of the canine enzyme. Finally, the structure of human MPO at 2.3 Å resolution was obtained and this structure has since been refined at 1.8 Å, using X-ray data recored at -180°C. Myeloperoxidase is a cationic 146 kDa dimer with a single disulfide bridge between symmetry-related halves (73 kDa), each of which contains two polypeptides of 14.5 and 58.5 kDa, the latter being glycosylated (figure 1-3). *(14)*

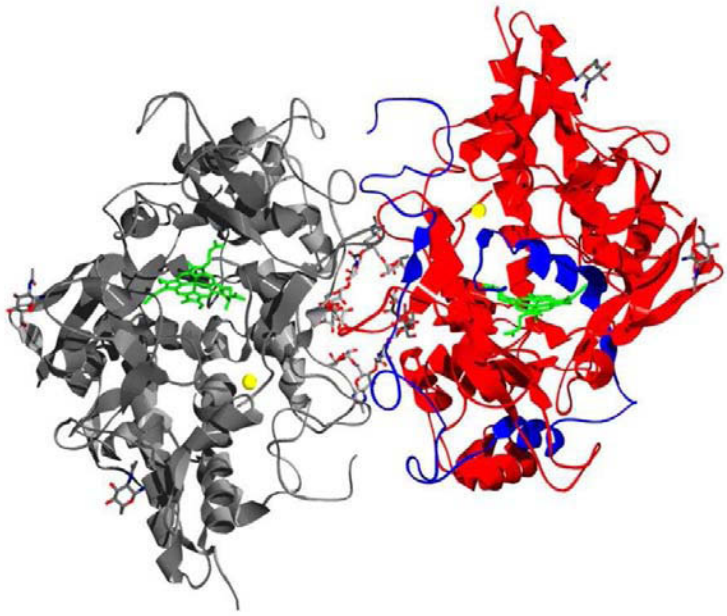


Figure 1-3: Structure of homodimeric MPO; the two monomers are linked via a disulfide bridge, each monomer comprise of a heme and consist of a heavy and a light chain

The small polypeptide is composed of 106 and the large of 467 amino acids. There are five additional intra-chain disulfides in the large polypeptide and one in the small. The secondary structure is predominantely α-helical with a total of 22 helices ranging in length from 4-29 residues that comprise 36% of the whole molecule. Each half molecule has a central heme-containing core composed of five α-helices (H2, H6, H7, H9, H10 and H14), five from the large polypeptide and one from the small. The bulk of the large polypeptide folds into five separate domains and one open loop that surround this core. The small polypeptide wraps around the surface of the molecule with its C-terminal α-helix (H2) penetrating the interior core. Two long amphipathic helices H6 and H7 joined by a short loop at the surface of the molecule, interact through largely hydrophobic interactions to form a prominent feature of the central core. The distal surface of the heme pocket is formed by H2 of the small polypeptide, while helices H9 and H10 from the large polypeptide form the proximal surface (figure 1-4). *(9,14)*

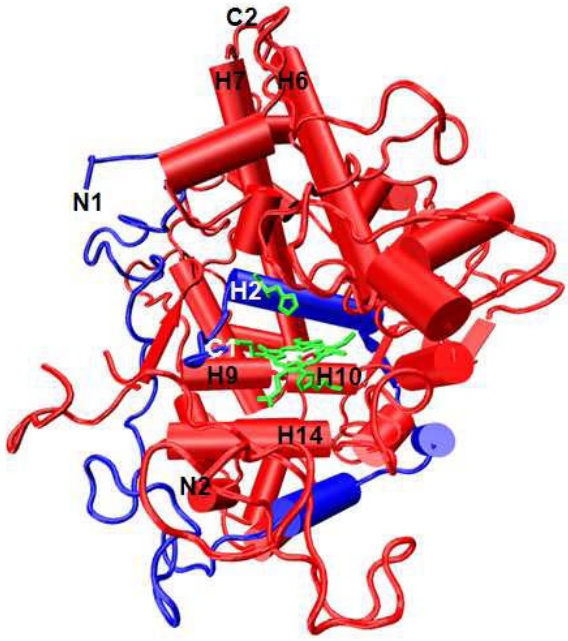


Abbildung 1-4: Heme core structure showing α helical fold

Five sites of asparagine-linked glycosylation (Asn-X-Ser and Asn-X-Thr) occur in the large polypeptide at residues 157, 189, 225, 317, 563 though the first and the last were not seen in the X-ray crystal structures. There is evidence from biosynthetic studies that Asn157 is also glycosylated. The sugar residues at Asn317 interact across the dimer interface and could play an important role in dimer formation.

Each half of dimeric MPO contains one iron, present as covalently bound heme, and one calcium. The calcium-binding site has typical pentagonal bipyramidal coordination. It is bound within a loop of eight conserved residues of the large polypeptide chain (Leu-Thr-Ser-Phe-Val-Asp-Ala-Ser) found in all mammalian peroxidases. The hydroxyl oxygen of Ser174 and the peptide carbonyl oxygen of Phe170 provide the axial ligands, whereas the other five ligands are arranged approximately co-planar. From these ligands only Asp96, which is adjacent to the distal His95, is on the small polypeptide. This suggests that the calcium ion plays not only a structural role in correctly orienting the distal histidine but also in the interaction between the small and heavy polypeptide within a monomer. This is underlined by the fact that removal of the calcium ion from MPO led to precipitation of the protein from solution. *(16)* Sequence alignment, modeling and experimental detection demonstrate that calcium binding is found in all mammalian peroxidases. *(14)*

The prosthetic group (modified heme *b*) is a protoporphyrin IX system of which the methyl groups on the pyrrole rings A and C have been modified. The modification is

needed to make an ester formation with the carboxyl groups of Glu242 and Asp94 possible. In MPO the heme is linked via a third linkage to the protein; a unique vinyl-sulfonium linkage. This link is formed by the sulfur atom of Met243 and the terminal β-carbon of the vinyl group on pyrrole ring A. See figure 1-5. *(13)*

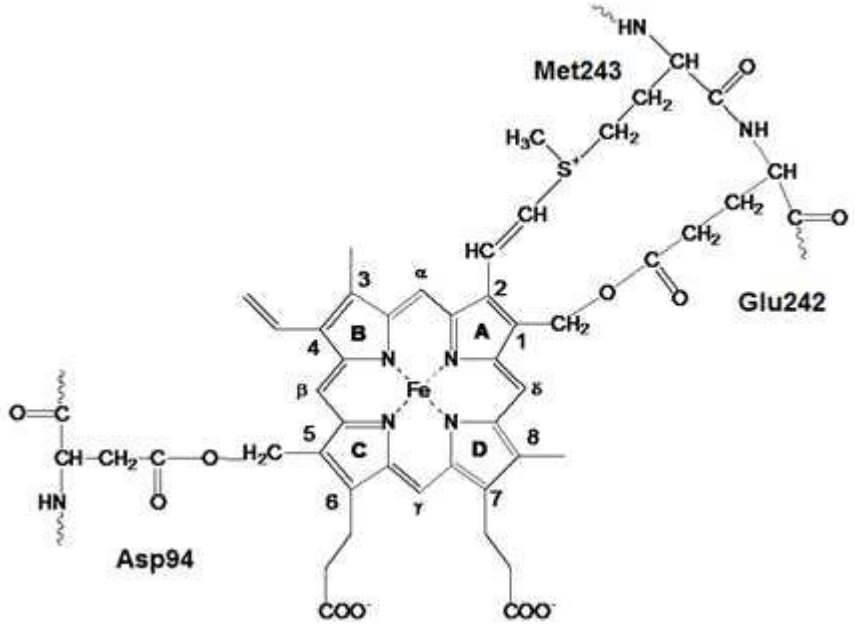


Figure 1-5: Structure of modified heme b with two ester links and one sulfonium ion link to the protein; the heme group consists of four pyrrol rings linked by methen bridges, 8 site chains are present (4 methyl, 2 vinyl and 2 propionate groups)

The third covalent heme to protein bond causes unique chemical, redox and spectral characteristics of the MPO. *(12)* It distorts the heme from planarity and causes a bow shaped structure. Therefore, the absorption maximum of the Soret peak of MPO is red-shifted (428 nm) in comparison with other mammalian peroxidases (e.g. LPO 412 nm). *(15)* A spectra comparison is shown in figure 1-6.

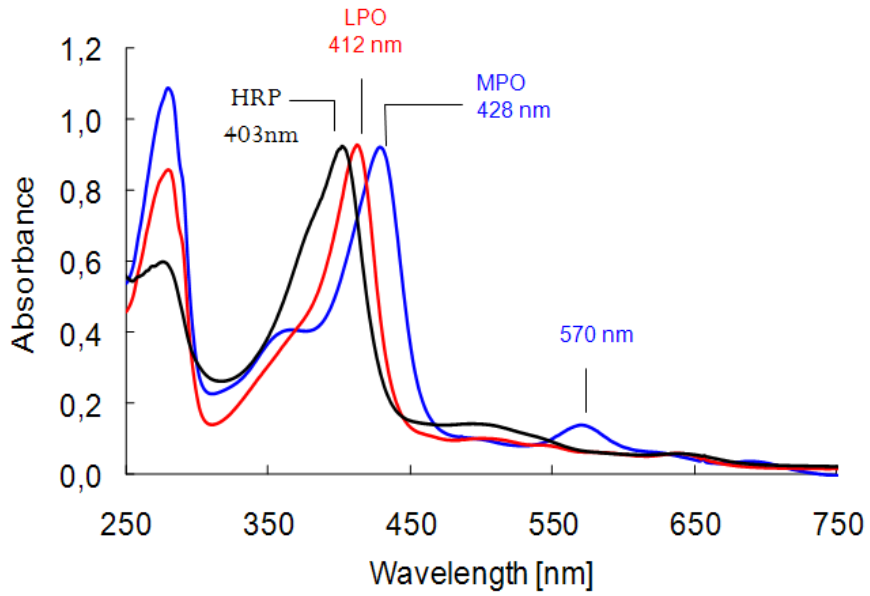


Figure 1-6: Comparision of the UV/VIS-spectra of HRP, LPO and MPO; HRP has no covalent link between the heme and the protein and a Soret maximum at 403 nm is seen. The Soret maxima of LPO and MPO are red-shifted. MPO (three covalent links) is more red-shifted than LPO (two covalent links)

1.2.4 Biosynthesis

MPO is expressed only in the myeloid cells and it is a gene 14 kb long, located on the long arm of chromosome 17. *(17)*

MPO is expressed as an 80 kDa precursor (preproMPO) which is released into the endoplasmatic reticulum (ER). The precursor is co-translationally modified by the addition of several sugars (N-glycosylation, $GlcNAc_2Man_9Glu_3$) and the removal of the signal peptide to yield the inactive apoproMPO. Glycosylation is important for the association with the chaperones calnexin, calreticulin and ERp57, which help the protein to fold properly *(18, 19)*

After binding of the prosthetic group (heme) the enzymatically active 90 kDa proMPO is formed. Further proteolytic cleavage includes excision of the 125 amino acid propeptide and of a small peptide between the small and the large subunit, finally forming the mature dimeric MPO. An overview of the biosynthesis is shown in figure 1-7. *(17)*

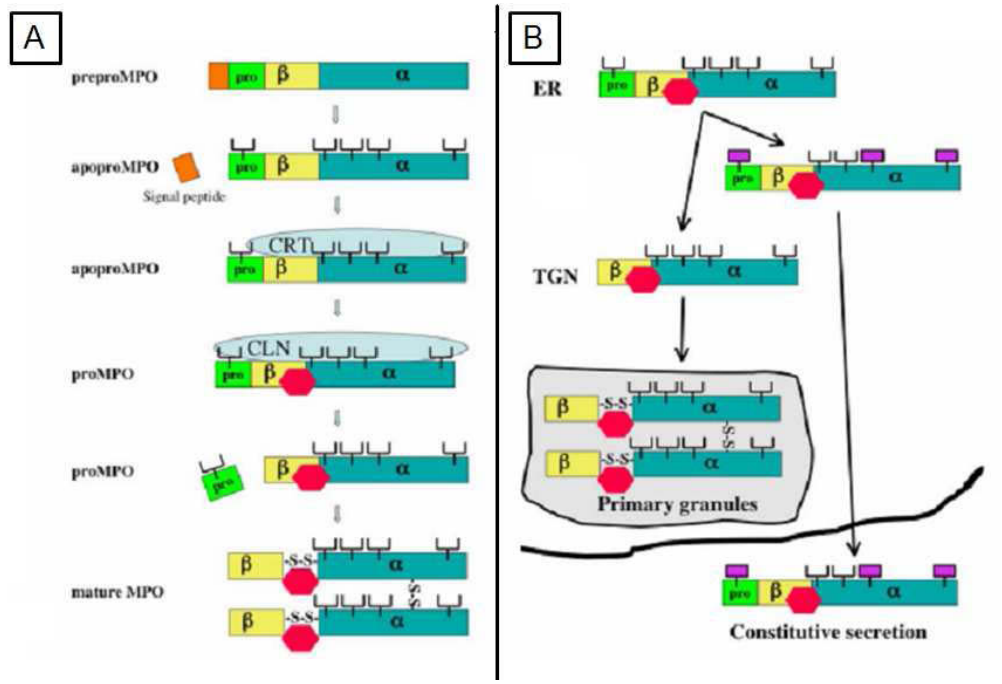


Figure 1-7: A: Biosynthesis of MPO; B: Storage in granules or secretion *(17)*

1.2.5 Reactions catalyzed by MPO

MPO is able to catalyze the oxidation various substrates. The most important feature of MPO is to oxidize small inorganic donor molecules (e.g. chloride). MPO is the only mammalian peroxidase which can oxidize chloride due to its unique redox potential. Furthermore bigger substrates can be oxidized in the peroxidase cycle and other reactions are seen as well. An overview of the reaction mechanism is shown in figure 1-8. *(20, 21, 22)*

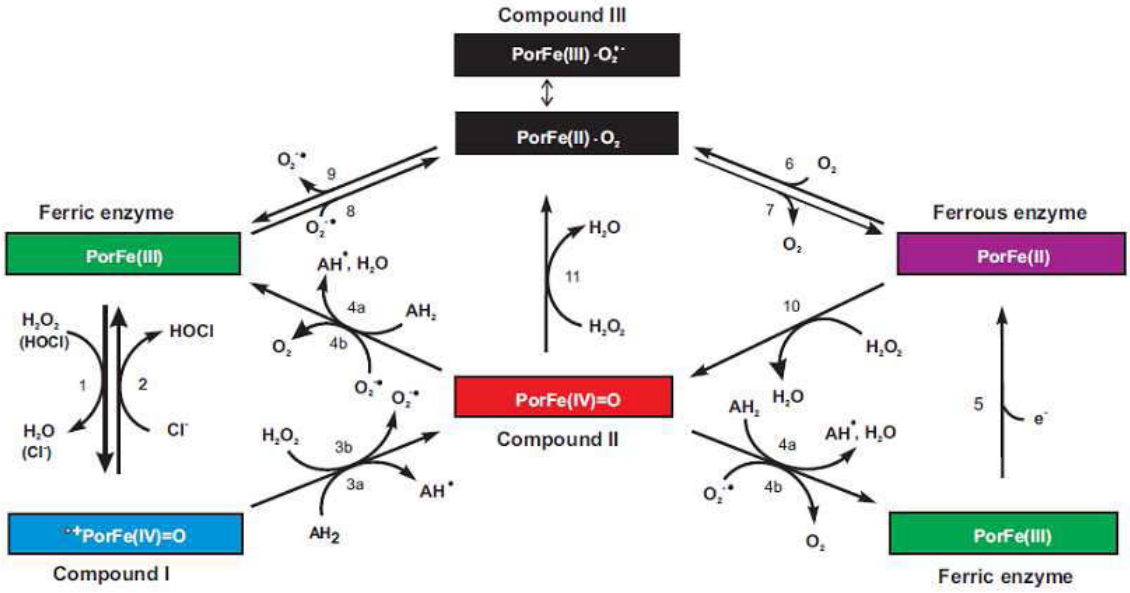


Figure 1-8: Reaction mechanism catalyzed by MPO

Both peroxidase and halogenation cycle starts by reaction of the Fe(III) form of the peroxidase with hydrogen peroxide to form compound I, which contains two oxidizing equivalents more than the resting enzyme (Reaction 1). One electron is removed from the iron to give the oxy-ferryl (Fe^{IV}=O) intermediate, and a second electron is removed from the porphyrin to give a porphyrin π-cation radical.

The absorption spectrum of native MPO is characterized by bands at 430 nm, 570 nm, 620 nm and 690 nm as well as shoulders at 370 nm and 496 nm. The Soret molar extinction coefficient at 430 nm is 91 000 M^{-1} cm^{-1} per heme. Upon addition of hydrogen peroxide compound I is formed within a few milliseconds, which has its Soret band still at 430 nm, but ε is about 50% lower than in native MPO.

Peroxidase reaction:

The general mechanism for a peroxidase reaction includes reactions 1, 3a and 4a (figure 1-8). In the peroxidase cycle compound I [Fe^{IV}=O…$Por^{\bullet+}$…aa] is reduced by two successive one-electron steps via compound II [Fe^{IV}=O…Por…aa]. In these one-electron oxidation reactions numerous substrates (AH) are oxidized to their corresponding radicals ($A^{\bullet}$). The reactivity of compound I as well as the reduction potential of the cI/cII couple is higher ($E^{0'}$ (cI/cII) = 1 350 mV, $E^{0'}$(cII/Fe^{III}) = 970 mV) and the reaction is faster. Thus the peroxidase cycle is dominated by compound II.

MPO can oxidize a wide range of substrates due to its large reduction potential of the cl/cII couple.

An overview of the peroxidase cycle is shown in figure 1-9.

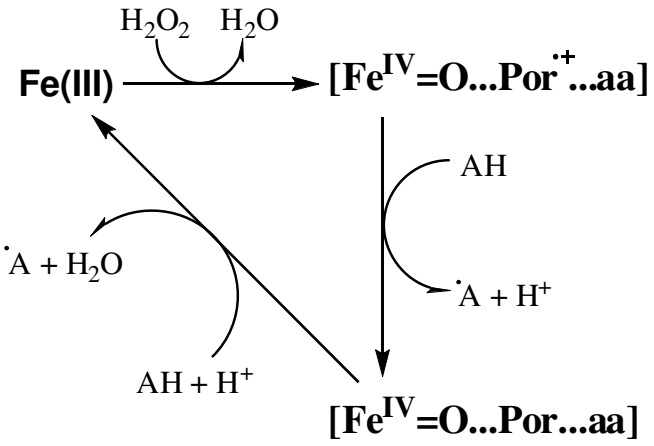


Figure 1-9: Peroxidase cycle: In the first step compound one is formed and in two one electron reactions the substrates are oxidized and the enzyme is reduced to yield the ferric enzyme again

Halogenation reaction:

The halogenations cycle includes the reactions 1 and 2 (figure 1-8). The first step is formation of compound I. Then compound I is reduced in a single two-electron reaction to yield the ferric enzyme again by oxidation of halides (and thiocyanate) to their corresponding hypohalous acid.

An overview of the halogenations cycle is shown in figure 1-10.

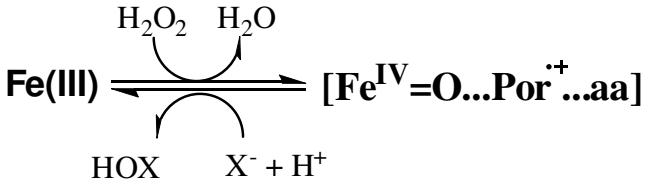


Figure 1-10: Halogenation cycle: In the first step compound I is formed and in a single two electron reaction the enzyme is reduced to its ferric form and halides are oxidized to their corresponding hypohalide acids

Pseudo catalytic activity of MPO:

In a catalatic cycle hydrogen peroxide acts as oxidant and reductant. But only monofunctional catalases and catalase-peroxidases follow this cycle and can oxidize H_2O_2 to molecular oxygen at high rates. Human peroxidases do not follow this reaction. Nevertheless, it is observed that in the absence of one-electron donors human peroxidases release molecular oxygen at low rates. An alternative cycle

involving ferrous peroxidase, compound II and compound III has been reported to be responsible for this reactivity. *(22)*

1.2.6 Establishing of mutants

For a better understanding of the reaction mechanism of MPO and the importance of single residues, different MPO variants were produced. Many mutants affecting the covalent links to the heme group or interacting with key residues in the active center were already designed and investigated. In this work the variants Glu91Thr and Arg333Ala/Lys were investigated.

1.2.6.1 Mutation of glutamine 91

Glutamine 91 was exchanged by threonine (Q91T). Threonine was chosen due to the fact that its hydroxyl group can form hydrogen bonds, like glutamine. Glutamine 91 is located at the distal site of the heme and conserved in all mammalian peroxidases. It might play a major role in binding of the small inorganic electron donor molecules (i.e. halides). Furthermore, it might be involved in correct heme insertion due to its hydrogen bonding to Glu 242. *(8)*

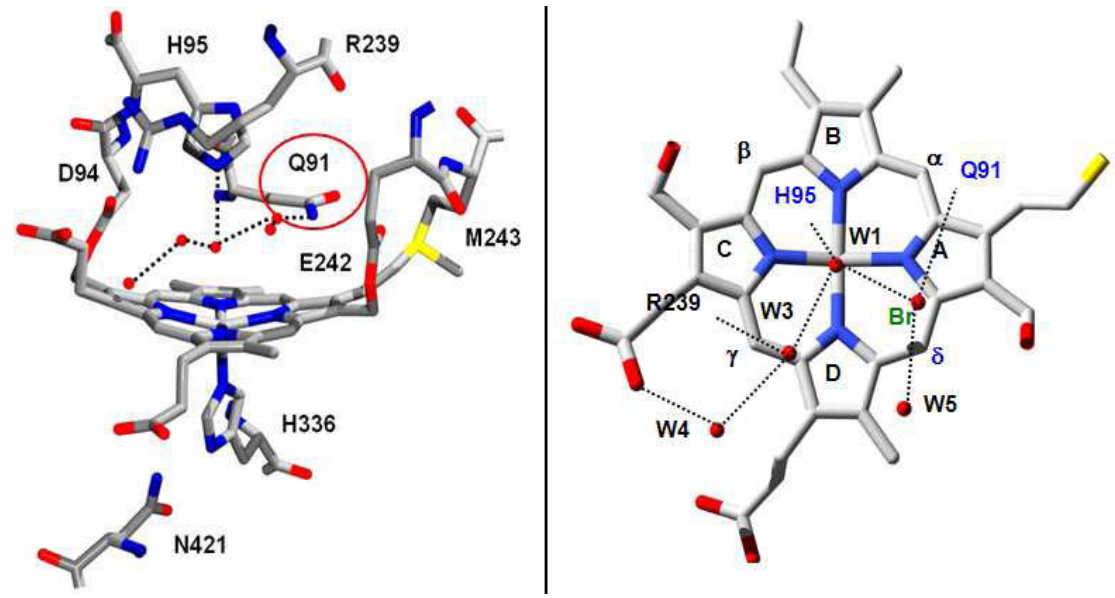


Figure 1-11: *left:* Active site structure of the MPO-Br^- complex structure; role of glutamine 91 at the distal site of the heme *right:* the hydrogen bonding network to bind small electron donors is shown. (8)

The structure of the MPO bromide complex shows a hydrogen bonding pattern including glutamine 91 (figure 1-11). His95 and Arg239 are also involved in the binding as well as several water molecules.

1.2.6.2 Mutation of arginine 333

Arginine 333 was exchanged by alanine (R333A) and lysine (R333K). Lysine was chosen due to its positive charge (like arginine) and alanine because it is a simple uncharged residue. Arginine 333 is located at the proximal heme site and part of the conserved triad together with His336 (proximal ligand of the heme iron) and Asn421. It interacts hydrogen bonding with Asn421 and by ionic interaction with a propionate group of the heme. Hence, Arg333 plays a major role in heme insertion and contributes to modulation of basicity of His336 and heme iron in general. *(13)*

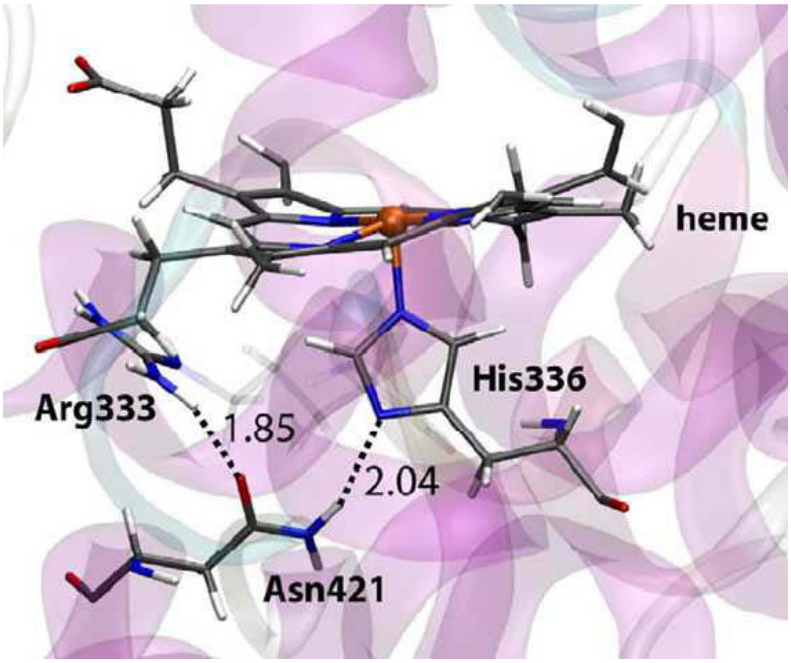


Figure 1-12: Conserved triad of His336, Asn421 and Arg333 at the proximal heme site

Former investigation showed the important role of Asn421. A molecular dynamics simulation was performed to determine the orientation of the carbonyl group of Asn421. The investigation clearly showed that the OArg position (when the carbonyl group of Asn421 points towards Arg333) is the most favorable orientation. *(13, 23)* The OArg position of Asn421 is shown in figure 1-13.

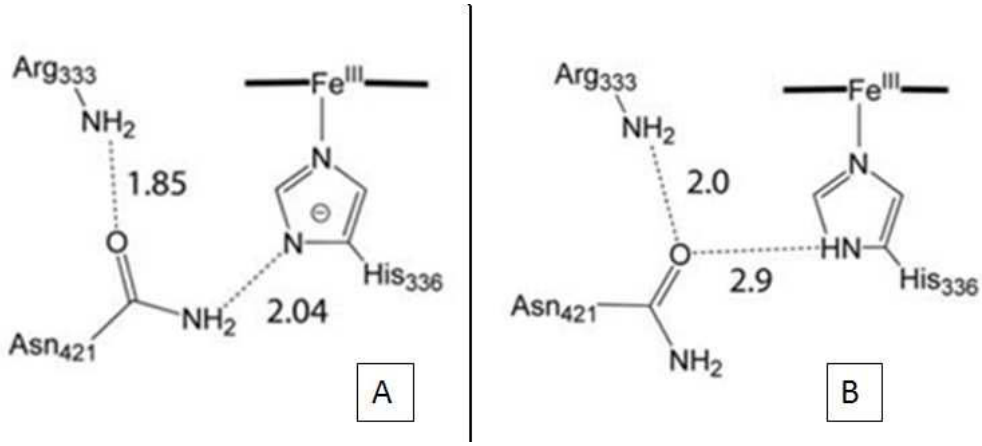


Figure 1-13: A: OArg orientation with anionic His336 B: OArg orientation with neutral His336 *(13)*

2 Aim of Study

CHO cells and HEK293 cells were already used for production of recombinant MPO and other mutants. Therefore media composition and purification protocols were available and established.

The Q91T mutant was already transfected into CHO cells and my work was to produce a sufficient amount of protein for biochemical characterization.

On the other hand both R333 variants had to be designed and stably transfected into HEK293 cells as well as produced and purified in adequate yield.

The recombinant proteins should be characterized biochemically and biophysically regarding spectral, redox and enzymatic properties. An important issue was the elucidation of the role of Glu91 in the halogenation activity of MPO.

3 Methods and Materials

3.1 Cloning of recombinant MPO variants

The cloning and transfection work of MPO mutant Q91T was already done before. Hence, starting with obtained cryogenic CHO cell culture of Q91T chapter 3.1 just deals with the plasmid preparation of R333 mutants.

3.1.1 DNA preparations

3.1.1.1 DNA vector

For transfection of the HEK cells the pcDNA3.1 vector (invitrogen) was used (figure 3-1). The vector contains a multiple cloning site, a strong CMV promoter and two antibiotic resistance genes for selective growth. Therefore cells carrying this plasmid can be selected by addition of ampicillin (*E. coli* cell culture) or geneticin (HEK293 cells) to the culture medium.

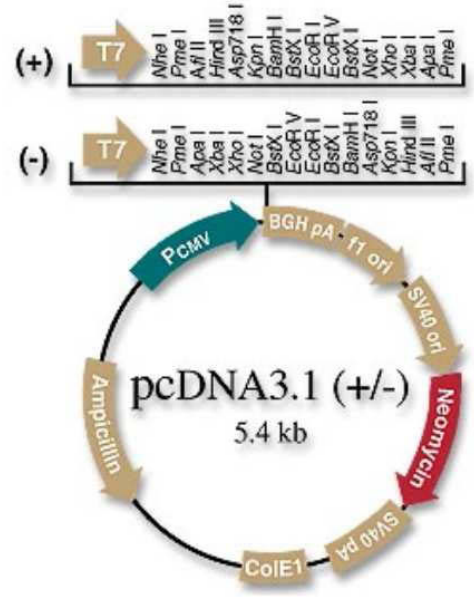


Figure 3-1: pcDNA3.1 vector from invitrogen, used for transfection of HEK cells, containing an ampicillin and a neomycin resistance for selective growth

3.1.1.2 Mutant strand synthesis reaction

The mutation at Position R333 was brought into the MPO containing plasmid by site direct mutagenesis using PCR. During PCR, the template DNA is denatured and 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 there will be sufficient amount of plasmid with the desired mutation for cloning into *E. coli*.

The primers were designed using Gene Runner. A mutagenesis primer should be G-C rich, not more than 40 base pairs long, the melting temperature T_m should be approximately 80°C, end at least 9 base pairs after the mutation and should contain as few loops, hairpins and dimers as possible. For each mutant 2 primers were needed for plasmid preparation and a PCR was performed.

Procedure:

The template DNA was purified with a Fermentas mini-prep kit from an over night culture of an *E. coli* strain with a MPO containing plasmid. The reaction mix as well as conditions of PCR are depicted in charts 3-1 and 3-2, respectively.

Chart 3-1: reaction mix for PCR

	concentration	volume
10 × buffer		5 µL
Template DNA	16.6 µg/µL	1.5 µL
dNTP mix		1 µL
Primer 1 | Primer 2 A	64.39 ng/µL | 59.69 ng/µL	2 µL
Primer 1 | Primer 2 K	67.91 ng/µL | 63.05 ng/µL	2 µL
dH_2O		38.5 µL
Pfu Polymerase		1 µL

Chart 3-2: PCR conditions

95°C	30 sec	
95°C	**30 sec**	
55°C	**1 min**	**16 cycles**
68°C	**8 min**	
72°C	10 min	
10°C	Hold	

Material and equipment:

Thermo cycler, Techne

10 × buffer, Fermentas

Pfu polymerase, Fermentas

QuickChange II Site-Directed Mutagenesis Kit

3.1.1.3 DpnI digest

A DpnI digest was performed before transformation to remove the parental DNA-template.

Procedure:

The PCR tubes were transferred on ice for two minutes and then 1 µL of DpnI (10 U/µL) was added. After down spinning the reaction mixture was incubated for 1 hour at 37°C.

3.1.2 Transformation of bacteria

For transformation *E. coli* XL1-Blue super competent cells were used. With the help of heat impulse, cell walls become permeable. Hence plasmid-DNA is able to move into the cell for tranformation. The vector pcDNA3.1 carries an ampicillin resistance gene allowing selection by addition of ampicillin to the medium.

Procedure:

Super competent *E. coli* XL1-Blue cells were thawed and 50 µL of the cell solution were pipetted into a tube. 25 µL of DpnI treated DNA was added and incubated for 30 minutes on ice. Afterwards the mixture was heated in a 42°C water bath for 45 seconds. The duration of the heat pulse is critical for maximum efficiency. After incubation on ice for 2 minutes, 0.5 mL pre-warmed (42°C) SOC-medium was added and cells were grown for one hour at 37°C with shaking at 250 rpm at the thermo-block. Finally 250 µL aliquots of the suspension were plated onto selective LB agar (two plates for each mutant) and grown for at least 16 hours at 37°C.

Materials and Equipment:

LB- medium:	5 g NaCl
	5 g Peptone
	2.5 g Yeast extract
	HQ- H_2O added to 500 mL
$LB\text{-}medium_{AMP}$:	LB- medium and addition of 100 µg / mL Ampicillin
Shaker	INFORS HT Ecotron

3.1.3 Screening

3.1.3.1 Miniprep

Miniprep refers to a small scale plasmid DNA preparation from bacterial cell suspensions. The basis of separation is an alkaline lysis of the cells, which denatures nucleic acids as well as proteins. It takes advantage of the fact that plasmid DNA is relatively small and highly supercoiled, whereas chromosomal DNA is long and less supercoiled. By addition of a neutralizing agent the plasmid DNA renatures and stays in solution, whereas chromosomal DNA and proteins precipitate.

For miniprep the Fermentas GeneJET™ kit was used.

Procedure:

1.5 mL of saturated bacterial cell suspension was transferred to a 1.5 mL tube and centrifuged at 10000 rpm for 5 minutes. The supernatant was discharged and the pellet resuspended in 250 µL resuspension solution (containing Rnase A). Then 250 µL Lysis solution were added to the tube and mixed by inverting 4-6 times. After the addition of 350 µL neutralization solution the tube was inverted again for 4-6 times. Upon centrifugation at 10000 rpm for 5 minutes, chromosomal DNA and proteins precipitate. Afterwards the supernatant (very carefully, the pellet was very lose) was loaded on GeneJET™ spin column. The spin column was washed with 500 µL of Wash solution and the flow-through was discharged. The washing step was done twice. The empty column was centrifuged for 1 minute at 10000 rpm to remove washing solution. Finally the column was incubated for 2 minutes with 50 µL elution buffer (or water) and centrifuged for 2 minutes at 10000 rpm. The flow-through contained the purified plasmid DNA.

3.1.3.2 DNA agarose gel electrophoresis

With agarose gel electrophoresis DNA can be separated according to its size. The separation is also dependent on the charge of the molecules, but due to its phosphate backbone all DNA samples are negative charged. Agarose gel electrophoresis of miniprep samples were made to probe whether the insert was built in correctly.

Procedure:

After pre-run of ready-to-use agarose gel 20 µL sample solution were loaded. In case of miniprep plasmid DNA 1 µL of the sample was mixed with 19 µL H_2O. The gadget was started and run for 30 minutes. The bands were visualized under UV light.

3.1.4 DNA sequencing

To assure accuracy of the final DNA construct, DNA sequencing is necessary. Comparison of the obtained with the expected sequence can confirm the correct integration of the mutation. For sequencing of the whole MPO DNA 5 primers are necessary.

Procedure:

DNA sequencing was carried out by an external company, AGOWA (Berlin, Germany). Therefore the samples (10 µL of plasmid DNA with 4 µL of the desired primer (5 pmol/µL)) were prepared and sent to the company.

Chart 3-3 lists the selected primers.

Chart 3-3: list of desired primers for DNA sequencing

Name of primer	Binding site
T7 promoter	863
MPO 4667_fw	1637
MPO 1291_fw	2392
MPO 5603_rv	2573
MPO 1720_rv	1733

3.1.5 Cryo cultures of clones

For long time storage of the selected clones, cryo cultures (-80°C) were made.

Procedure:

900 µL of an over night culture were pipetted into a cryogenic tube. Afterwards a cryogenic protectant (900 µL 30% glycerol) was added. The tubes were frozen at -80°C.

Material and Equipment:

30% Glycerol

Shaker, INFORS HT Electron

LB-medium

3.1.6 Endotoxin free mediprep

Endotoxins are lipopolysaccharids, elements of the outer membrane of gram negative bacteria. They can cause fever and are therefore also called pyrrogens.

During cell lysis of the plasmid preparation endotoxins are set free. These remain in solution with the isolated plasmid DNA. The endotoxin free plasmid separation is necessary before transfection in animal cell culture to protect the cells. The endofree kit contained an extra buffer for removing of endotoxins.

Procedure:

100 mL saturated *E. coli* culture were grown out of a starter culture. The bacterial suspension was centrifuged for 15 minutes at 6000 g at 4°C. The supernatant was discharged and the pellet was resuspented in 10 mL of buffer P1 (including Rnase A). Then 10 mL of buffer P2 were mixed by inverting 4-6 times and incubated for 5 minutes at room temperature. Afterwards 10 mL chilled buffer P3 was added and again inverted 4-6 times. The lysate was poured into a QIAGENfilter Cartridge and incubated for 10 minutes at room temperature. Afterwards the lysate was filtered into a 50 mL tube and 2.5 mL of buffer ER were added. The tube was inverted approx. 10 times and incubated for 30 minutes on ice. In the meantime a QIAGEN-tip 500 was equilibrated with 10 mL QBT (gravity flow). The filtered lysate was applied on the tip and allowed to enter the resin by gravity flow. Then the QIAGEN-tip was washed two times with 30 mL buffer QC and the DNA was eluted with 15 mL buffer QN. 10.5 mL isopropanol were added for DNA precipitation and immediately centrifuged at 15000 g for 30 minutes at 4°C. The supernatant was decanted carefully. Afterwards the pellet was washed with 5 mL of 70% ethanol, centrifuged at 15000 g for 10 minutes at 4°C followed by carefully decanting of the supernatant. Finally the DNA pellet was air-dried for 5 minutes and dissolved in 100 µL buffer TE.

Material and equipment:

Endofree plasmid maxi kit, QIAGEN
LB-Medium
Shaker, INFORS HT Electron
Centrifuge, Sorvall RC5C
Micro centrifuge, Sigma 1-15

3.1.7 Quantification of purified DNA

For determination of DNA concentration UV-VIS-spectroscopy was used. DNA has a maximum absorbance at 260 nm and the concentration can be easily calculated (50 µg/mL equals an absorption of 1).

To estimate the purity of the probe the wavelengths 230 nm, 280 nm and 320 nm are measured as well. Proteins have an absorption maximum at 280 nm due to the aromatic amino acids. Pure double stranded DNA has a typical ratio of A_{260} / A_{280} of

2. Impurities like phenols, peptides, carbohydrates and aromatic hydrocarbons would show a peak at 230 nm. Therefore the ratio A_{230} / A_{260} should be around 0.6. Due to the fact that neither nucleic acids nor proteins absorb at 320 nm the absorption at this wavelength should be ideally 0.

Procedure:

The DNA sample was diluted (1:10 to 1:90 with TE buffer) giving a final absorbance between 0.1 and 2.0. The photometer was calibrated with TE buffer as reference. Finally DNA samples were measured and purity numbers were calculated.

Material and equipment:

Photometer, Zeiss

TE buffer, QIAGEN

DNA sample

3.2 Protein production in animal cell culture

3.2.1 Animal cell culture

3.2.1.1 Laminar flow hood

To prevent contaminations handling of cells was performed in a laminar flow hood. Before and after working in the laminar flow hood the UV-lamp and the fan were turned on for at least half an hour. The working place and every material inserted into the laminar flow hood were cleaned with 70% ethanol. The whole laminar flow hood was cleaned with bacilol once a week. All disposable material which was in contact with the cells was autoclaved and discarded. Reusable material (Pasteur pipettes, media bottles, …) was autoclaved before and after using and cleaning.

Material and equipment:

Laminar Flow Hood: Heraeus

Ethanol: Merck

UV-Lamp: Osram 15V

bacillol

3.2.1.2 Stock solution preparation

Solid chemicals (Penicillin, Streptomycin, Amphetoricin B and Geneticin) were weighted outside of the laminar flow bank and dissolved in ddH_2O (apart from hemin

and hematin which were dissolved in 0.1 M NaOH). Afterwards, they were sterile filtered inside of the laminar with disposable syringe-filters and aliquots were stored at -20°C (hemin and hematin had to be freshly produced each time).
Liquid supplements (FBS and L-glutamine) were thawed, aliquoted and stored at -20°C.

Material and equipment:

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

3.2.1.3 Media preparation

The right medium and supplements for every cell line is very important. A defined medium has to contain a carbon source, vitamins, amino acids, inorganic salts and micronutrients as well as a buffer-system, usually realized by a hydrogencarbonat buffer. The used media (DMEM and α-MEM) also contained phenol red as pH indicator, that changes the color from yellow (acidic) to red/violet (basic). The complex supplement FBS (fetal bovine serum) was added to supply the cells with growth factors, adhesion factors and hormones. Penicillin (destroys the cell wall of gram positive bacteria) and streptomycin (binds to bacterial ribosome) were added to prevent contaminations.

Procedure:

Solid α-MEM medium and 2.2 g/L $NaHCO_3$ were dissolved in HQ-H_2O and the pH value was adjusted to 6.8 with 1 M NaOH or 1 M HCl. Afterwards the medium was filtered with bottle top filters in autoclaved bottles (for large scale production batches of 2 L). The pH value increases during filtration for approximately 0.3-0.4 pH units. The airtight closed bottles were stored at 4°C. Supplements (pre-assembled stock solutions; 1% Penicillin/Streptomycin, 10% FBS, 1-2% L-glutamine and (for production only) 5 µg/mL hematin/hemin) were added aseptically before use.
The preparation of DMEM was similar to α-MEM medium, except 3,7g/L $NaHCO_3$ were added to the powdered medium and no L-glutamine had to be added.
Before use the medium was equilibrated to 37°C by using a water bath.

Materials and equipment:

Water bath, Julabo

Bottle top filters

(PES membrane, 0.2 µm), Nalgene

pH- Meter, Radiometer Copenhagen

$NaHCO_3$, Merck

3.2.1.4 Cultivation condition of the cells

Chinese Hamster Ovary (CHO) cells were grown in α-MEM medium supplemented with penicillin and streptomycin (100 µg/mL), 10% FBS and 1-2% L-glutamine. The cells were maintained at 37°C in a humidified atmosphere (95%) containing 5% CO_2. Human Embryo Kidney (HEK) cells were cultivated in DMEM (containing Glutamax®) supplemented with penicillin and streptomycin (100 µg/mL) and 10% FBS within the same growth conditions.

3.2.1.5 Trypsinization of cells

Both cell lines used for protein production were adherent cells. For detaching them carefully from the surface of the culture flasks trypsin, a serine-protease, is used. FBS contains trypsin inhibitors, for that reason the culture medium has to be removed before treatment with trypsin. After a few minutes the trypsinization should be stopped (with FBS containing media), because trypsin can cause irreversible cell damage.

Procedure:

The medium was discarded and the cells were washed with PBS to remove FBS. Then the cells were covered with trypsin solution. After an incubation time of a few minutes at 37°C and 5% CO_2 the flask was controlled with a microscope. At the time when most of the cells were detached and had a round shape the cells were knocked off in the laminar flow hood. With addition of media the trypsinization process was stopped. If some cells stayed adherent they could be removed by pipetting. The cell suspension was now ready to be transferred in new flask and incubated at 37°C in the incubator. After trypsinization the cells should not be manipulated for at least 24 hours.

Chart 3-4 used amounts of PBS (for washing) and trypsin

Cell culture flask	PBS [mL]	Trypsin [mL]
T25	5	0,5

T75	15	1
T175	25	2

Methods 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 Changing of media

Cell media had to be changed due to nutrient consumption and accumulation of toxic metabolites. The changing frequency depended on the growth rate, cell number and metabolism. Unstable components (e.g. antibiotics and L-glutamine max. 4 days at 37°C) made it necessary to change the media every 3-4 days.

Procedure:

The old medium was poured off and if needed for protein purification stored at 4°C. If necessary the adherent cells were washed with PBS. Then the new medium containing all supplements (37°C) was added and the flask was incubated again.

3.2.1.7 Passaging of cells

Monolayer cell culture should be transferred in larger culture vessels when they have reached a confluency of 80-90%, because they stop growing due to contact inhibition if the whole surface is occupied. The dilution factor of 1:3 – 1:10 depends on the growth rate of the cells. If the growth rate is very high a high dilution factor should be chosen (and vice versa).

Chart 3-5: used cell culture vessels and surface size

Cell culture vessels	Surface [cm^2]
96 well	0.28
12 well	3.9
6 well	10
Petri dish (diameter 60 cm)	20
T25	25
Petri dish (diameter 90 cm)	58
T75	75
T125	125

T175	175
Triple flask	Approx. 500
Cell factory	6320

Procedure:

The cells were treated with trypsin and transferred with a pipette in a new cell culture vessel according to the desired dilution. During incubation at 37°C the cells should not be manipulated within the following 24 hours.

3.2.1.8 Freezing of cells

For long-time storage cells were frozen at -80°C (1 year) and in liquid nitrogen at -196°C. To prevent cell damage a cryogenic protectant (10% Dimethylsulfoxid (DMSO)) was added. The freezing rate should be slow enough to enable DMSO to substitute cell water. It is important to use healthy and proliferating cells (confluence ~80%). To achieve the desired concentration of 2-5 × 10^5 cells/cryogenic vial T25-flasks (one cryogenic culture) or T75-flasks (three cryogenic cultures) were used.

Procedure:

Cells were detached using trypsin and after addition of new medium cell suspension was transferred into a sterile centrifugation tube. After centrifugation for 10 minutes with 170 g the supernatant was removed and the cell pellet carefully resuspended in the pre-cooled freezing medium (normal growing media with 10% DMSO). The cell suspension was portionated in prechilled cryogenic vials and frozen at -80°C. After 24 hours some of the vials were transferred to the liquid nitrogen tank.

Material and Equipment:

Cryogenic vials	*Nalgene*
DMSO	*Sigma*
Centrifuge	*Hettich*

3.2.1.9 Thawing of cells

Cells should be thawed rapid, but cautious to prevent any cell damage. After transferring the cell suspension in the culture vessel dilution of the cryogenic media is necessary to decrease DMSO concentration to improve cell anchorage to the surface.

Procedure:

The cryogenic culture was thawed in the hand without shaking. When thawing was completed the suspension was pipetted carefully with a single use pipette into a new T25 flask with 7 mL medium (37°C). Cells were incubated without moving or manipulating for the next 24 hours. Finally, the medium was changed to remove the DMSO.

Material and equipment:
single use pipette *Roth*

3.2.1.10 Stable transfection

HEK cells were transfected via Ca^{2+} co-precipitation. CHO cells have been transfected via lipofection was done before.

Ca^{2+} co-precipitation:

Cultured cells can uptake DNA co-precipitates via endocytose. Part of uptaken DNA is able to escape from endosomes or lysosomes and get transferred from the cytosol to the nucleus.

Procedure:

For transfection cells with 40-50% confluence in a 12 well plate were used. The plates were pretreated with 25 µg/mL polyethylenimine (PEI) to enhance adherence on the surface. Four hours before transfection the cells had to be treated with 25 µM chloroquine. One µg DNA was diluted in 35.5 µL 0.25 M $CaCl_2$, finally 35.5 µL HEPES-buffered saline pH 7.05 was added during vortexing. The complete solution was added carefully to the cells and incubation was performed for for 24 hours. Afterwards the medium was changed.

Material and Equipment:
Polyethylenimine
Chloroquine

3.2.1.11 Selection of transfected cell clones

28 hours after transfection cells were subcultured in selective media, containing geneticin. Geneticin is an antibiotic which harms eukaryotic cells, therefore only positive transfected cells can survive, due to neomycin resistance.

Isolation of single cell colonies have been achieved either by picking of single cells or by serial dilution.

Procedure:

Selective media containing geneticin had to be exchanged every 2-3 days. After one week just positive cell clones should have survived and dead cells were discarded by washing.

If single cell clones had grown apart from each other manual picking was possible. Therefore single cell clones were marked under microscope, detached with a pipette dip and resuspended into wells of a 96-well plate.

If manual picking was not possible, serial dilution steps in a 96-well plate were done. Thereby 100 µL of media were pipetted in each well of a 96-well plate, apart from the well A1 in which 200 µL of the cell suspension (5×10^3 - 1×10^4 cells) were transferred. 100 µL of the cell suspension of A1 were transferred via pipetting to well B1. The suspension in B1 was carefully mixed and 100 µL were transferred to well C1. This dilution step was repeated all over the whole first column. The last 100 µL were discarded. 100 µL fresh medium were added to the first column. Afterwards 100 µL from each column were transferred to the following column with a multichannel pipette, whereas the final 8 x 100 µL were discarded again. Before cells were incubated at 37°C 100 µL fresh medium was added to each well.

Material and Equipment:

Multichannel pipette *Gibson*

3.2.1.12 Screening of positive cell clones

For selection of the best producing cell clones two screening methods were used, immune detection and ABTS screening.

Immune detection:

To detect small amounts of protein immune detection can be used. Thereby a specific antibody binds to an epitope of MPO. The specific antibody is captured by a

secondary antibody which is connected with a phosphatase that reacts with 5-brom-4-chlor-3-indolylphosphate (BCIP) and nitroblue tetrazolium (NBT) to form a colorful compound indicating the presence of the target protein.

Procedure:

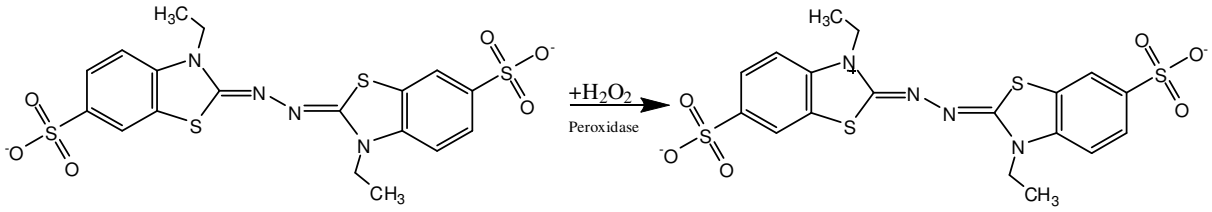
Very little amounts (1-2 µL) of the crude medium were dotted on a nitrocellulose membrane. After drying the membrane was incubated for at least 1 hour in 10 mL blocking solution at room temperature. Then the dot blot was incubated in binding solution, containing the primary antibody for 2 hours. After two washing steps with binding solution the membrane was incubated for 2 hours in 10 mL binding buffer containing 0.5 µL of the secondary antibody. All incubation steps were done with permanently shaking. Before staining the blot was washed four times for 10 minutes with binding buffer. Afterwards the membrane was incubated in 5 mL AP-buffer with 33 µL NBT and 16.5 µL BCIP. If protein was present a colorful dot developed after approximately 20 minutes.

Material and Equipment:

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

ABTS-screening:

MPO can oxidize a wide range of substrates by consumption of hydrogen peroxide. When ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)) is oxidized a colorful (green) product is formed. This can be used to demonstrate the presence of peroxidase activity.



Procedure:

The medium supernatant was transferred in a new 96-well plate. For screening of the positive cell clones 2 µL of 10 mM H_2O_2, 10 µL of 200 mM phosphate buffer, pH 5.0 and 10 µL of 50 mM ABTS were added to the medium. Additionally, a negative and a positive control for easier evaluation of the color change were performed.

Figure 3-2: example for ABTS screening in a 96-well plate

Material and equipment:

ABTS	*Sigma*
H_2O_2	*Sigma*
Microtiterplate	*Nunc*

3.2.1.13 Selection of cell clones for production

12 cell clones of each R333 mutant were chosen for detailed determination of the expression performance and enzyme activity, based on the results of ABTS oxidation and immune detection. Picked clones were transferred into 12 well plates to identify the best clone by determination of specific peroxidase activity. Due to the fact that no activity could be measured, all clones had to be transferred into T25 cell culture flask and 50 mL medium supernatant of each clone was purified (see chapter protein

purification). Again no definitive conclusion could be made, but three clones of each mutant were chosen for up-scaling. Already in this early stage of investigation it became clear that the mutation at R333 had a strong impact on heme integration and enzyme activity.

3.2.1.14 Large scale cultures

To produce high amount of protein closed multilayer systems are used. Triple flasks (used for R333 mutant production) have three layers and are much smaller than cell factories (used for Q91T) with 10 layers. The triple flask (150-300 mL medium) is handled like a normal cell culture vessel.

A cell factory has two outlets, one is used for medium exchange and the other one is closed with a sterile filter for gas exchange.

Figure 3-3: Triple flask

Figure 3-4: Cell factory

Procedure:

The cell factory was filled with the suspended cells of 6 T175 flasks. All chambers had to be filled with the same amount of media. The total volume of the medium was approximately 2.5 L. The first two batches were grown without hematin for better cell attaching, whereas in the following batches 5 µg/mL hematin was added for a better heme insertion. The medium was changed twice a week and the supernatant was filtered through a bottle top filter. Bottles were stored at 4°C.

Material and Equipment:

Cell factory	*Nunc*
Sterile air filter	*Nunc*
Sterile funnel	*Nunc*
Triple flask	*Nunc*
Bottle top filter	*micropore*

3.2.1.14.1 Handling of contaminated cell factories

Despite sterile working in laminar flow hood, in some cases a contamination occurred. If that happened elimination of yeast cells was tried by cautious washing of the cells with PBS and adding amphotericin B (an antibiotic against yeast and fungi) to the new medium. In most of the cases it was not possible to rescue the mammalian cells or the protein expression levels decreased.

Procedure:

The contaminated media were discharged and cells were washed with PBS. The new media contained 2.5 µg/mL amphotericin B and no hematin or hemin.

Material and equipment:

PBS

Amphotericin B,Sigma-Aldrich

3.3 Protein purification

The protein is purified from the supernatant using a cation exchange chromatography. The pooled fractions are desalted, using a gel filtration and afterwards concentrated with centripreps. For long time storage the protein is lyophilized and frozen at -80°C.

3.3.1 Cation exchange chromatography

Using cation exchange chromatography proteins can be separated due to their charge. MPO is a highly positively charged protein. For that reason it is possible to separate it with a weak cation exchange gel (Carboxylmethyl-Sepharose CM). The main principle of the purification is the reversible interaction between the charged protein and the oppositely charged matrix. Bound proteins can be eluted by increasing salt concentration or change of pH.

Procedure:

The gel is stored in 20% ethanol to prevent microbiological growth. After packing the column ethanol is washed away with RO-water at a low flow rate. Then the column is equilibrated with 20 mM phosphate buffer, pH 7.0, (5 times the volume of the gel). The cell culture supernatant was diluted 1:2 with RO-water and loaded on the column. Afterwards the column was washed with equilibration buffer again, until no absorption at 280 nm was detectable. For protein elution a linear gradient of 0-1 M NaCl in 20 mM phosphate buffer, pH 7.0, was used. The eluate was collected in

fractions of 3-5 mL and analyzed spectrophotometrically to estimate the protein content.

Finally, the column was washed with high concentration of NaCl to remove all bound proteins and RO-water. It was stored within 20% ethanol.

By the time the separating capacity of the column decreased. Regeneration of the gel was possible by washing it in opposite direction with 1 M NaOH (NaOH should not remain for long time at the gel to prevent damage).

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, pH 7.0
Ultrasonic bath Sonorex Super RK510H
Filter HVLPO4600, pore size 0.45 µm *Millipore*

3.3.2 Concentration of the protein

To concentrate the protein solution Centripreps YM-30 were used. Centripreps consist of three parts; a sample container, a filtrate collector (with a membrane) which is put in the sample container and a cap. The Centriprep containing protein solution is centrifuged and due to an increasing hydrostatic pressure molecules which are smaller than 30 kDa are forced through the membrane. Bigger proteins remain in the sample container.

Figure 3-5: centriprep

Procedure:

The protein solution was pipetted in the sample container and centrifuged with 2500 rpm for 20 minutes at 4°C. Then the filtrate from the filtrate collector was discharged and concentration steps were repeated till the desired concentration was reached.

3.3.3 Desalting

Due to high NaCl concentration after chromatography a desalting step was necessary. Therefore a PD-10 column, containing a size exclusion gel, was used. Big molecules like proteins are faster than small molecules (e.g. chloride, sodium) due to the fact that they cannot enter small pores of the gel. Therefore big molecules are eluted with the void volume.

Procedure:

The column had to be equilibrated with the desired buffer (20 mM phosphate buffer, pH 7.0). The protein solution was loaded onto the column and eluted with the desired buffer. Fractions of the desalted protein were collected and pooled.

3.3.4 Lyophilization and freezing for long time storage

The stability of MPO (and especially of the mutants) in aqueous solution, even at 4°C, is limited. Due to that fact concentrated protein solutions are lyophilized and frozen at -80°C.

Procedure:

Small portions of highly concentrated protein solutions were portioned and lyophilized. After lyophilization they were frozen at -80°C for long time storage.

Material and equipment:

Phosphate Buffer pH 7		Centriprep	YM-30 Amicon
Centrifuge	Du Pont Instruments Sorvall, RC-5C	Speed Vac	SVC 100 H SAVANT Instruments

3.4 Protein characterization

3.4.1 Spectral properties

The absorption spectra of MPO (and its mutants) show a very characteristic peak beside the protein peak at 280 nm, the so called Soret peak. The maximum of the Soret peak depends on the chemical environment and modification of the heme group. The ratio between the Soret peak and the protein peak is called the purity

number (RZ) of the protein solution. The sample concentration can be calculated with Lambert-Beer`s law.

Lambert-Beer`s law: $A = \varepsilon \times d \times c$ $\quad RZ = \frac{absorption\ soret\ peak}{absorption\ protein\ peak}$

A… absorbance
ε… molar extinction factor [M^{-1} cm^{-1}]
d… diameter of the cuvette [cm]
c… concentration [mol/L]
RZ… purity number

Procedure:

First a reference spectrum with the same buffer was measured. In order to reduce background absorbance the enzyme solution was centrifuged or filtered before measuring. Then 0.3-1 mL protein solution was pipetted into a quartz cuvette and a wavelength scan from 200 to 800 nm was performed.

3.4.2 SDS-PAGE

SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis, is a technique to separate proteins according to their molecular weight, shape and charge. In an electric field charged molecules are able to migrate. The electrophoretic movement depends also on the ionic strength, viscosity and temperature of the medium. Therefore a marker (containing defined proteins) should be used to determine the relative molecular weight.

SDS binds to proteins and gives them a highly negative charge. Thus all proteins are charged identical and the separation is only dependent on molecular mass and shape of the proteins. Due to the fact that SDS has denaturating effects as well and mercaptoethanol is reducing cysteine bridges all proteins are random coils and have similar shape.

Electrophoresis is carried out in an inert homogenous carrier like a polyacrylamide gel, which porosity can be determined by the concentration of acryl amide and methylenebisacrylamide. For visualization of the protein bands the easy and fast Coomassie blue method was used.

Procedure:

The gel consisted of a stacking and separation gel. Solutions were mixed as shown in chart 3-6. Before TEMED and APS were added the solution was degassed in an ultrasonic bath for 7 minutes.

Chart 3-6: composition of stacking and separation gel

	Separation gel; 10% monomer conc. (for 2 gels)	Stacking gel; 4% monomer conc. (for 2 gels)
H_2O	3.96 mL	2.97 mL
1.5 M Tris-HCl	2.5 mL	-
0.5 M Tris-HCl	-	1.25 mL
Acryl amide/ Bis	3.33 mL	670 µL
10% SDS	100 µL	50 µL
10% APS	100 µL	50 µL
TEMED	10 µL	10 µL

First the separation gel was made and covered with 2-butanol in order to achieve a plane surface. After fully polymerization the 2-butanol was removed and the stacking gel was casted on the separation gel. Before the stacking gel polymerized a comb was set in order to form the slots. After polymerization of the stacking gel, the gels were transferred into the electrophoresis chamber, which was filled with running buffer. Afterwards the comb was removed and the samples, which were mixed with sample buffer, loaded with a Hamilton syringe. The electrophoresis chamber was connected with the electrodes and electrophoresis was started (running conditions: 200 V and 70 mA). When the bromphenolblue front reached the end of the gel electrophoresis was stopped and the gel was either stained or blotted (Western Blot). For staining the gel was incubated for 30 minutes on a shaker in Coomassie blue staining solution at room temperature. Afterwards the gel was transferred into a destaining solution, until all bands were clearly visible.

Material and Equipment:

5x Electrophoresis buffer: 15 g/L Tris
72 g/L glycine
5 g/L SDS
HQ- H_2O added to 1000 mL

2x Sample buffer: 900 µL SB- Stock
100 µL β-mercaptoethanol

1x Electrophoresis buffer: 200 mL 5 x Electrophoresis Buffer HQ- H_2O added to 1000 mL

Acrylamide/Bis: 500 ml
46.0 g acrylamide
4.0 g Bis

1.5 M Tris-HCl, pH 8.8: 300 ml
54.45 g Tris Base
pH adjustment with 6 M HCl

0.5 M Tris-HCl, pH 6.8: 100 ml
6.0 g Tris Base
pH adjustment with 6 M HCl

Power supply BioRad
Electrophoresis Equipment BioRad

3.4.3 Western Blot

Western Blotting is a frequently used method to detect small amounts of proteins. The proteins (or fragments of proteins) already separated with SDS-PAGE are transferred from the polyacrylamide gel to a nitrocellulose membrane. For achieving the transfer filter pads, filter paper, the gel and a nitrocellulose membrane are placed together like a sandwich between two electrodes. To remove SDS and enhance the binding of proteins to the membrane methanol is added to the transfer buffer. For specific detection of MPO immune detection is performed (chapter 3.2.1.12).

Procedure:

The filter papers, filter pads, the gel and nitrocellulose membrane were incubated together in precooled running buffer. The blot was assembled in the cassette without bubbles between the gel and the membrane as shown in figure 3-6. Then the cassette was transferred into the electrode bracket. The buffer chamber was filled with transfer buffer and a magnetic stirrer was added. For assignment running conditions were set to 100 V at 4°C for at least one hour.

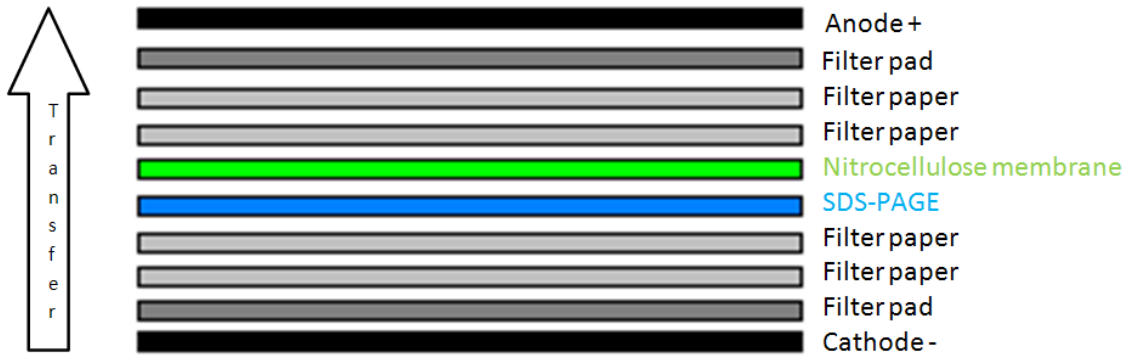


Figure 3-6: assembly of a Western Blot

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 Peroxidase activity

Two different assays were used to determine the peroxidase activity, the ABTS assay and the guaiacol assay.

3.4.4.1 ABTS assay

MPO catalyzes the reduction of H_2O_2 to water by oxidation of a wide range of substances. When ABTS is oxidized (chapter 3.2.1.12) the absorption at 414 nm increases and can be measured.

$\varepsilon_{414} = 36000\ M^{-1}\ cm^{-1}$

Procedure:

All solutions of table 3-7 were mixed together in a cuvette with a stirring bar (except H_2O_2). The cuvette was set into the light beam of the spectrophotometer and the measurement was started. After about 10 seconds the reaction was started with the addition of H_2O_2.

Chart 3-7: reaction conditions for ABTS assay

	Concentration	Volume [µL]	Concentration in cuvette
Phosphate buffer pH 5.0	200 mM	500	100 mM
ABTS	50 mM	20	1 mM
Enzyme		50 (or 100)*	
RO water		420 (or 370)*	
H_2O_2	10 mM	10	100 µM
Total		1000	

*depending on Enzyme concentration and activity

3.4.4.2 Guaiacol assay

Another way to determine the peroxidase activity is the guaiacol assay. Thereby guaiacol is used as a substrate and forms a dimer (3,3'-dimethoxy-4,4'-biphenoquinone) upon oxidation. In this assay the change of absorbance at 470 nm is measured (pH 7.0, 25°C).

$\varepsilon_{470} = 26600\ M^{-1}\ cm^{-1}$

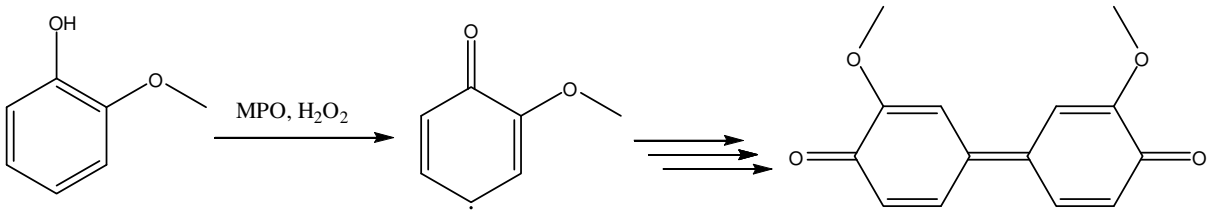


Figure 3-7: Reaction of guaiacol

Procedure:

All solutions of chart 3-8 were mixed together in a cuvette with a stirring bar (except H_2O_2). The cuvette was set in the light beam of the spectrophotometer and the measurement was started. The reaction was started with the addition of H_2O_2.

Chart 3-8: reaction mix for guiaicol assay

	Concentration	Volume [µL]	Concentration in cuvette
Phosphate buffer pH 7.0	200 mM	500	100 mM
Guaiacol	1 mM	100	100 µM
Enzyme		50 (or 100)*	
RO water		340 (or 290)*	
H_2O_2	10 mM	10	100 µM
Total		1000	

*depending on Enzyme concentration and activity

Material and equipment:

Guaiacol Sigma

Stirred quartz cuvette

3.4.5 MCD halogenation assay

MPO is able to oxidize halides (like chloride or bromide) to their corresponding hypohalide acids (e.g. HOCl or HOBr). The activity of halogination can be measured with the monochlorodimedon (MCD) assay. Like ABTS or guaiacol assay the change of absorbance is measured with a photometer (at 290 nm, ε = 19000 M^{-1} cm^{-1}). MCD gets oxidized by HOCl or HOBr and forms dichlorodimedon or monochloromonobromodimedon.

Procedure:

All solutions of table 3- were mixed together in a cuvette with a stirring bar except H_2O_2. The cuvette was set in the light beam of the spectrophotometer and the

measurement was started. After some seconds the reaction was started by addition of H_2O_2.

Chart 3-9: reaction mix for MCD assay

	concentration	Volume [µL]	Conc. in cuvette
Phosphate buffer pH 5 or 7	200 mM	500	100 mM
MCD	1 mM	100	100 µM
Enzyme		50 (or 100)*	
NaCl or KBr	1 M	100	100 mM
RO water		340 (or 290)*	
H_2O_2	10 mM	10	100 µM
Total		1000	

*depending on Enzyme concentration and activity

Material and Equipment:

MCD Sigma

4 Results

4.1 Cloning of MPO mutants R333

4.1.1 Primer design for site directed mutagenesis

Primer for design of mutant R333A: calculated (Gene Runner) melting temperature of 82.7°C, GC percentage of 58.1%, length of 43 base pairs, two hairpinloops and five putative dimeric regions.

Primer for design of R333K: melting temperature of 79.7°C, GC percentage of 55%, length of 40 base pairs, 2 hairpinloops and five putative dimeric regions.

Primer for design of mutant R333A

5' CTTCACCAATGCCTTCGCTTACGGCCACACCCTCATCCAACCC 3'

MPOR333A_1

5' GGGTTGGATGAGGGTGTGGCCGTAAGCGAAGGCATTGGTGAAG 3'

MPOR333A_2

Primer for design of mutant R333K

5' CACCAATGCCTTCAAATACGGCCACACCCTCATCCAACCC 3'

MPOR333K_1

5' GGGTTGGATGAGGGTGTGGCCGTATTTGAAGGCATTGGTG 3'

MPOR333K_2

Triplett highlighted in red indicates point mutation

4.1.2 Characterization of plasmid DNA

For verification of cloning a DNA electrophoresis of purified plasmid DNA was performed. The size of the plasmid was calculated to be 8 kb. As shown in figure 4-1, a band at approximately 10000 bp was found. Nevertheless, it was decided to disclaim a restriction enzyme digest for screening, due to the fact that DNA sequencing was made to confirm the correct arrangement of the plasmid.

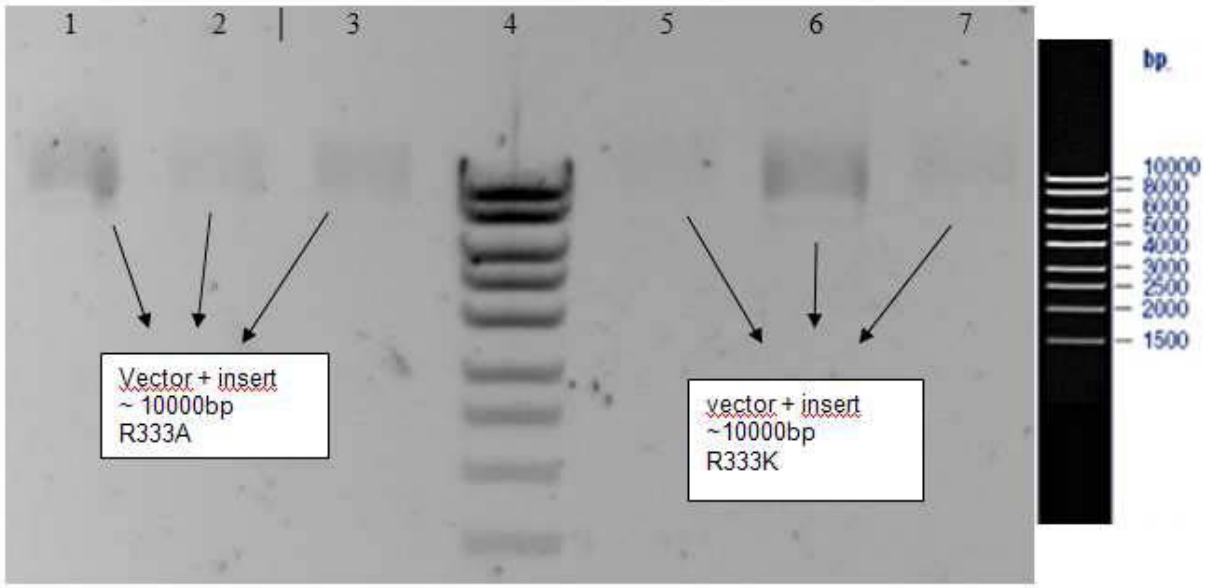


Figure 4-1: DNA a gel of minipreps; lane 1, 2 and 3: three different minipreps of the plasmid DNA encoding R333A; lane 4: the DNA ladder; lane 5, 6 and 7: three different minipreps of the plasmid DNA encoding R333K

4.1.3 DNA-Sequencing

In order to verify the mutated DNA sequence, DNA sequencing was performed. Thereby, the actual and theoretical sequence of the mutants R333A and R333K were compared with the sequences of wild type MPO. No unexpected DNA mutation was found. Small sequence sections showing the mutation in R333A and R333K are shown in figure 4-2 and figure 4-3.

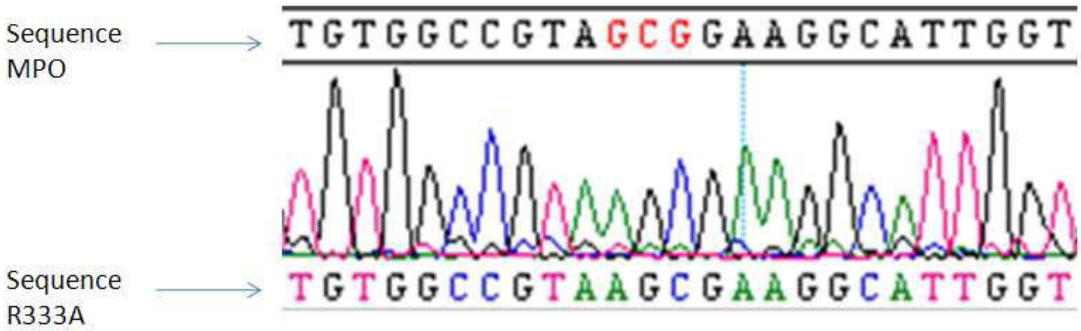


Figure 4-2: Detail of DNA sequence comparing wild-type and R333A of the region of mutation

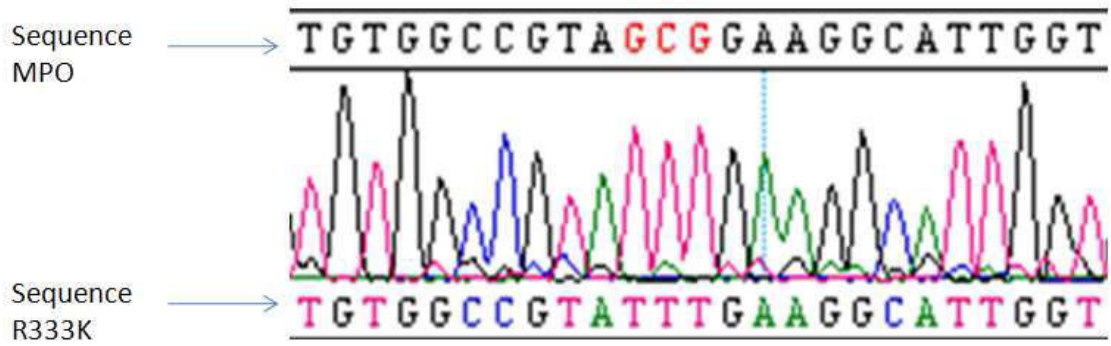


Figure 4-3: Detail of DNA sequence comparing wild-type and R333K of the region of mutation

4.1.4 Maxiprep and purification of DNA

In chart 4-1 the DNA concentrations as well as OD ratios of the purified plasmids are listed. These DNA templates were used for transfection into HEK cells.

Chart 4-1: Results of the maxiprep plasmid purification

	Theoretical values	R333A	R333K
A_{260} / A_{280}	~ 1.8	1.8	2.1
A_{230} / A_{260}	< 0.6	0.5	0.5
A_{320}	< 0.00	0.004	0.0057
concentration		350 µg/mL	125 µg/mL

4.2 Production of MPO mutants

4.2.1 Production of MPO mutant Q91T

4.2.1.1 Cultivation of CHO cells

CHO cells were cultivated in α-Mem medium with 10% FBS, 1% penicillin-streptomycin stock solution, 450 µg/mL geneticin (as selection marker) and 1% L-glutamine. For protein production a medium without geneticin but with a higher L-glutamine concentration (2%) was used.

No problems occured with adherence of the cells to the surface of the cell culture vessels. Freezing and thawing of the cell colonies were without any difficulty. A monolayer with spread cells was formed. Most of the time the cells were split 1 to 10, adapted to their growing behavior (media change 2-3 times a week).

4.2.1.2 Large scale production

For large scale production cell factories were used. The cells formed a monolayer and a static culture, however yeast contaminations occurred. For this reason overall yield and purity number of the Q91T mutant were poor, although some of the batches showed a reliable heme insertion. The yield of two cell factories is listed in charts 4-2 and 4-3. The change of α-mem medium to DMEM caused worse growing and production. Another approach was to change the hematin concentration, however it did not increase the purity number or protein yield. In chart 4-4 the overall yield and purity numbers of two cell factories are listed.

Chart 4-2: yields of the first cell factory; batches of identical color have been pooled

	Medium	Hematin	Amph. B	cont.	heme	amount [mg]	RZ
1.Batch	α-MEM	5 µg/mL	-	-	no		
2.Batch	DMEM	5 µg/mL	-	yeast	no		
3.Batch	α-MEM	5 µg/mL	2.5 µg/mL	-	yes	0.49	0.23
4.Batch	DMEM	5 µg/mL	2.5 µg/mL	-	no		
5.Batch	DMEM	5 µg/mL	2.5 µg/mL	-	no		
6.Batch	DMEM	5 µg/mL	-	-	yes	0.46	0.10
7.Batch	DMEM	5 µg/mL	-	-	yes		
8.Batch	DMEM	5 µg/mL	-	-	yes		
9.Batch	DMEM	5 µg/mL	-	yeast	no		
					SUM	0.95	

Chart 4-3: yields of the second cell factory; batches of identical color have been pooled

	Medium	Hematin	Amph. B	cont.	heme	amount [mg]	RZ
1.Batch	α-MEM	5 µg/mL	-	-	no		
2.Batch	DMEM	5 µg/mL	-	-	yes	0.55	0.14
3.Batch	DMEM	5 µg/mL	-	-	yes		
4.Batch	DMEM	5 µg/mL	-	-	yes	0.46	0.13
5.Batch	DMEM	5 µg/mL	-	-	yes		
6.Batch	α-MEM	5 µg/mL	2.5 µg/mL	-	yes	0.68	0.16
7.Batch	α-MEM	5 µg/mL	-	-	yes	0.43	0.20
8.Batch	DMEM	5 µg/mL	-	-	yes		
9.Batch	α-MEM	5 µg/mL	-	-	yes	3.32	0.18
10. Batch	α-MEM	5 µg/mL	-	-	yes		
11. Batch	α-MEM	10 µg/mL	-	-	yes		
12. Batch	α-MEM	10 µg/mL	-	-	yes	0.77	0.12
12.Batch	α-MEM	10 µg/mL	-	-	yes	0.33	0.16
					SUM	6.53	

Chart 4-4: Overall yield and purity number of mutant Q91T

Overall yield	7.48 mg
Overall purity number	0.16

4.2.2 Production of MPO mutants R333A and R333K

4.2.2.1 Cultivation of HEK cells

HEK cells were grown on DMEM medium with supplements (10% FBS, 1% penicillin/streptomycin and 900 µg/mL geneticin as selection marker).

A monolayer with spread cells (even more spread than CHO cells) was formed (see figure 4-4). Usually the cells were passaged 1 to 10 into a new cell culture vessel, dependent growing behavior (media change 2-3 times a week).

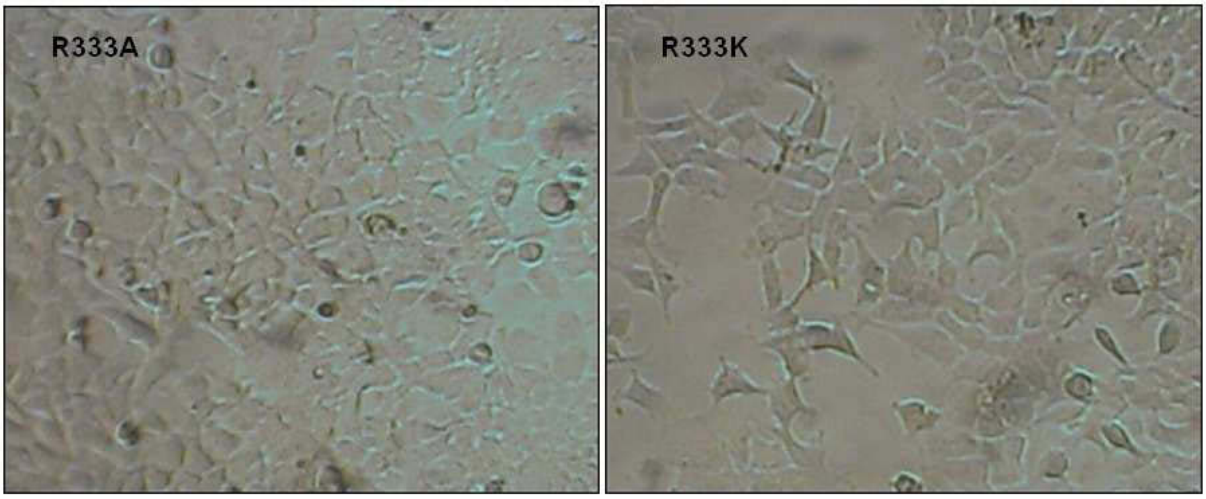


Figure 4-4: Picture of adherent HEK293; left: MPO variant R333A, right: MPO variant R333K

4.2.2.2 Screening of positive transformed clones

Selection of the right cell clone was difficult to perform, because the measured peroxidase activity in the supernatant was extremely low. Hence all cell clones had to be grown till the amount of supernatant was high enough to carry out protein purification (about 50 mL supernatant). Both heme insertion and the measured activity were poor, but clear differences could be seen. Finally 2 clones (B2 and C2) for production of R333K variant and 3 clones (A4, B1 and C1) for production of R333A variant were chosen for scale up.

4.2.2.3 Large scale production

For large scale production triple flasks were used. As described above two clones of the R333K variant and three clones of the R333A variant were chosen. The yields of active protein (with correct heme incooparation) were very low. Therefore, it was tried to increase heme insertion by adding supplements like hematin (5 µg/mL) or hemin (5 µg/mL). An increase of the purity numbers occurred, but the yields remained poor. The observed purity numbers were low (0.1 - 0.2) and the Soret maximum of both variants was significantly blue-shifted.

4.3 Protein characterization

4.3.1 Spectral properties

4.3.1.1 Spectral properties of the MPO variants R333A/K and Q91T

The overall purity number of mutant Q91T was about 0.16, although media supplements like hematin were used to improve heme insertion. The Soret band was blue shifted to 416 nm.

The purity numbers of the R333 mutants were very low (0.1 – 0.2) and without media supplements like hematin or hemin no heme insertion was seen. The Soret maximum of R333A was found to be at 405 nm, whereas from R333K it was even more blue-shifted (401 nm) (figure 4-5).

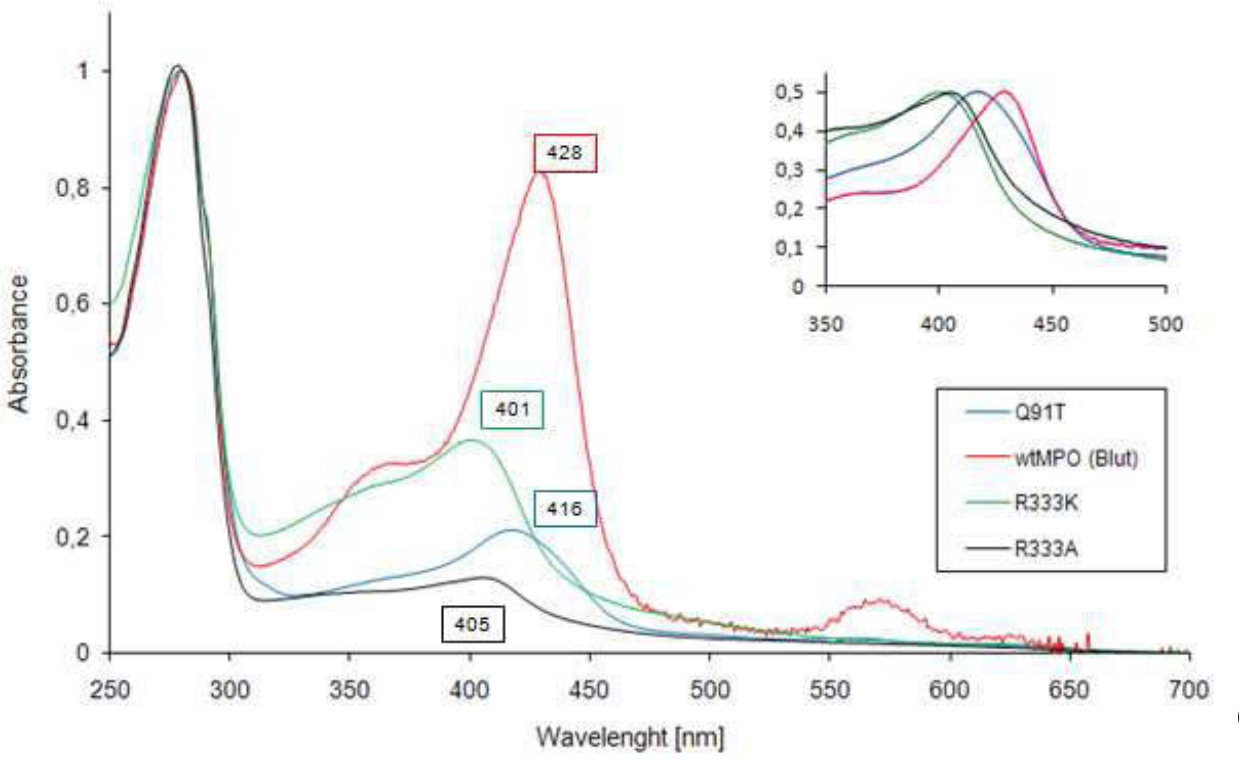


Figure 4-5: comparison of spectral properties of wild-type MPO and MPO mutants Q91T and R333A/K in 20 mM phosphate buffer, pH 7 at RT,

4.3.2 SDS-PAGE and Western Blot

4.3.2.1 Characterization of Q91T by SDS-PAGE and Western blot

Purified Q91T was analyzed by SDS-PAGE (using a 10% acrylamide gel) and Western blotting. In the SDS-Page (figure 4-6) an expected band at approximately 73 kDa is seen. The SDS-PAGE showed that the enzyme solution is rather pure.

The Western blot, using a polyclonal antibody raised against MPO, showed a prominent band at approximately 73 kDa (shown in figure 4-7) and verifies the assumption made for the SDS-PAGE.

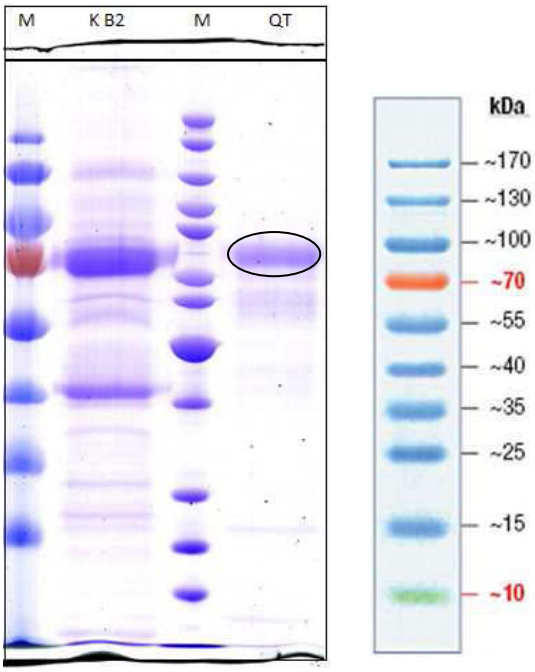


Figure 4-6: SDS-PAGE of the protein preparations; lane M Marker; lane K B2 MPO mutant R333K; lane M marker and lane QT MPO mutant Q91T; Samples were purified by a cation exchange gel, concentrated with centripreps and desalted using a PD10 column. The pooled samples were applied to the gel (10 µg Protein)

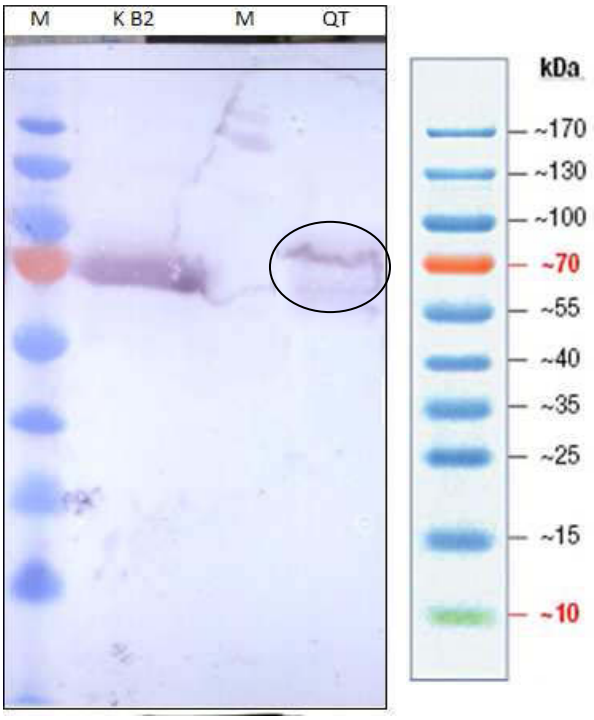


Figure 4-7: Western Blot of the protein preparations; lane M Marker; lane K B2 MPO mutant R333K; lane M marker and lane QT MPO mutant Q91T; Samples were purified by a cation exchange gel, concentrated with centripreps and desalted using a PD10 column. The pooled samples were applied to the gel (10 µg Protein) and the detection of the Western blot was performed with a polyclonal antibody raised against MPO

4.3.2.2 Characterization of R333A and R333K by SDS-PAGE and Western blot

Similarly purified R333A and R333K were analyzed by SDS-PAGE and Western blot. With both proteins the expected molar mass at 73 kDa was seen.

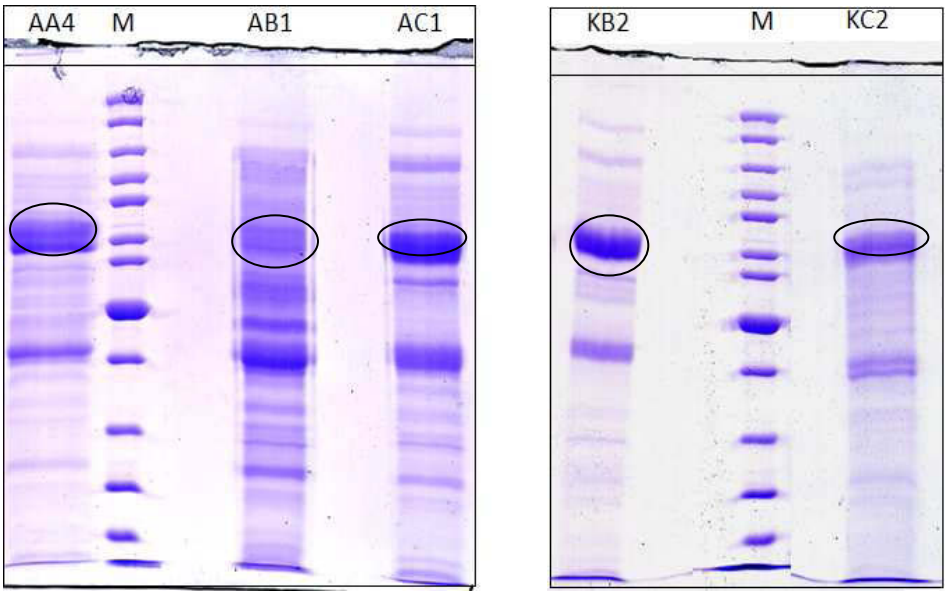


Figure 4-8: SDS-PAGE of the protein preparations; Samples were purified by a cation exchange gel, concentrated with centripreps and desalted using a PD10 column. The pooled samples were applied to the gel (10 µg Protein)

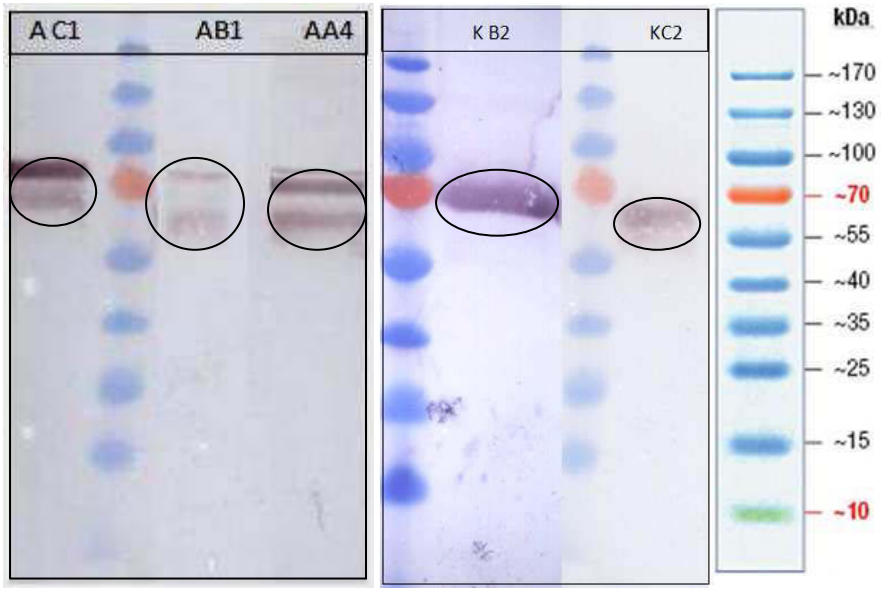


Figure 4-9: Western Blot of the protein preparations; Samples were purified by a cation exchange gel, concentrated with centripreps and desalted using a PD10 column. The pooled samples were applied to the gel (10 µg Protein) and the detection of the Western blot was performed with a polyclonal antibody raised against MPO

4.3.3 Enzymatic activity

4.3.3.1 Peroxidase activity

For determination of peroxidase activity two different assays were used (guaiacol and the ABTS assay). Guaiacol and ABTS are artificial electron donors of MPO that change their absorption maxima upon oxidation. Typical conditions for the guaiacol assay were as follows: 100 µM guaiacol, 100 µM hydrogen peroxide and enzyme in 100 mM phosphate buffer, pH 7.0. The reaction was photometrically at 470 nm. Activity measurements with ABTS were performed using 1 mM ABTS, 100 µM hydrogen peroxide and enzyme in 100 mM phosphate buffer at pH 5.0. The change of absorbance at 414 nm was monitored.

4.3.3.1.1 Guaiacol assay Q91T

The specific peroxidase activity using guaiacol as electron donor is summarized in chart 4-5 in comparison with wild-type MPO. The specific activity was found to be 11.7% of the wild-type MPO.

Chart 4-5 Specific activity with guaiacol of Q91T compared with recombinant wild type MPO

	rMPO	QT
Slope/min	0.643	0.161
Concentration [mg/L]	15.8	33.7
U [µM/min]	24.184	6.053
U/mg	1.531	0.179
percentage	100%	11.71%

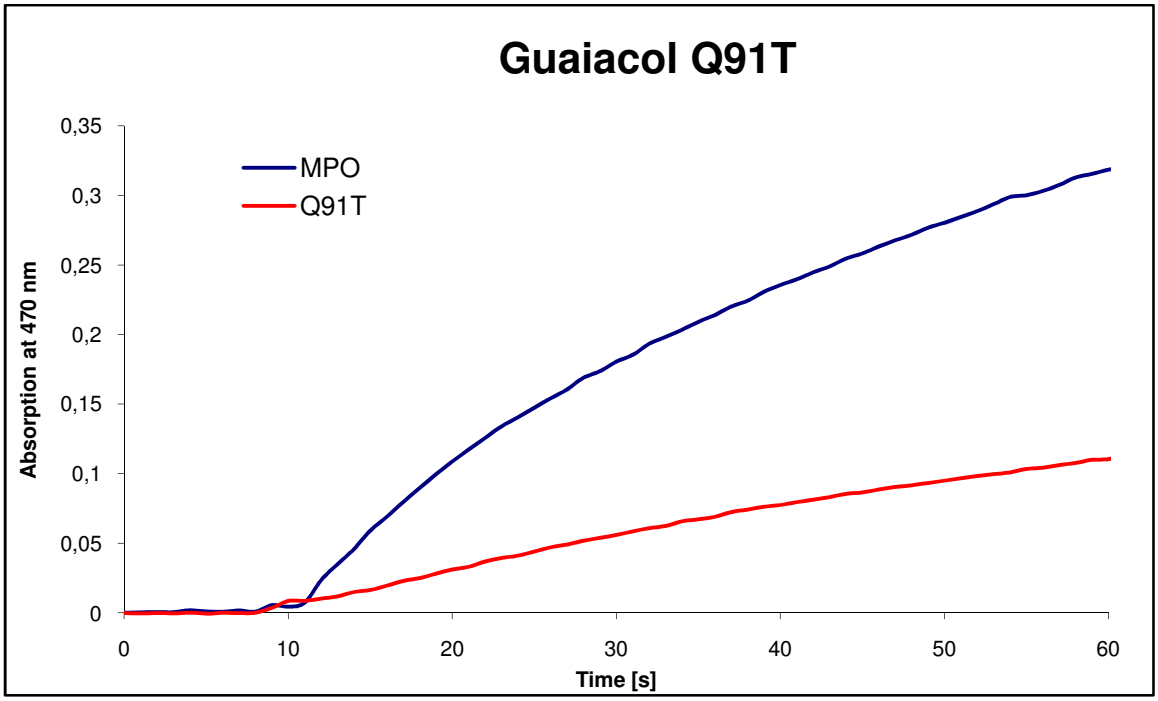


Figure 4-10: Guaiacol assay for Q91T compared with wild type MPO; reaction conditions: 100 µM guaiacol, 100 µL of 2 µM (wild-type) or 4 µM (Q91T) enzyme solution in 100 mM phosphate buffer, pH 7, at RT; 100 µM H_2O_2 was added to start the reaction after 10 seconds, the change of absorbance at 470 nm was monitored

4.3.3.1.2 ABTS assay R333A

The specific peroxidase activity using ABTS as electron donor is summarized in chart 4-6 in comparison with wild-type MPO. The specific activity was found to be negligible.

Chart 4-6: Specific activity with ABTS of R333A mutants compared with recombinant wtMPO

	rMPO	R333A C1	R333A B1	R333A A4
Slope/min	31,28	0,041	0,022	0,108
Slope/min	28,15	0,043	0,026	0,106
Average	29,7	0,042	0,024	0,107
Concentration [mg/L]	9.085	39.405	12.345	25.263
U [µM/min]	825.417	1.158	0.674	2.972
U/mg	90.855	0.029	0.055	0.118
percentage	100%	0,03%	0,06%	0,13%

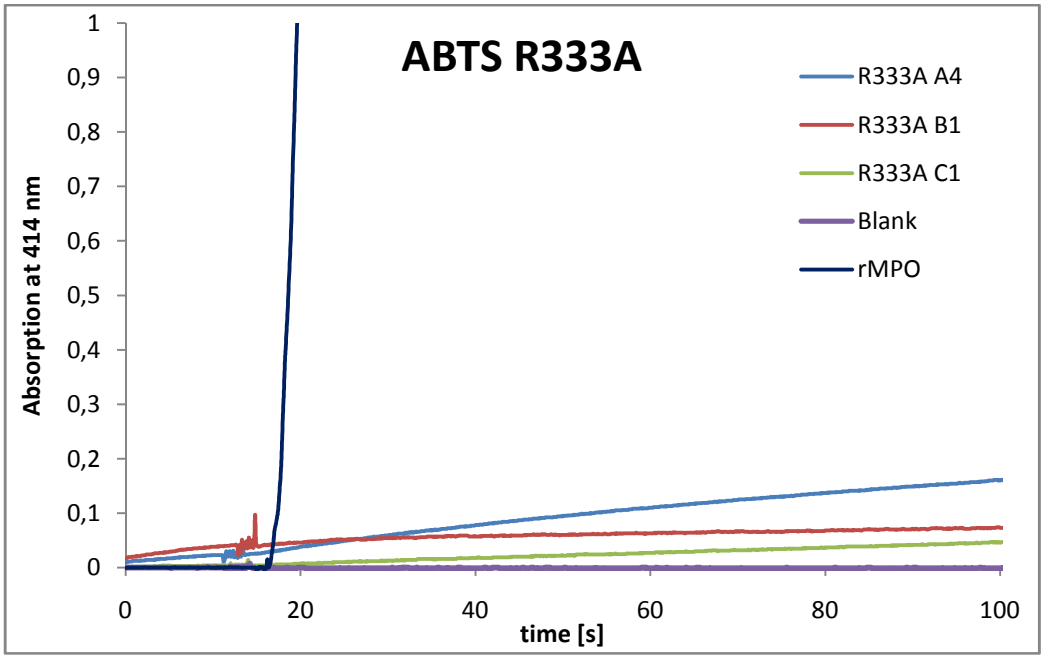


Figure 4-11: ABTS assay for R333A compared with wild type MPO; reaction conditions: 1 mM ABTS, 50 or 100 µL enzyme solution (50 µL of wild-type, 100 µL of R333A) of different concentrations (wt: 2.28 µM, C1: 5 µM, B1: 1.56 µM, A4: 3.2 µM) in 100 mM phosphate buffer, pH 5, at RT; 100 µM H_2O_2 was added to start the reaction after 10 seconds, the change of absorbance at 414 nm was monitored

4.3.3.1.3 ABTS assay R333K

The specific peroxidase activity using ABTS as electron donor is summarized in chart 4-6 in comparison with wild-type MPO. The specific activity was found to be negligible.

Chart 4-7: Specific activity with ABTS of R333K mutants compared with recombinant wtMPO

	rMPO	R333K B2	R333K C2
Slope/min	32.03	0.246	0.081
Slope/min	27.37	0.273	0.085
Average	29.7	0.260	0.083
Concentration [mg/L]	9.085	16.963	18.040
U [µM/min]	825.000	7.211	2.317
U/mg	90.809	0.425	0.128
percentage	100%	0.47%	0.14%

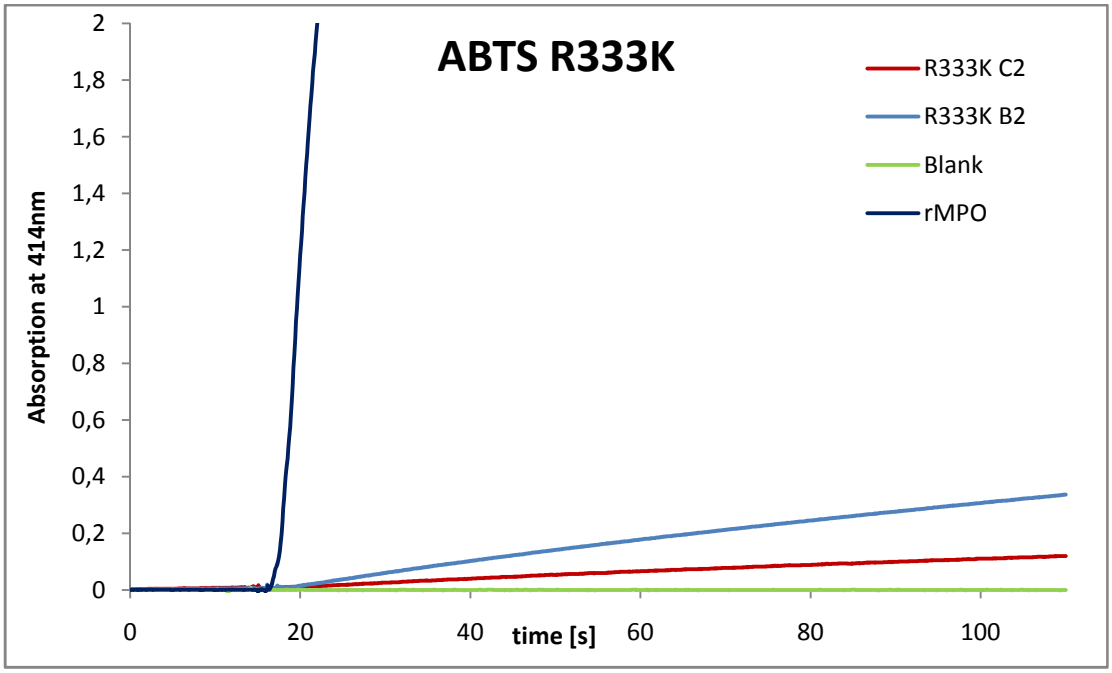


Figure 4-12: ABTS assay for R333K compared with wild type MPO; reaction conditions: 1 mM ABTS, 50 or 100 µL of approximately 2 µM enzyme solution (50 µL of wild-type, 100 µL of R333K) in 100 mM phosphate buffer, pH 5, at RT; 100 µM H_2O_2 was added to start the reaction after 10 seconds, the change of absorbance at 414 nm was monitored

4.3.3.2 Halogination activity

4.3.3.2.1 MCD assay Q91T

The specific halogenation rate of Q91T was found to be extremely poor by using both chloride and bromide as two-electron donors. For bromide the rate was 1.6% of the wtMPO and the rate using chloride was smaller than 1%.

Bromide as two-electron donor:

Chart 4-8: Specific activity with MCD and bromide as two electron donor of the Q91T variant compared with recombinant wtMPO

	MPO	Q91T
Slope/min	3.503	0.4905
Concentration [mg/L]	7.900	67.541
U [µM/min]	184.368	25.816
U/mg	23.338	0.382
percentage	100%	1.64%

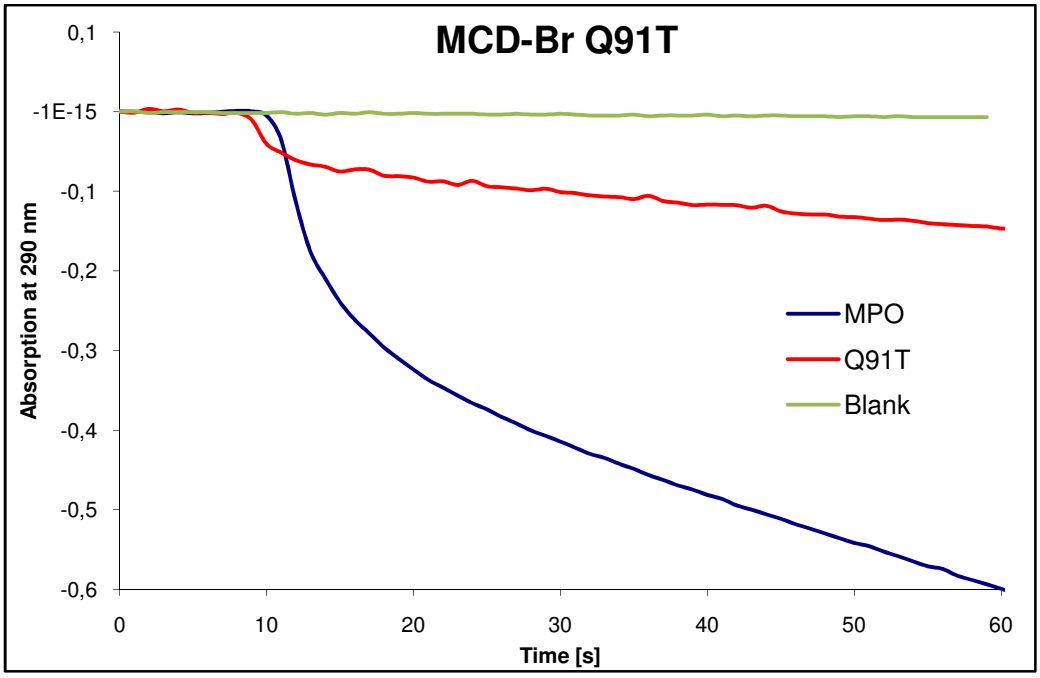


Figure 4-13: MCD assay with bromide as two-electron donor for Q91T in comparison with recombinant wild-type MPO; reaction conditions: 100 µM MCD, 100 mM KBr, 50 or 200 µL of 2-4 µM enzyme solution (50 µL of 2 µM wild-type, 200 µL of 4 µM Q91T), in 100 mM phosphate buffer, pH 7, at RT, 100 µM H_2O_2 was added to start the reaction after 10 seconds, the change of absorbance at 290 nm was monitored

Chloride as two-electron donor:

Chart 4-9 Specific activity with MCD and chloride as two-electron donor of the Q91T variant compared with recombinant wtMPO

	MPO	Q91T
Slope/min	3.0122	0.1041
Concentration [mg/L]	7.900	67.541
U [µM/min]	158.537	5.476
U/mg	20.068	0.081
percentage	100%	0.40%

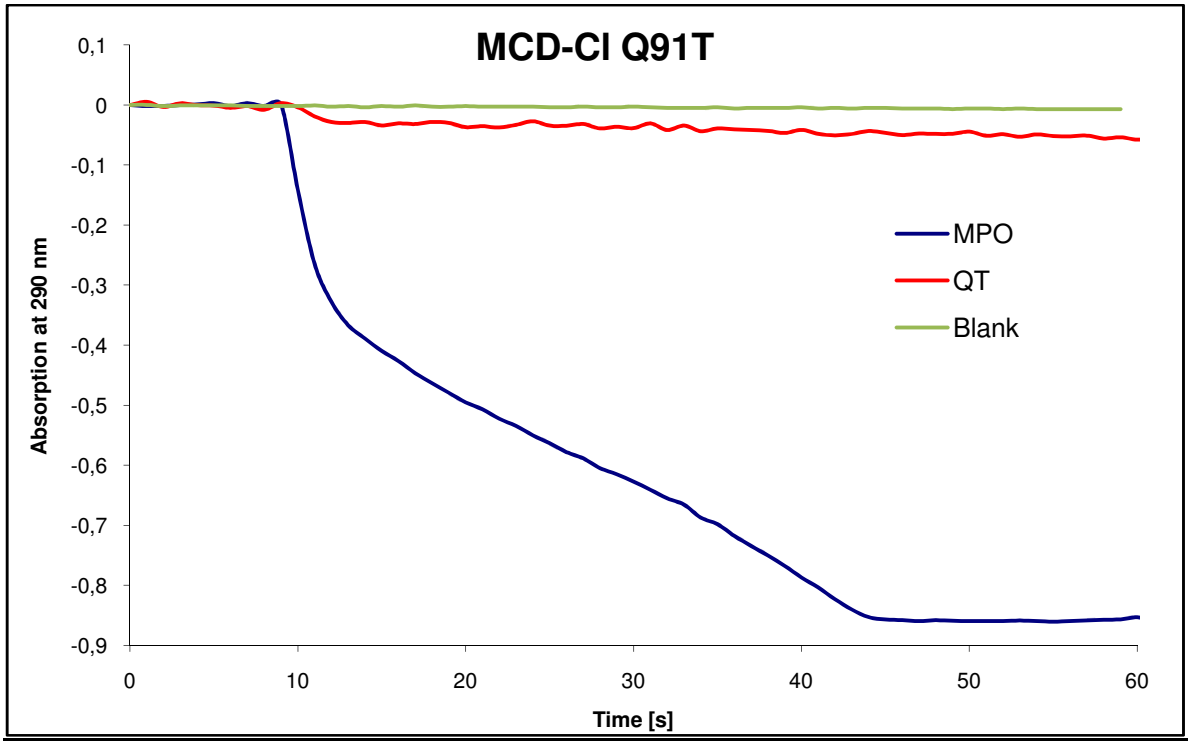


Figure 4-14 MCD assay with chloride as two-electron donor for Q91T in comparison with recombinant wild-type MPO; reaction conditions: 100 µM MCD, 100 mM NaCl, 50 or 200 µL of 2 µM enzyme solution (50 µL of 2 µM wild-type, 200 µL of 4 µM Q91T), in 100 mM phosphate buffer, pH 7, at RT, 100 µM H_2O_2 was added to start the reaction after 10 seconds, the change of absorbance at 290 nm was monitored

4.3.3.2.2 MCD assay R333A

The MCD-assay was performed with bromide as two-electron donor. For all clones of R333A the specific halogenation rate was drastically reduced. As shown in figure 4-15 and chart 4-10 this MPO variant almost lost the ability for halogentation.

Chart 4-10: Specific activity with MCD and bromide as two-electron donor of the R333A variant compared with recombinant wtMPO

	rMPO	R333A C1	R333A B1	R333A A4
Slope/min	11,645	0,075	0,1301	0,049
Slope/min	-	0,0648	0,1045	0,041
Average	11,6450	0,0699	0,1173	0,0450
Concentration [mg/L]	9.024	24.690	78.809	50.525
U [µM/min]	612.895	3.679	6.174	2.368
U/mg	67.917	0.149	0.078	0.047
percentage	100%	0,22%	0,12%	0,07%

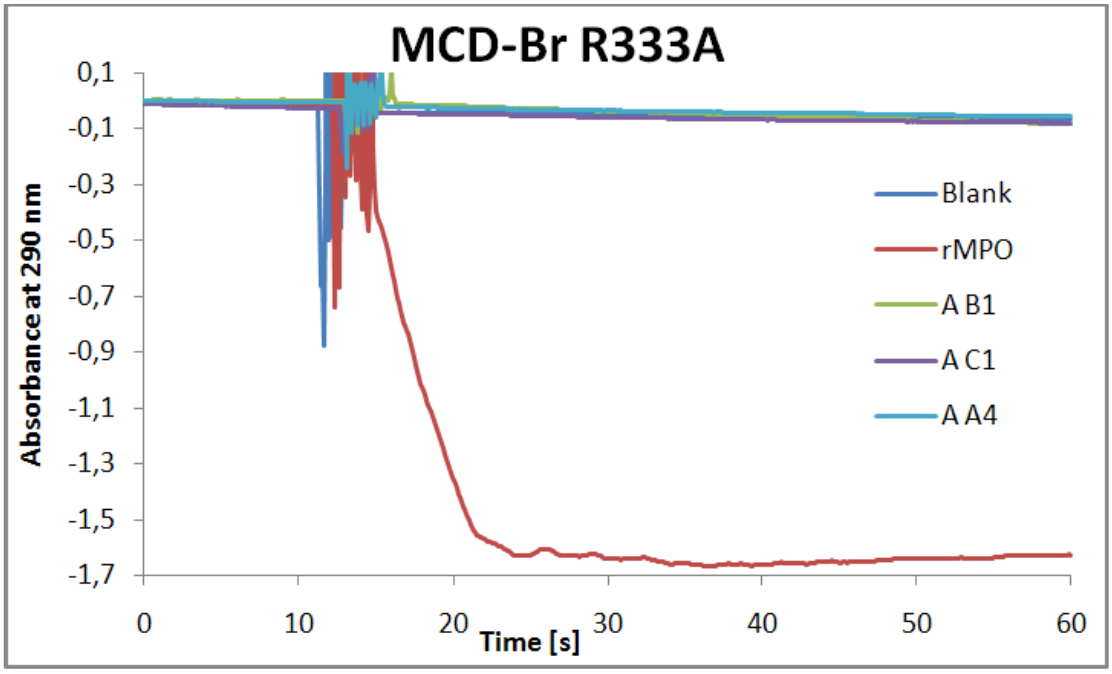


Figure 4-15 MCD assay with bromide as two-electron donor for R333A in comparison with recombinant wild-type MPO; reaction conditions: 100 µM MCD, 100 mM KBr, 50 or 200 µL enzyme solution (50 µL of wild-type, 200 µL of R333A) of different concentrations (wt: 2.28 µM, C1: 5 µM, B1: 1.56 µM, A4: 3.2 µM), in 100 mM phosphate buffer, pH 7, at RT, 100 µM H_2O_2 was added to start the reaction after 10 seconds, the change of absorbance at 290 nm was monitored

4.3.3.2.3 MCD assay R333K

The MCD assay was performed using bromide as two-electron donor. The results of the MPO variant R333K were alike the results of the alanine mutant. Halogenation activity is almost lost (figure 4-16 and chart 4-11).

Chart 4-11: Specific activity with MCD and bromide as two-electron donor of the R333K variant compared with recombinant wtMPO

	rMPO	R333K B2	R333K C2
Slope/min	11,645	0,1879	0,0204
Slope/min	-	0,1602	0,0208
Average	11,6450	0,1741	0,0206
Concentration [mg/L]	9.024	33.927	36.080
U [µM/min]	612.895	9.161	1.084
U/mg	67.917	0.270	0.030
percentage	100%	0,40%	0,04%

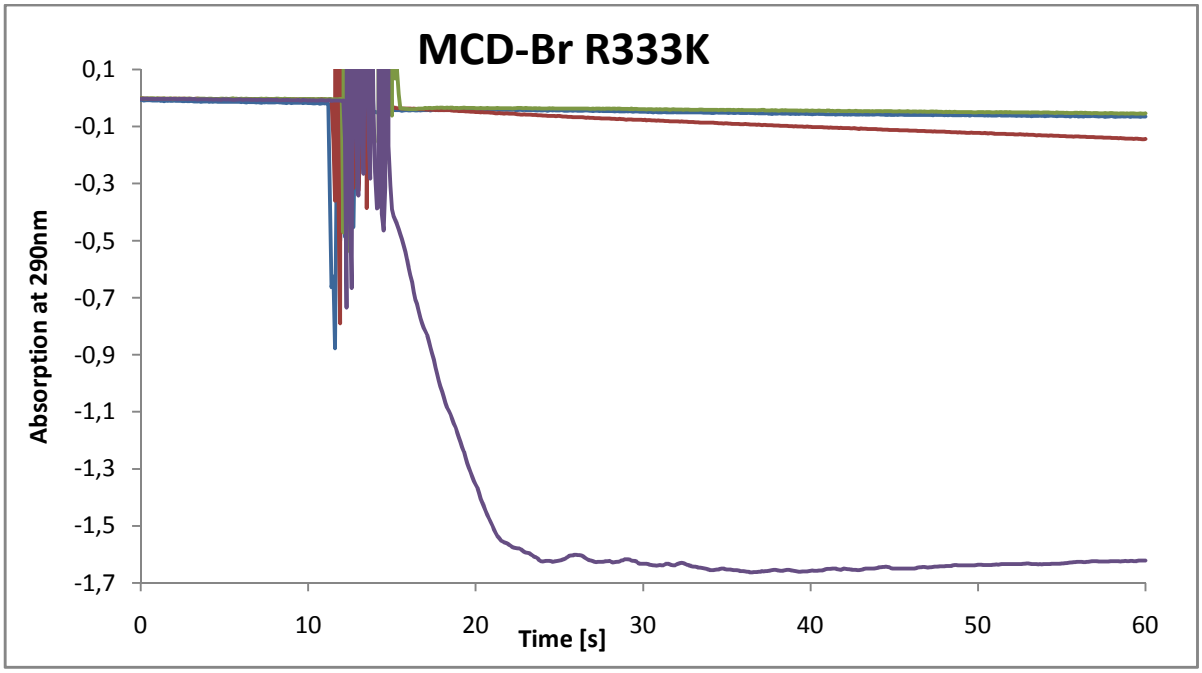


Figure 4-16 MCD assay with bromide as two-electron donor for R333K in comparison with recombinant wild-type MPO; reaction conditions: 100 µM MCD, 100 mM KBr, 50 or 200 µL of approximately 2 µM enzyme solution (50 µL of wild-type, 200 µL R333K), in 100 mM phosphate buffer, pH 7, at RT, 100 µM H_2O_2 was added to start the reaction after 10 seconds, the change of absorbance at 290 nm was monitored

5 Discussion

5.1 Cloning

Regarding Q91T stable transfection of CHO cells was already done before.
For design ofR333A/K site directed mutagenesis using PCR and specific primers was performed for cloning in *E. coli*. After sequence controll plasmid DNA was used for transfection of HEK293 cells.

5.2 Protein production

5.2.1 Production in CHO cells (Q91T)

CHO cells were grown in α-MEM medium with several additives. The yield of produced protein and the purity number was low. Medium change to DMEM did not improve the yield. For better heme insertion hematin or hemin (both 5 µg/ml) were added. However, a higher hematin concentration (10 µg/mL) had no effect on protein yield or purity number and additionally impaired the viability of the cells.
Since previous experiments with MPO variants produced in both CHO and HEK293 cells have shown differences in yield and heme insertion, the other variants were produced in HEK293 cell culture. To increase the rate of stable and active Q91T variant, exchange of the expression system would be an interesting alternative.

5.2.2 Production in HEK293 cells (R333A/K)

Transfection of HEK293 cells were performed by Ca^{2+}-phosphate-coprecipitation. The cells were grown on selective media (containing geneticin) and single cell colonies were picked manually. Selection of promising cell colonies to scaled-up for production was quite difficult, because of inserted mutation significantly decreased the enzymatic activity and thus hampered detection. Nevertheless 3 clones of the alanine variant and 2 clones of the lysine variant were chosen because of reliable cell growth, protein expression level and enzyme activity.
HEK293 were grown in DMEM medium with several supplements. The protein yield and purity number of both mutants were very low despite addition of hematin or hemin. Exchange of arginine 333 seemed to hamper stable protein production.

5.3 Protein purification

For both MPO variants the established purification protocol for recombinant MPO was used. It is a rather simple protocol because of the high isoelectric point of the enzyme. Upon using cation exchange chromatography (CM-Sephadex) MPO can be purified. At pH 6.8 MPO is among few other proteins of the cell supernatant that bind to the gel. At lower pH (6.5) BSA of the medium would bind.

Due to the fact that MPO is eluted with high salt concentrations the enzyme solutions had to be desalted after chromatography and concentrated with centripreps. For the desalting step two methods were used. Desalting using centripreps seemed rather practical due to the fact that centripreps are also used for concentration, but the loss of protein using this method was quite high. The second approach was to use PD 10 columns. This was the faster method and loss of protein was negligible. Therefore, PD 10 columns were used for all subsequent desalting steps.

For long time storage protein solutions were lyophilized and stored at -80°C. Storage had no effect on the enzyme quality, but small amounts of protein were lost during freezing and thawing. After storage at -80°C the purity number usually increased, suggesting that correct heme insertion is relevant for both enzymatic activity and conformational stability of the protein.

5.4 Protein characterization

Based on previous data the three covalent links of MPO involving Asp94, Glu242 and Met243 of MPO are responsible for the low symmetry and the bowed heme structure as well as the peculiar enzymatic activity. Compared to other mammalian peroxidases that lack the sulfonium ion linkage (e.g. LPO) the Soret maximum is red-shifted to 428 nm. Generally loss of one of these three links causes a blue shift of the Soret maximum.

MPO variant Q91T

The mutant exhibited a blue shifted Soret maximum at 416 nm and weak bands at 560 nm and 620 nm. By contrast, wild type MPO exhibits a Soret maximum at 428 nm and additional bands at 498, 570, 622 and 690 nm. This suggests that the mutation had an impact on the correct insertion of the heme group as formation of the covalent bond. The blue shift of the Soret band indicates a rearrangement of the bowed heme structure. To rule out that no major structural rearrangements have

occurred a CD-spectrum was made. It showed no difference in secondary structure compared with wild type MPO. This result exclude that the differences in heme insertion and activities are due to structural changes.

The peroxidase activity of the Q91T mutant was drastically decreased. The specific activity of guaiacol oxidation was only 11% compared with the wild type enzyme. The decrease of the specific activity with ABTS as a substrate was even higher (2% of wild-type activity). Both chlorination and bromination activity was negligible.

Glutamine 91 is believed to be involved in the binding of small inorganic donor molecules. The MPO-Br^--complex structure shows a defined hydrogen-bonding pattern including glutamine 91.

The present results confirmed its prominent role in halide oxidation. Since purity numbers and heme occupancy were low glutamine 91 might also play a role in heme insertion. This assumption is supported by the fact that the Soret maximum is blue shifted.

MPO variant R333A/K

The spectral properties of the R333A/K mutants differed even more in comparison with the wild type enzyme. The Soret band was shifted to 405 nm (R333A) and 401 nm (R333K). Additional peaks in the long wavelength region could not be detected.

The drastically changed spectral properties show a major impact of the arginine residue on the correct insertion of the heme. To prove that major structural rearrangements have occurred a CD-spectroscopy should be performed, but the amount of enzyme was too low.

The peroxidase and halogenation activity was almost lost for both mutants. All specific activities measured for the mutants were less than 1% compared with wild-type MPO.

Arginine 333 is a member of the conserved triad His336-Asn421-Arg333, which is located at the proximal site of the heme. Arg333 has an impact on the Asn421-His336 interaction and thus contributes to modulation of basicity of His336 (proximal ligand of heme iron) and heme in general (via interaction with propionate side-chain).

The complete loss of activity (halogention and peroxidase), the drastically shift of the Soret maxima, the low purity numbers and the low heme content underline the

improtant impact of arginine 333 on the correct insertion of the prosthetic group and on protein folding.

6 References

1. T.T. Puck, S.J. Cieciura, A. Robinson (1958): *Genetics of somatic mammalian cells. III. Long-term cultivation of euploid cells from human and animal subjects*. J. Exp. Med. 108(6):945-956
2. K.P. Jayapal, K.F. Wlaschin, M.G.S. Yap, W-S. Hu (2007): *Recombinant protein therapeutics from CHO cells - 20 years and counting*. Chem. Eng. Prog. 103(7):40-47
3. Thomas, P., and Smart, T. G. (2005) *HEK293 cell line: a vehicle for the expression of recombinant proteins*, J Pharmacol Toxicol Methods 51, 187-200.
4. Davies, M. J., Hawkins, C. L., Pattison, D. I., and Rees, M. D. (2008) *Mammalian heme peroxidases: from molecular mechanisms to health implications*, Antioxid Redox Signal 10, 1199-1234
5. Arnhold J, Flemmig J. (2010) *Human myeloperoxidase in innate and acquired immunity*. Arch Biochem Biophys. 2010 Aug 1;500(1):92-106.
6. Klebanoff, S. J. (1990) *Myeloperoxidase: Occurence And Biological Function*, in Peroxidases in Chemistry and Biology, Volume I (Grisham, M. B., Everse, K. E., and Everse, J., Eds.), CRC Press.
7. Hampton, M. B., Kettle, A. J., and Winterbourn, C. C. (1998) *Inside the neutrophil phagosome: oxidants, myeloperoxidase, and bacterial killing*, Blood 92, 3007-3017
8. Taurog A. (1999) *Molecular evolution of thyroid peroxidase.* Biochimie. 1999 May;81(5):557-62
9. Furtmüller PG, Zederbauer M, Jantschko W, Helm J, Bogner M, Jakopitsch C, Obinger C. (2006) *Active site structure and catalytic mechanisms of human peroxidases*, Arch Biochem Biophys. 2006 Jan 15;445(2):199-213.
10. Klebanoff, S. J. (1999) *Myeloperoxidase*, Proc Assoc Am Physicians 111, 383-389.
11. Andrews, P. C., and Krinsky, N. I. (1981) *The reductive cleavage of myeloperoxidase in half, producing enzymically active hemi-myeloperoxidase*, J Biol Chem 256, 4211-4218.
12. Blair-Johnson, M., Fiedler, T., and Fenna, R. (2001) *Human myeloperoxidase: structure of a cyanide complex and its interaction with bromide and thiocyanate substrates at 1.9 A resolution*, Biochemistry 40, 13990-13997.
13. Carpena, X., Vidossich, P., Schroettner, K., Calisto, B. M., Banerjee, S., Stampler, J., Soudi, M., Furtmuller, P. G., Rovira, C., Fita, I., and Obinger, C. (2009) *Essential role of proximal histidine-asparagine interaction in mammalian peroxidases*, J Biol Chem 284, 25929-25937.
14. Fiedler, T. J., Davey, C. A., and Fenna, R. E. (2000) *X-ray crystal structure and characterization of halide-binding sites of human myeloperoxidase at 1.8 A resolution*, J Biol Chem 275, 11964-11971.
15. Wever, R., and Plat, H. (1981) *Spectral properties of myeloperoxidase and its ligand complexes*, Biochim Biophys Acta 661, 235-239.
16. Shin K, Hayasawa H, Lönnerdal B. (2001) *Mutations affecting the calcium-binding site of myeloperoxidase and lactoperoxidase*. Biochem Biophys Res Commun. 2001 Mar 9;281(4):1024-9.
17. Hansson, M., Olsson, I., and Nauseef, W. M. (2006) *Biosynthesis, processing, and sorting of human myeloperoxidase*, Arch Biochem Biophys 445, 214-224.
18. Nauseef, W. M. (1998) *Insights into myeloperoxidase biosynthesis from its inherited deficiency*, J Mol Med 76, 661-668.
19. J. M. Berg, J. L. Tymoczko, L Stryer (2003) *Biochemie* 5.Auflage ISBN 3-8274-1303-6
20. Furtmüller PG, Obinger C, Hsuanyu Y, Dunford HB. (2000) *Mechanism of reaction of myeloperoxidase with hydrogen peroxide and chloride ion*. Eur J Biochem.;267(19):5858-64.
21. Zederbauer M, Furtmüller PG, Ganster B, Moguilevsky N, Obinger C. (2007) *The vinyl-sulfonium bond in human myeloperoxidase: impact on compound I formation and reduction by halides and thiocyanate*. Biochem Biophys Res Commun. 2007 May 4;356(2):450-6.
22. Jantschko, W., Furtmuller, P. G., Zederbauer, M., Jakopitsch, C., and Obinger, C. (2004) *Kinetics of oxygen binding to ferrous myeloperoxidase*, Arch Biochem Biophys 426, 91-97.
23. M. Soudi (2010) *Characterization of the myeloperoxidase mutant Asn421Asp produced in HEK and CHO cells*
24. Benedict RG, Schmidt WH, Coghill RD. (1946) *The stability of penicillin in aqueous solution,* J Bacteriol. 1946 Mar;51:291.

Danksagung

In erster Linie möchte ich meinen Dank Christian Obinger und Paul Georg Furtmüller aussprechen, für die Möglichkeit diese Diplomarbeit erfolgreich in der Arbeitsgruppe abzuschließen. Paul möchte ich besonders für die Unterstützung bei der Arbeit und Christian für die schnelle und gründliche Korrektur der Diplomarbeit danken.

Großer Dank geht an Johanna Stampler, die mich in die Laborarbeit eingeführt und mich während meiner praktischen Arbeit betreut hat. Sie war stets für Fragen offen und motivierte mich für meine Arbeit. Außerdem möchte ich ihr für die Planung der Experimente und Besprechung meiner Ergebnisse danken.

Mein Dank geht auch an alle Arbeitskollegen für ihre Unterstützung im Labor und für das ein oder andere After-Work Bier.

Meinen Eltern Elisabeth und Klaus möchte ich dafür danken, dass sie mir mein Studium ermöglicht und mich während meiner Studienzeit immer unterstütz haben.

Zu guter Letzt möchte ich meinen Freunden und Studienkollegen danken, die mir stets zur Seite gestanden sind. Besonders hervorheben möchte ich Margit Lanzinger, die auf der BOKU kennen und schätzen lernte, Christopher Lehner, ein Volksschulfreund der jederzeit bereit war mich von Prüfungen abzulenken, Michael Hajek, mit dem ich besonders zu Beginn des Studiums oft gemeinsam lernen war und meinem Bruder Philipp.