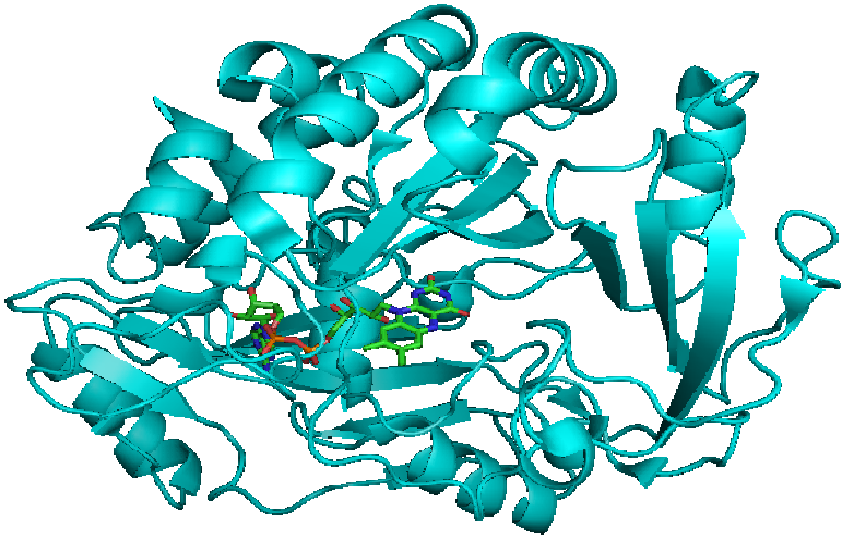
The creation and characterisation of an oxidase-peroxidase chimera of Alditol Oxidase



10-week Bachelor thesis
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Abstract

This project aimed to prepare an oxidase-peroxidase chimera of Alditol Oxidase (AldO). This was achieved by mutating several amino acids to the CXXCH-heme binding motif at various locations in the AldO sequence. Heme could be bound covalently to this sequence by the cytochrome *c* maturation (Ccm) system. Four different mutants of the same loop were prepared. Two other hemeloop mutants and two mutants containing N-terminal mutations were analyzed as well, these constructs were provided by Drs. R. T. Winter. This project showed that an oxidase-peroxidase chimera of AldO could be formed. This was however only possible for the N-terminal mutants, because the cytochrome *c* maturation system could only bind heme to the CXXCH-motif of protein regions without secondary structure. The mutant TatCXXCHAldOStrep (ThAS) was cultured on a large scale (3 L) yielding 0.20 mg of the desired protein. ThAS displayed oxidase, peroxidase and coupled oxidase-peroxidase activity. It was shown that it contained both covalently bound FAD and heme, but that heme was only attached to approximately 15.4 % of the protein. Kinetic analysis of the oxidase activity of ThAS yielded the following values: K_M = 0.35 mM and k_{cat} = 12.2 s^{-1}, which agreed well with the literature values of AldO (K_M = 0.32 mM and k_{cat} = 13 s^{-1}) (2). Two hemeloop mutants, mutants TAS 232-236 and TSA 232-236 showed higher periplasmic AldO expression, but they could not bind heme.

Table of contents

Introduction

Enzymes are important for biological systems because they catalyze specific reactions. Many enzymes require cofactors for their catalytic activity (1). This cofactor could either be a metal or a small organic molecule, a coenzyme. An enzyme catalyzes only a specific reaction for specific substrates. It is however interesting and possible to change an enzyme into a chimera, a protein with two different cofactors and two different functions. Two different functions can give the enzyme very interesting properties and promising applications such as a biosensor. Biosensors detect substrates in a more easy way because of the coupling of two reactions. The first functionality of the biosensor converts the substrate for example to a colourless product while the second functionality converts this product to a coloured end product, which could be detected by sight or with an UV/Vis spectrophotometer.

This project attempts to change *Streptomyces coelicolor* A3(2) Alditol oxidase (AldO) into an oxidase-peroxidase chimera. AldO is a recently discovered member of the Vanillyl Alcohol Oxidase (VAO) family (2)(3). Every protein within this family contains a covalently bound flavin (FAD) which enables it to perform redox chemistry. AldO is capable of performing regio-selective oxidations on different polyol substrates, of which xylitol and sorbitol are preferred. In order to convert AldO into an oxidase-peroxidase chimera several residues were mutated to create the CXXCH-motif which can covalently bind heme (4) (figure 1).

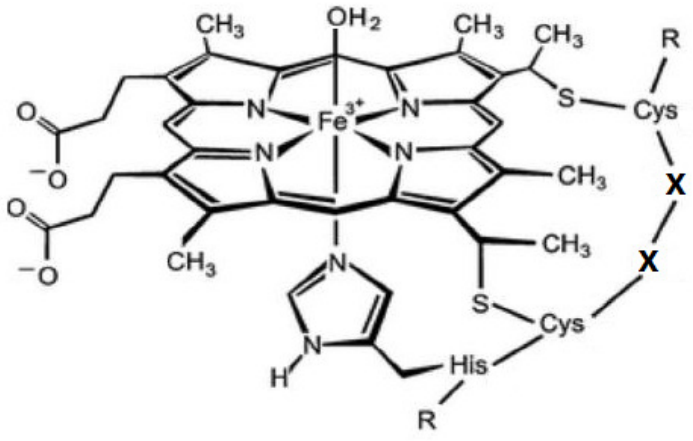

Figure 1: Heme bound to the CXXCH-motif. Modified from an article by Spector et al (2000) (5).

Heme will be bound covalently to the CXXCH-motif by the cytochrome *c* maturation (Ccm) system (6). This system is found in the periplasm of many Gram negative bacteria, including *E. coli*. This system is located within the inner membrane and periplasm of the cell

and transports heme over the inner cell membrane to the periplasm were it binds heme covalently through the formation of two disulfide bonds to the CXXCH-motif of apoproteins within the periplasm. AldO(CXXCH) needs to be transported over the inner cell membrane to the periplasm to make the heme attachment by the Ccm-system possible. In order to transport AldO(CXXCH) over the inner cell membrane the Twin-arginine translocation (Tat) system was used. This system transports properly folded proteins containing the Tat-signal sequence over the inner membrane. The Tat-signal sequence is cleaved from the protein after transport. van Bloois et al (2008) attached the Tat-sequence to AldO and showed that AldO could be transported over the inner cell membrane into the periplasm in an active confirmation (7). Besides being essential to obtain covalent heme binding, translocation to the periplasm is desirable because it is a first purification step, as the periplasm of *E. coli* contains less protein than the cytoplasm. The periplasm was separated from the cytoplasm by cell fractionation by the osmotic shock method (8).

Due to the expected low yields of the chimera a Strep-tag was attached to the protein to enable selective purification (9). The Strep-tag is a short peptide sequence which contains only 8 amino acids (10), (figure 2). This tag has a high affinity for a Strep-Tactin column while other proteins cannot bind to the column, making highly selective purification possible.

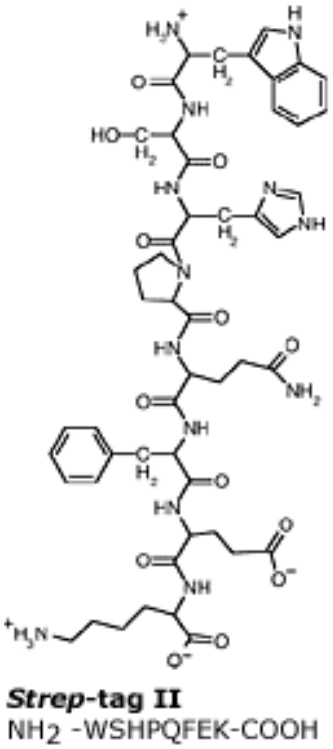

Figure 2: Strep-tag II, picture obtained from the website of IBA BioTagnology (11).

AldO(CXXCH) was expressed in *E.coli* strain BL21(DE3)/pEC 86. This strain contains plasmid pEC86, which constitutively expresses the Ccm-system (12). This system transports heme over the inner cell membrane to the periplasm were it binds heme to the CXXCH-motif of proteins. This plasmid contains a chloramphenicol resistance marker. AldO(CXXCH) was expressed in pBAD, another vector. Different pBAD vectors were used to express different mutations of AldO, all containing a Strep-tag and a Tat-sequence. The mutations were performed in the pBADTatAldOStrep vector which is shown in figure 3. In this vector the Tat-signal sequence is attached to the N-terminus and the Strep-tag to the C-terminus of the gene of interest (AldO in this case). Other mutations were performed on the pBADTatStrepAldO vector where the Strep-tag is directly C-terminal of the Tat-sequence. The Strep-tag is fused to the C-terminus of AldO in the first vector (pBADTatAldoStrep) and to the N-terminus in the second vector (pBADTatStrepAldO). Both vectors were used because of the possible influence of the Strep-tag on the binding of heme and vice versa. The heme might for instance hinder the capability of the Strep-tag to bind to the Strep-Tactin column making the Strep-tag purification less efficient. Or the Strep-tag might influence the capability of the cytochrome *c* maturation (Ccm) system to bind heme to the CXXCH-motif of AldO. Besides the AldO gene, the Strep-tag and the Tat-signal sequence (TorA-PSS) both vectors contain an ampicillin resistance and an arabinose recognition site. The protein will be expressed in the presence of arabinose.

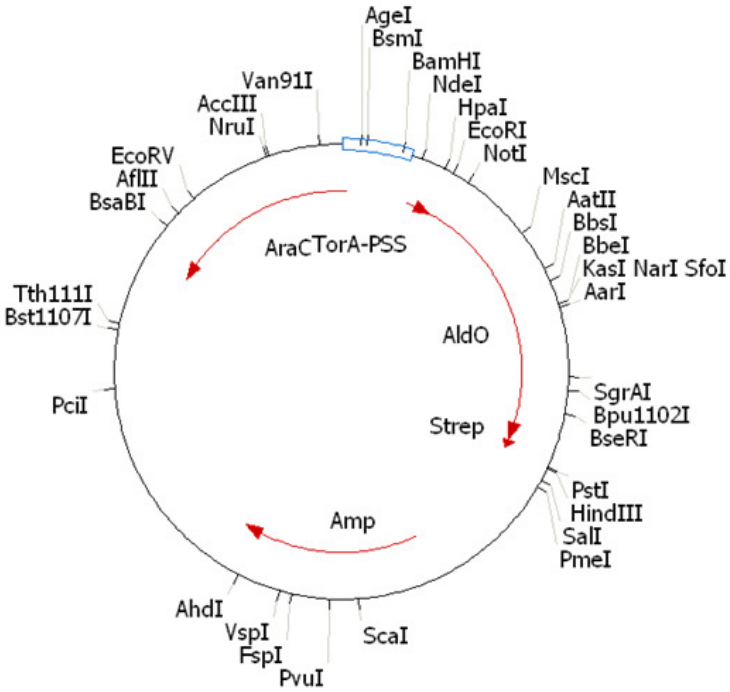

Figure 3: Map of plasmid pBADTatAldOStrep

During this project a surface loop of AldO was mutated to the CXXCH-motif by QuikChange Polymerase Chain Reaction (PCR). Amino acids 232-236 and 235-239 of AldO were chosen to be changed because of the high flexibility of the loop. AldO is shown in figure 4. Figure 5 shows a b-factor representation of AldO, clearly showing the high flexibility of the loop containing amino acids 232-239. Both AldO mutants: AldO Hemeloop 232-236 and AldO Hemeloop 235-239 were created for both above mentioned vectors (pBADTatAldOStrep and pBADTatStrepAldO) because of the possible influence of the Strep-tag. The mutated vectors were introduced into *E. coli* strain BL21(DE3)/pEC86 by chemical transformation.

Four other mutants were also studied. In two of them amino acids 280-284 were changed to the CXXCH-motif. In the two other mutants a region near the N-terminus of AldO was changed to the CXXCH-motif, see figure 4. Both the N-terminus and loop 280-284 mutations were for the reason mentioned above prepared in both vectors pBADTatAldOStrep and pBADTatStrepAldO. All eight constructs mentioned above were expressed in *E.coli* strain BL21(DE3)/pEC86.

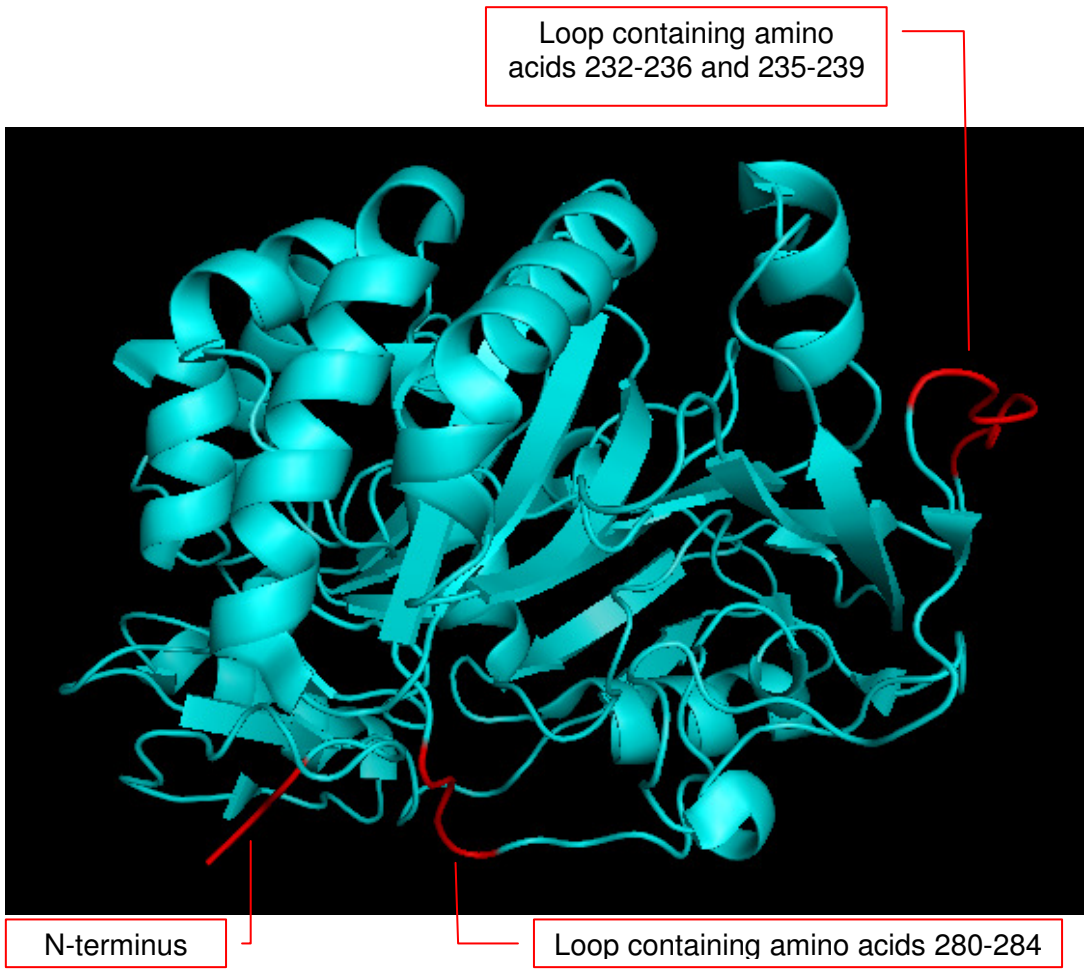

Figure 4: AldO with the N-terminus and changed loops marked

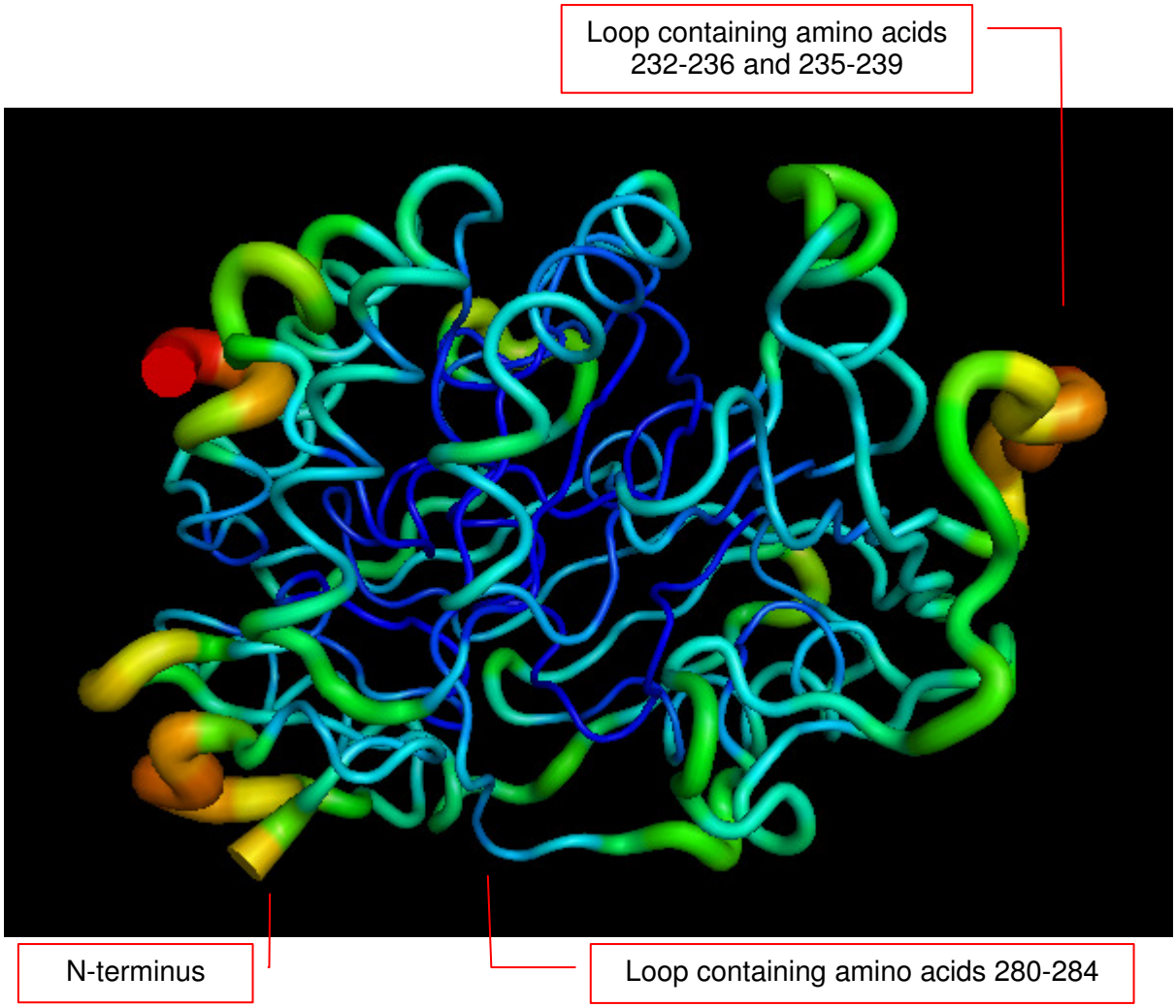

Figure 5: AldO shown in b-factor representation. High flexibility shown in broad yellow and red lines. Low flexibility shown in small dark blue lines.

All constructs were checked for oxidase and peroxidase activity with a 3,5-dichloro-2-hydroxybenzenesulfonic acid (DCHBS)/ 4-aminoantipyrine (AAP) assay (2). After SDS-PAGE all fractions were stained for heme, fluorescence and total protein content. The most promising mutant, TatCXXCHAldOStrep was purified, its heme and FAD content was quantified and its kinetic parameters for xylitol were determined.

During this project the AldO mutant CXXCHAldOStrep (ThAS) showed oxidase, peroxidase and coupled activities, showing that it is possible to incorporate a peroxidase functionality into an oxidase and thus creating an oxidase-peroxidase chimera of AldO.

Materials and methods

Mutagenesis

Plasmids pBADTatAldOStrep and pBADTatStrepAldO were mutated by the QuikChange polymerase chain reaction (PCR) in order to change a loop of AldO to the CXXCH-motif. A

loop comprising amino acids 232-239 was changed in two different ways to obtain the mutants; AldO Hemeloop 232-236 and AldO Hemeloop 235-239. These mutants were prepared by changing the following amino acids. The respective codons, which were changed by mutagenesis, are shown in brackets. For AldO Hemeloop 232-236 R232C(cgg→tgc), P233G(ccg→ggg), D235C(gac→tgc) and G236H(ggt→cat) were changed, while for AldO Hemeloop 235-239 D235C(gac→tgc, P238C(ccg→tgc) and Y239H (tac→cac) were changed. In order to obtain these mutations above plasmids were changed by PCR with the following primers, see table 1. The primers were produced by Sigma-Aldrich.

Table 1: Primers used in this study. The changed residues are shown in red.

Primer	Sequence
Hemeloop 232-236_fw	5'-GCGGCGCACCGACTGCGGGCTGTGCCATTTCCCGTACGCGG
Hemeloop 232-236_rv	5'- CCGCGTACGGGAAATGGCACAGCCCGCAGTCGGTGCGCCGC
Hemeloop 235-239_fw	5'-CCGACCGGCCGCTGTGCGGTTTCTGCCACGCGGCCCCGGCCG
Hemeloop 235-239_rv	5'-CGGCCGGGGCCGCGTGGCAGAAACCGCACAGCGGCCGGTCGG

The primers were first dissolved in 10 mM Tris-HCl, pH 8.5 to a final concentration of 100 µM. 10 µL of these 100 µM primer stock solutions were further diluted by the same buffer to a final concentration of 10 µM. Four different mutants were prepared by PCR; pBADTatAldOStrep Hemeloop 232-236, pBADTatAldOStrep Hemeloop 235-239, pBADTatStrepAldO Hemeloop 232-236 and pBADTatStrepAldO Hemeloop 235-239. A negative control was performed for one of the constructs by following the same protocol as described below but without the addition of polymerase. Five 0.5 mL eppendorfs were filled with 2.0 µL 10 µM desired hemeloop forward primer (final concentration: 0.4 µM), 2.0 µL 10 µM desired hemeloop reverse primer (final concentration: 0.4 µM), 1.0 µL d-NTPs (stock of 10 mM of each, final concentration: 0.2 mM), 5 µL Pfu polymerase buffer (10x, Stratagene), 1.0 µL pBADTatAldOStrep or pBADTatStrepAldO, 1.5 µL DMSO, 37.0 µL distilled H_20 and 1.0 µL Pfu Turbo polymerase (2.5 U, Stratagene) yielding a final volume of 50.0 µL. No Pfu Turbo polymerase was added to the negative control.

The PCR programme consisted of an initial denaturation period of 5 min at 94°C, a denaturation step of 1 min at 94°C, annealing for 30 sec at 60°C and an extension of 13 min at 68°C. The denaturation, annealing and extension repeated 18 times after which a final extension was performed at 68°C for another 5 minutes.

Transformation

0.5 µL of plasmid DNA was added to 200 µL $CaCl_2$-competent *E. coli* strain BL21(DE3)/pEC86, provided by Drs. R.T. Winter, University of Groningen. This mixture was incubated on ice for 30 min and subsequently heat-shocked for 45 sec in a thermo block at 42°C. After the cells were stored on ice for 5 min 800 µL sterile Luria-Bertani Broth medium (LB-medium, contents L^{-1} d-H_2O: 10 g bactotryptone, 5 g yeast extract and 10 g NaCl) was added. This mixture was incubated at 37°C and shaken for 60 min. The mixture was subsequently centrifuged for 1 min at max speed in a table top centrifuge. The harvested cells were resuspended in ~100 µL of the supernatant and transferred to a LB agar plate (1.5 % agar) containing the antibiotics ampicillin (50 µg mL^{-1}) and chloramphenicol (34 µg mL^{-1}). The inverted plates were incubated at 37°C overnight and subsequently stored at 4°C.

Expression

After overnight culturing on an LB-agar plate, a preculture was grown by transferring one colony to 5 mL LB-medium containing the antibiotics ampicillin (50 µg mL^{-1}) and chloramphenicol (34 µg mL^{-1}). The preculture was grown overnight to saturation (~ 17 h) at 37°C, 200 rpm. Subsequently, 50 µL of preculture (100x dilution) was transferred to 5 mL Terrific Broth (TB-medium, contents see below) containing ampicillin (50 µg mL^{-1}), chloramphenicol (34 µg mL^{-1}), heme precursor δ-aminolevulinic acid (δ-Ala) (0.5 mM) and expression inducer arabinose (0.2 % w/v). The TB culture was incubated over the weekend (~ 65 h) at 17°C, 200 rpm.

Terrific Broth (L^{-1}): 24 g yeast extract, 12 g bactotryptone, 4 mL glycerol, 2.31 g KH_2PO_4 and 16.43 g $K_2HPO_4 \cdot 3\ H_2O$ dissolved in distilled H_2O. To avoid salt precipitation the medium was autoclaved in two parts. The first part containing yeast extract, bactotryptone and glycerol dissolved to a volume of 900 mL and the second part containing KH_2PO_4 and $K_2HPO_4 \cdot 3\ H_2O$ dissolved to a volume of 100 mL. The mixtures were added together after cooling down. When it was used for plating 15 g agar (1.5 %) was added.

To collect more protein some of the strains were expressed on a larger scale. Precultures with the same content as described above were used. A part of these precultures were transferred and grown in 50 mL, 400 mL or 3 L TB-medium, containing equal concentrations of the preculture, ampicillin, chloramphenicol, δ-Ala and arabinose as described above. These large(r) scale cultures were also incubated over the weekend (~ 65 h) at 17°C, 200 rpm.

Periplasmic fractionation

OD_{600} of the grown TB cultures was recorded. 20 OD_{600} units of each culture were collected by centrifugation at 11,000 x *g* for 1 min making use of a table top centrifuge. The harvested cells were washed once with half of the harvested volume of ice-cold 10 mM Tris-HCl, pH 7.4. The cells were collected again by centrifugation at 11,000 g for 1 min. The supernatant was discarded after which the pellet was gently resuspended in 80 µL 10 mM Tris-HCl, pH 8.0 + 25 % sucrose, 10 µL 20 mM EDTA, pH 8.0 and 10 mL 5 mg mL^{-1} lysozyme dissolved in 10 µM Tris-HCl, pH 7.4. The cells were harvested by centrifugation for 15 min at 12,000 x *g* and 4°C after which they were incubated on ice for 20 min. The resulting supernatant (the periplasm) was collected. The pellet containing the spheroplasts was subsequently resuspended in 100 µL 10 mM Tris-HCl, pH 7.4. The resuspended spheroplasts were disrupted by sonication for 2 sec at 70 % making the solution less viscous. The ruptured spheroplasts were centrifuged for 10 min at 17,000 g and 4°C. The supernatant containing the combined cytoplasmic and total membrane proteins was collected, the pellet was discarded.

The large(r) scale cultures were fractionated with the same procedure. In these cases the whole culture instead of 20 OD_{600} units was collected. The amount of the solutions used in above procedure was adjusted to the harvested amount of OD_{600} units. For the largest two cultures (400 mL and 3 L) only the periplasm was collected because the desired protein will (because of its Tat-sequence) be transported to the periplasm by the Tat-system.

Purification of the periplasmic extract

The amounts used in the protocol below were changed to fit the collected periplasmic volume. 1 mL periplasmic extract was purified by the following procedure. In the first step aggregates were removed from the periplasmic extract by centrifugation at 14,000 rpm and 4°C for 5 min. The supernatant was transferred to a Strep-Tactin Sepharose column with a volume of 200 µL. This column was first equilibrated with 5 column volumes of washing buffer W containing 100 mM Tris-HCl, pH 8 + 150 mM NaCl. The mixture of resuspended column material and periplasmic extract was incubated at 4°C for 2 h under gentle rotation. After the mixture was within the column allowed to settle again the flow through was collected. The column was subsequently washed with 5 times 200 µL (one column volume) of washing buffer W, all five fractions were collected separately. Finally the column was eluted

with 6 times 100 µL (half a column volume) of elution buffer E, containing 100 mM Tris-HCl, pH 8 + 150 mM NaCl + 2.5 mM D-desthiobiotin. The eluted fractions were collected separately.

Analytical methods

Characterization by 12 % SDS-PAGE

The flow through, wash, elution, periplasmic and combined cytoplasmic and total membrane protein fractions were analyzed by 12 % SDS-PAGE (sodium dodecyl sulfate - polyacrylamide gel electroforese). Two 12 % SDS gels were prepared using the following amounts. The 12 % running gel was prepared with 2.0 mL running buffer (4x), 3.2 mL 30 % N,N'-Methylenebisacrylamide (final concentration 12 %), 2.8 mL milliQ water, 40 µL 10% ammonium persulfate (APS) and 5 µL N,N,N',N'-tetramethylenediamine (TEMED). The 4 % stacking gel was prepared with 0.4 mL 30 % N,N'-Methylenebisacrylamide (final concentration 4 %), 0.75 mL stacking buffer (4x), 1.85 mL milliQ water, 15 µL 10% APS and 5 µL TEMED. The samples were prepared in 4x SDS loading buffer without DTT. The samples were loaded on two gels. After the gels were runned for ~45 min at 200 V the gels were analyzed. One gel was stained to analyze the heme content, it was stained with 50 mg *o*-dianisidine (= 3,3'-dimethoxybenzidine diHCl) dissolved in 50 mL distilled H_2O and 1.2 mL 30 % H_2O_2 (13). A picture of the gel was taken. The other gel was analyzed for both fluorescence and total protein content. This gel was first soaked in 5 % acetic acid for 5 min and after viewing on a UV bench it was washed twice with distilled H_2O, heating in a microwave in between washes. The gel was subsequently stained with SimplyBlue Safe Stain (Invitrogen) according to the manufacturer's protocol.

Oxidase-peroxidase activity essay

The oxidase, peroxidase and coupled activity of the periplasmic, cytoplasmic and total membrane protein, flow through, wash and elution fractions were analyzed using a DCHBS/AAP assay. DCHBS is an abbreviation for 3,5-dichloro-2-hydroxybenzensulfonic acid and AAP for 4-aminoantipyrine. The 3 types of activities were tested by loading wells of a microtiter plate with a total volume of 100 µL containing 10 µL sample and 50 mM KPi-buffer pH 7.5. The oxidase activity was checked in wells filled with the following substances: 1 mM DCHBS, 0.1 mM AAP, 0.01 U horseradish peroxidase (HRP) and 10 mM xylitol. The peroxidase activity was checked in wells containing: 1 mM DCHBS, 0.1 mM AAP and 5 mM

H_2O_2. And the coupled activity in wells containing: 1 mM DCHBS, 0.1 mM AAP and 10 mM xylitol. A well coloured pink in the case of activity.

Spectrophotometry

The absorption spectra of CXXCH-AldOStrep and StrepAldo(232-236) were measured between 200 and 800 nm in order to determine the presence of FAD and heme in the protein. Quartz 1 mL cuvettes were used during the procedure. Destilled H_2O was used as auto-zero. 50 mM KPi, pH 7.5 was used as a blank measurement.

Size Exclusion Chromatography (SEC)

The CXXCH-AldOStrep was analyzed by size exclusion chromatography (SEC) in order to determine the oligomeric state of the protein. During this procedure an ÄKTA fast protein liquid chromatography system (ÄKTA FPLC) was used in combination with a Bioworks SEC column with a void volume of 4.3 mL. During the measurement a constant flow rate of 1 mL min^{-1} of PBS buffer was used. Spectra of 280 nm (protein content), 450 nm (FAD content) (2) and 600 nm were taken. In order to estimate the molecular weight of the protein the elution volume of the following five proteins was measured and a standard curve prepared: apoprotein (6.5 kDa), RNAse (13.7 kDa), carbonic anhydrase (29 kDa), ovalbumin (43 kDa) and Conalbumin (75 kDa).

Protein concentration determination by Bradford

The total protein concentration within the sample was determined making use of the Bio-Rad Protein assay (14) which is based on the Bradford method (15). During this method the Bio-Rad Bradford stock was diluted 5 times with distilled H_2O and subsequently filtered over a paper filter. The following dilutions were prepared from a 1 mg/mL stock solution of bovine serum albumin (BSA): 0, 0.2, 0.4, 0.6, 0.8 and 1 mg/mL in distilled H_2O. 10 µL of each of these dilutions was pipetted to plastic 1 mL cuvettes, subsequently 990 µL filtered Bradford solution was added. After these mixtures were allowed to stand in the dark for 10 minutes the absorption at 595 nm was measured. A calibration curve was made by plotting the used BSA concentrations in mg/mL against the measured absorbance. The protein concentrations of the Strep-tag purified and desalted protein was subsequently estimated by preparing a cuvette with 10 µL diluted protein with 990 µL filtered Bradford solution. After it was allowed to

stand in the dark for 10 min and the absorbance at 595 nm was measured, the concentration was estimated making use of the BSA calibration curve.

Protein concentration determination by the Waddell method

Beside the Bradford method the Waddell method was used to determine the total protein content of the Strep-tag purified and desalted protein (16). This method determines the protein concentration making use of the absorbance at 215 and 225 nm, see equation 1. The concentration of the Strep-tag purified and desalted protein was measured within a quartz cuvette making use of 10 times dilution in 50 mM KPi, pH 7.5.

$$Concentration(mg/mL) = Abs(215nm) - Abs(225nm) * 0.413 \qquad \text{(eq. 1)}$$

FAD concentration determination

The FAD concentration within an oxidase-peroxidase chimera could be determined by measuring the difference between the absorbance of the oxidized and reduced state of the chimera. This method is necessary because it is impossible to determine the FAD concentration from the height of the FAD peak at 451 nm (2), because the heme (Soret band at 410 nm (17)) absorbs at 451 nm too.

The FAD concentration within the sample was determined making use of a 1 mL quartz cuvette with a screw cap and a spectrophotometer which determined the absorbance between 200 and 800 nm. First a blank of 100 µL 10 mM 4-ethylphenol (final concentration of 1 mM) in 900 µL 50 mM KPi, pH 7.6 was taken. Subsequently the oxidized spectrum of Strep-tag purified, desalted and concentrated ThAS (CXXCH-AldOStrep) was taken by adding 132 µL concentrated ThAS, 100 µL 10 mM 4-ethylphenol and 758 µL 50 mM KPi, pH 7,6 to a cuvette, giving a total volume of 990 µL. 5 µL dissolved VAO was added to the wall of the cuvette above the surface of the liquid. The cuvette was closed with a septum. After the measurement the cuvette was transferred and attached to a nitrogen gas source to replace the air by nitrogen to obtain anaerobic conditions. After flushing, 10 µL 1.5 M xylitol in 50 mM KPi, pH 7.6 was added. It was added by opening the septum. The cuvette was subsequently shaken to absorb and mix the 5 µL dissolved VAO through the mixture. The VAO was added to extract still present oxygen from the solution by oxidizing 4-ethylphenol. The reduced spectrum was taken after the mixture was flushed for another couple of minutes. The concentration was determined making use of the Lambert-Beer law (eq. 2) in which the

extinction coefficient is given by ε (M^{-1} cm^{-1}), the path length by l (cm) and the concentration by c (M).

$$Absorbance = \varepsilon \cdot l \cdot c \qquad \text{(eq. 2)}$$

In this experiment it is impossible to determine the FAD content direct from the oxidase peak, as mentioned above. Therefore the difference in the absorption at 451.1 nm ($\Delta Abs_{451.1}$) was determined. The concentration was calculated making use of the difference of the extinction coefficient between the oxidized and reduced state of mbpAldO, $\Delta\varepsilon = 9.43$ mM^{-1} cm^{-1}, determined by Drs. R.T. Winter, 20 may 2009 (unpublished data). Δε is expected to be the same for mbpAldO and ThAS because the environment of the flavin is unchanged in both proteins.

Pyridine hemochrome assay

The heme content of ThAS (CXXCH-AldOStrep) was determined through a pyridine hemochrome assay described by Berry et al (1986) (18) for cytochrome *c* and by Yumoto et al (2000) (19) for pyridine hemochrome *b*. These procedures are based on work by Fuhrhop et al (1975) (20). The absorption between 200 and 800 nm was measured making use of a spectrophotometer and a quartz cuvette. The spectrophotometer was first auto-zeroed with 1 mL distilled H_2O. Subsequently a blank measurement of 100 µL pyridine (final concentration of 10 vol/vol%, 1.24 M), 100 µL 2 M NaOH (final concentration of 200 mM) and 800 µL distilled H_2O was recorded. Subsequently, 3 mg sodium dithionite was added and gently dissolved by pipetting, trying to prevent aeration. Another absorption spectrum was recorded. Finally ThAS was measured. 150 µL ThAS (12 µM), 100 µL pyridine (final concentration of 10 vol/vol%, 1.24M), 100 µL 2 M NaOH (final concentration of 200 mM) and 650 µL distilled H_2O were added to a cuvette giving a total volume of 1 mL. 3 mg sodium dithionite was added just before the sample was measured. The blank spectrum was subtracted from the sample spectrum.

The heme concentration within the samples were calculated making use of the absorption at 550 or 557 nm, the Lambert-Beer law (eq. 2) and the extinction coefficients for hemochrome *b* at 557 nm ($\varepsilon_{557} = 34.53$ mM^{-1} cm^{-1}) (19) or for cytochrome *c* at 550 nm ($\varepsilon_{550} = 30.27$ mM^{-1} cm^{-1}) (18). The extinction coefficient of cytochrome *c* was used for ThAS because both contain a covalently bound heme.

Determination of the kinetic parameters

The steady-state kinetic parameters K_m and k_{cat} of ThAS (CXXCH-AldOStrep) were determined as described in (2). The kinetic parameters were determined by measuring the initial reaction rate at different substrate concentrations. The following concentrations of xylitol were used: 0.05, 0.1, 0.5, 1, 2.5, 5 and 15 mM in 50 mM KPi, pH 7.6. Beside xylitol the 1 mL cuvettes contained; 0.1 mM AAP, 1.0 mM DCHBS, 0.004 U/mL HRP and 50 mM KPi, pH 7.6. They were filled to a total volume of 990 µL. The initial reaction rates (dA_{515nm}/dt) of each concentration were determined by measuring the absorbance of the pink product formed at 515 nm ($\varepsilon = 26\ mM^{-1}\ cm^{-1}$). Distilled water was used as auto-zero. After a blank was taken from a solution with a specific xylitol concentration, 10 µL 10x diluted Strep-tag purified, desalted and concentrated enzyme was added after which the absorbance measurement was continued. The obtained initial rates were plotted against the concentrations yielding a Michaelis-Menten plot. The curve was fitted to the Michaelis-Menten equation (a hyperbola, eq. 3) to estimate the kinetic parameters K_M and k_{cat}. The initial rates are given by v_0 and the substrate concentrations by [s].

$$v_0 = \frac{v_{max}[s]}{K_m + [s]} \qquad \text{(eq. 3)}$$

Results

Mutagenesis, culture growth and activity analysis

Plasmids pBADTatAldOStrep 232-236 (TAS 232-236), pBADTatStrepAldO 232-236 (TSA 232-236), pBADTatAldOStrep 235-239 (TAS 235-239) and pBADTatStrepAldO 235-239 (TSA 235-239) were successfully formed by mutagenesis and subsequently used to transform *E. coli* strain BL21(DE3)/pEC86. These strains were cultured along with those provided by Drs. R.T. Winter: BL21(DE3)/pEC86 & pBADTatAldOStrep 280-284 (TAS 280-284) and BL21(DE3)/pEC86 & pBADTatStrepAldO 280-284 (TSA 280-284). As described in materials and methods these strains were grown in different volumes of TB medium to which arabinose was or was not added. An approximately equal OD_{600} of 11.5-13 was measured for cultures grown with or without arabinose. This observation was unexpected because *E. coli* BL21(DE3) can use arabinose as a carbon source (21) and grows better in the presence of it (9).

There was however another difference between the cultures with and without arabinose. Only the cultures without arabinose displayed grey coloured sedimentation, which was resuspended before the OD_{600} was measured. The reason for this phenomenon is unknown.

The periplasmic and cytoplasmic fractions of these cultures were obtained during the fractionation of the cultures as described in the Methods and Materials section. The fractions were analyzed on 12 % SDS-PAGE gels using a fluorescence and total protein stain. The gels stained for total protein content are shown in figure 6. These gels show for all cytoplasmic fractions a higher protein content than for the periplasmic fractions, which was expected. The cytoplasm contains more protein than the periplasm. This difference was more clearly visible for the cultures to which arabinose was added.

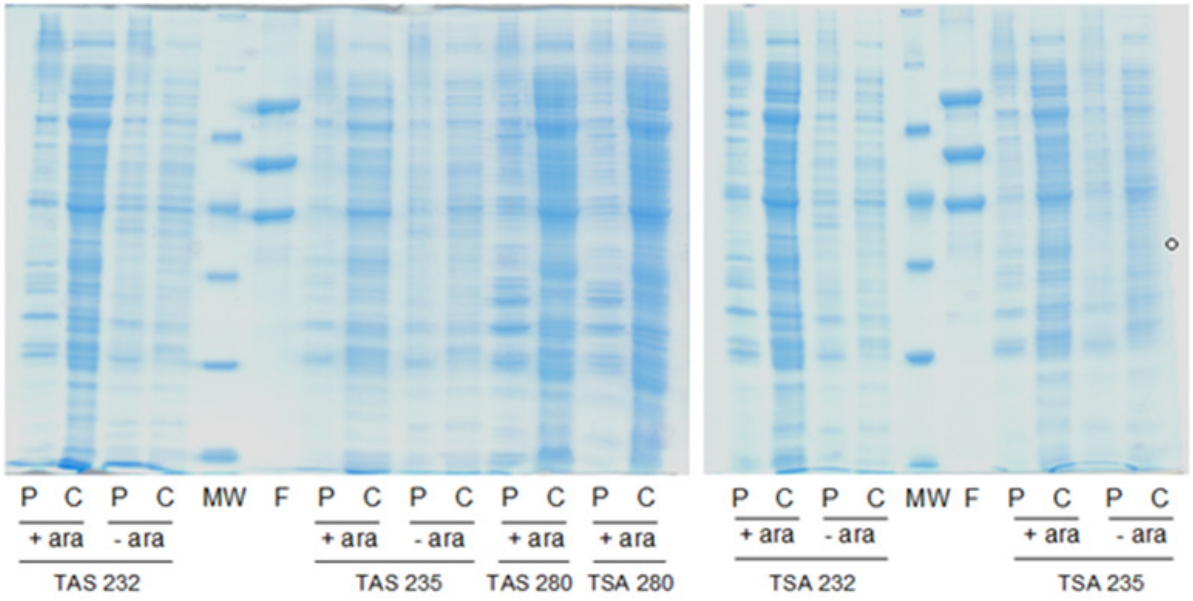

Figure 6: Total protein stain. Left gel contains TAS 232-236, TAS 235-239, TAS 280-284 and TSA 280-284. Right gel contains 232-236 and TSA 235-239. Abbreviations: p: periplasm, c: cytoplasm, + ara: with arabinose, - ara: without arabinose, MW: low molecular weight marker, F: fluorescence marker containing AldO (45.7 kDa, lower band), EUGO (59.5 kDa, middle band) and MBP-AldO (88.1 kDa, higher band).

The gels were also stained for fluorescence with 5% acetic acid (before they were stained for total protein content), see figure 7. Both TAS 232-236 and TSA 232-236 showed a fluorescent band for both the periplasmic and cytoplasmic fractions. Both fractions contain fluorescent AldO, and depending on its location both a Strep-tag and a Tat-sequence (cytoplasm) or only a Strep-tag (periplasm). The protein secreted to the periplasm no longer contains a Tat-sequence because the Tat-sequence will be cleaved from the protein after the correctly folded protein is transported over the inner cell membrane into the periplasm.

The periplasmic fraction of TAS 280-284 showed a fluorescent band at the expected height too. All other fractions of the other constructs did not show fluorescence, because the

expression was too low to be detected. This was expected for the cultures which were grown without arabinose, because arabinose induces protein expression. The constructs with a CXXCH-motif for amino acids 232-236 show a higher (periplasmic) expression of the desired protein than the constructs with a CXXCH-motif within amino acids 235-239 or 280-284.

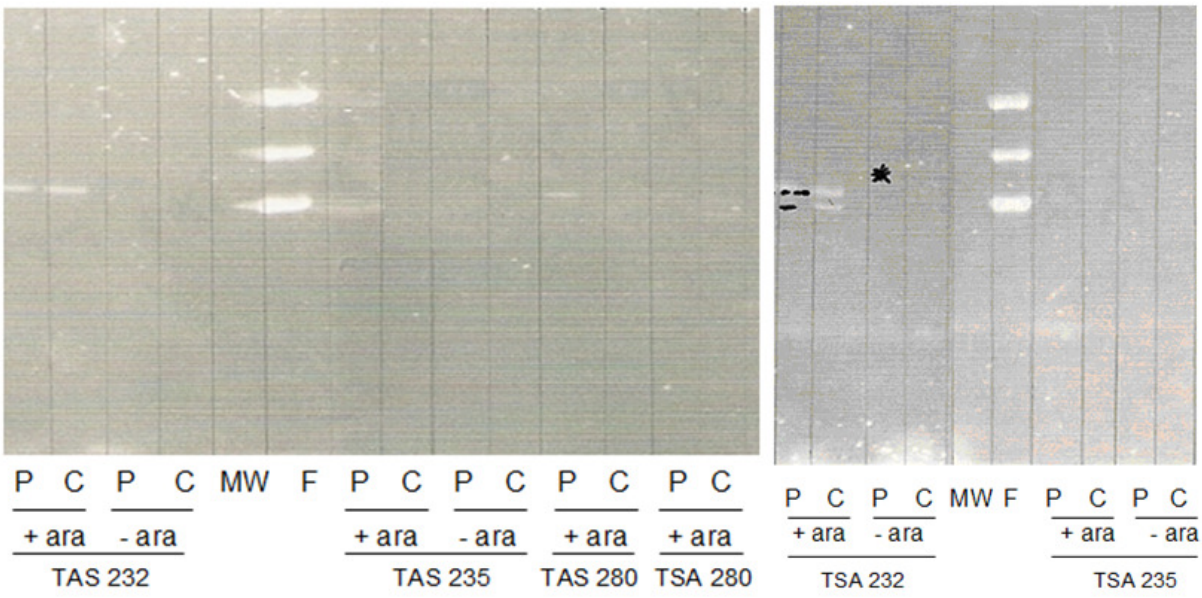

Figure 7: Fluorescent stain of the gels shown in figure 6. Left gel contains TAS 232-236, TAS 235-239, TAS 280-284 and TSA 280-284. Right gel contains TSA 232-236 and TSA 235-239. Abbreviations: p: periplasm, c: cytoplasm, + ara: with arabinose, - ara: without arabinose, MW: low molecular weight marker, F: fluorescence marker containing AldO (45.7 kDa, lower band), EUGO (59.5 kDa, middle band) and MBP-AldO (88.1 kDa, higher band). * marks the cytoplasmic fraction of TSA 232-236 +ara which contains an unexpected second band.

For the cytoplasmic fraction of TSA 232-236 + ara something unexpected was shown, the fluorescent picture showed two bands. This result was reproducible. One band at the same height and the other one with clearly a lower molecular weight. The molecular weight of the proteins within these bands was determined by fitting the distance of the fluorescence marker to the log of the molecular weight of the marker proteins. Subsequently the mass of the two bands were calculated after the travelled distance was measured, resulting to molecular weights of 50.2 and 45.6 kDa. The higher weight is very close to the expected value of 50.5 kDa of TatStrepCXXCHAldO, the difference will be due to the measurement of the distance. The other weight was unexpected and further analyzed by purification of the cytoplasmic fraction over a Strep-Tactin column. The protein with the lower molecular weight did not bind to the column. The protein with the higher molecular weight bound to the column and was eluted with D-desthibiotin. Hence, within the cytoplasm a portion of the expressed protein has been cleaved C-terminal of the Strep-tag. The periplasmic fraction of this construct only showed the high molecular weight protein, so the smaller molecular weight

protein was not transported over the inner membrane by the Tat-system. This result shows that within the cytoplasm a part of the N-terminus of the protein containing both the Tat-sequence and the Strep-tag has been cut off of the protein. The molecular weight of StrepCXXCHAldO and CXXCHAldO are 46.3 and 45.1 kDa respectively. Compared to the measured molecular weight of 45.6 kDa the suggested cleavage in or near the Strep-tag could be possible.

The oxidase and peroxidase activity of the periplasmic and cytoplasmic fractions of above cultures were tested by an oxidase-peroxidase assay based on AAP/DCHBS. In figure 8 the oxidase and peroxidase activity of TAS 232-236, TSA 232-236, TAS 235-239 and TSA 235-239 with and without arabinose are shown, each time with the culture grown without arabinose below the culture grown with arabinose. The oxidase and peroxides activities of TAS 280-284 and TSA 280-284 are visible in the last column. The activities of the periplasm and cytoplasm of TAS 280-284 are shown in the first four wells while the activities of TSA 280-284 are shown in the last four wells.

The activity assay showed oxidase activity for both the periplasmic and cytoplasmic fractions of all cultures grown with arabinose. The cultures which were grown without arabinose did not show oxidase activity. This was expected because the protein is expressed within the cytoplasm after induction by arabinose and subsequently transported to the periplasm by the Tat-system. None of the cultures grown with arabinose showed peroxidase activity, so the CXXCH-motif did not bind heme or with a too low efficiency to be recognized by this assay. The cultures grown without arabinose however did show peroxidase activity within the periplasm, which was highly unexpected but reproducible. An explanation for this unexpected result might be that another peroxidase active protein is suppressed in the presence of arabinose but is expressed in the absence of it. Hassan and Fridovich (1978) had a similar observation for glucose (22). They showed that glucose suppresses the synthesis of peroxidase and catalase in *E. coli* by repressing the catabolic pathway. Perhaps arabinose suppresses the peroxidase synthesis too because *E. coli* B, from which the in this study used *E.coli* strain is a derivative, could use D-arabinose instead of glucose as a sole carbon and energy source (21).

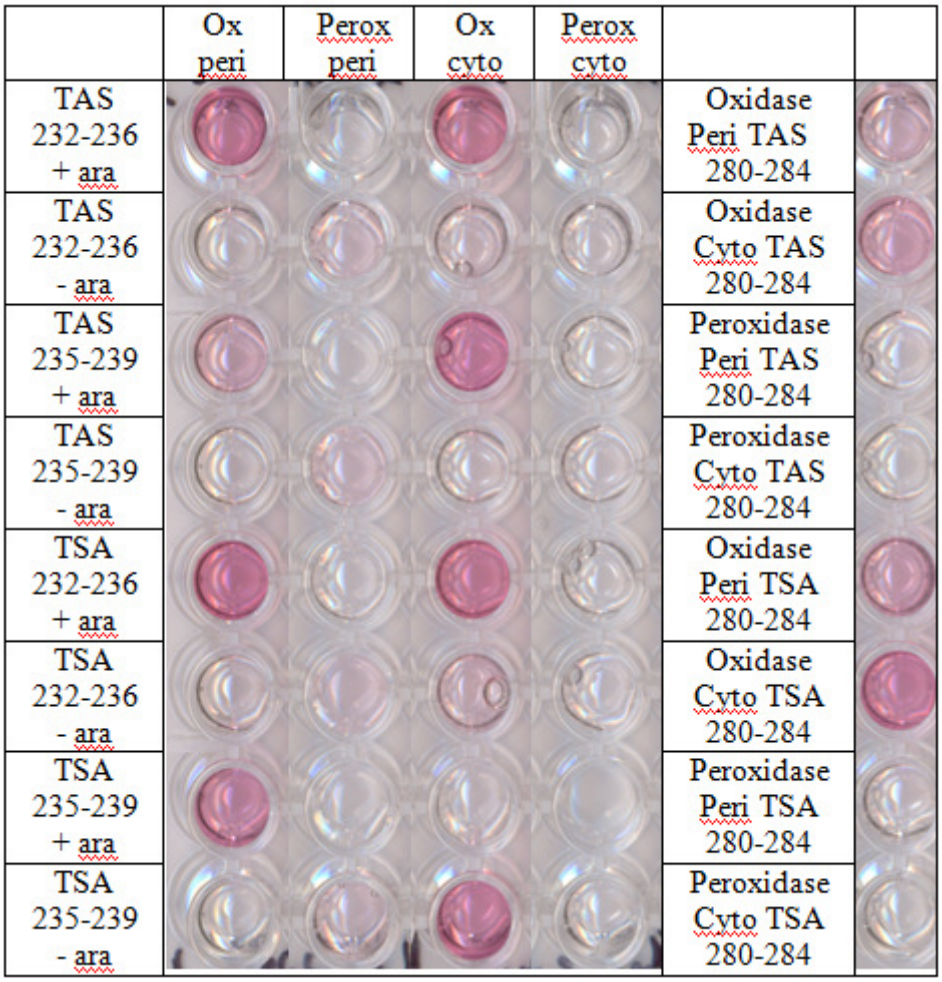

Figure 8: Oxidase-peroxidise activity assay of TAS 232-236, TSA 232-236, TAS 235-239, TSA 235-239, TAS 280-284 and TSA 280-284. Abbreviations: Ox: oxidase activity, Perox: peroxidase activity, ara: arabinose, Peri: periplasm and Cyto: cytoplasm.

Beside these constructs two other constructs were analyzed. Both constructs contained a region near the N-terminus which was changed to the CXXCH-motif. They were expressed in BL21(DE3)/pEC86 & pBADTat-CXXCH-AldOStrep (ThAS) and BL21(DE3)/pEC86 & pBADTatStrep-CXXCH-AldO (TShA). The periplasm of both constructs was purified by a Strep-Tactin column. All flow through, wash and elution fractions showed oxidase activity as expected. Only the elution fractions of ThAS showed peroxidase and coupled activity. So ThAS was transported to the periplasm where it bound heme, because the purified protein contained both activities. The flow through however showed peroxidase activity as well. This might be due to the above mentioned peroxidase activity or because the column was overloaded. The latter is unlikely however, as the binding capacity of the used Strep-Tactin column was expected to be enough to bind all of the desired protein.

TShA did not show any peroxidase activity within the elution fractions although the for total protein content stained 12 % agarose SDS-PAGE gel showed a band within the elution fractions at the expected height. Hence TShA did not bind heme. This could be due to the

Strep-tag which is attached to the N-terminus of this construct. The Strep-tag might be too close to the CXXCH-motif which is located within the N-terminus of the protein. The flow through however showed a higher peroxidase activity than ThAS. This suggests that this activity is not only due to the above mentioned peroxidase but that a part of TShA binds heme. Hence the portion of TShA which does bind heme could not bind to the Strep-Tactin column because the Strep-tag and the CXXCH-motif are too close to each other.

ThAS and TSA 232-236 were expressed on a large scale by growing BL21(DE3)/pEC86 & pBADTat-CXXCH-AldOStrep and BL21(DE3)/pEC86 & pBADTatStrepAldO 232-236 in respectively 3 L and 0.4 L TB medium, as described in the Materials and Methods part above. These cultures were chosen to be expressed on large scale because of their desired properties. ThAS showed the desired coupled oxidase-peroxidase activity and TSA 232-236 showed as well as TAS 232-236 a higher protein expression than the other hemeloop mutants. TSA 232-236 shows however two fluorescent bands within the cytoplasm, but this will not influence the experiment because the correct construct was purified from the periplasm. The periplasm showed as mentioned above only one band at the desired height on the fluorescence stained gel. TSA 232-236 was chosen to be expressed on a large scale to obtain more protein and determine whether a small fraction of the protein contained both heme and FAD.

After the cells were harvested and fractionated the periplasm was purified making use of a Strep-tag purification. The flow through, wash fractions, elution fractions and a sample of the combined and concentrated elution fractions were analyzed on two 12 % SDS-PAGE gels. One of the gels was stained for both fluorescence and total protein content while the other gel was stained for heme content, as described in the Materials and Methods section. The total protein and heme stained gels are shown in figure 9 and 10 for respectively ThAS and TSA 232-236 while the fluorescence stained gels are shown in figure 11. The total protein content stained gels show a good purification of the desired protein. TSA 232-236 shows within the elution fractions a second band with a higher molecular weight. This band is visible on the fluorescence stained gel as well and might be due to aggregation/dimerization of our protein, see figure 11. Both gels show the desired protein within the second wash fraction and more intense bands at the same height within the flow through and first wash fraction. The fluorescence stained gels shows for all fractions one (or two) band(s) at the desired height. These observations suggests that both columns were slightly overloaded or that the protein bound less tightly to the column than would be expected, which could be due to the protein or

due to the column which had been used before (which is possible for a Strep-Tactin column after it is regenerated).

The heme stained gel of ThAS shows in fractions E4-E7 and Ec a yellow-brown band at the same height as the bands that were visible in the elution fractions of the gel stained for fluorescence and total protein content. So ThAS contains both a heme and a flavin. TSA 232-236 however did not show heme content even though the total amount of protein on gel was significant; see the difference between the elution fractions of TSA 232-236 and ThAS.

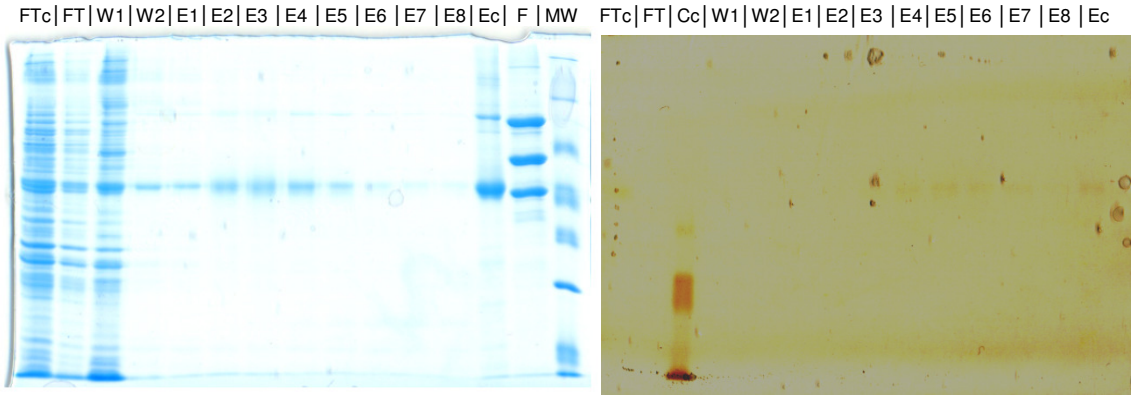

Figure 9: **Total protein and heme stained gel of ThAS.** Left: total protein stain of ThAS. Right: heme stain of ThAS. Abbreviations: FT(c): flow through (concentrated), W: wash fraction, E: elution fraction, Ec: concentrated elution fraction, F: fluorescence marker containing AldO (45.7 kDa, lower band), EUGO (59.5 kDa, middle band) and MBP-AldO (88.1 kDa, higher band), MW: low molecular weight marker and Cc: heme containing cytochrome c.

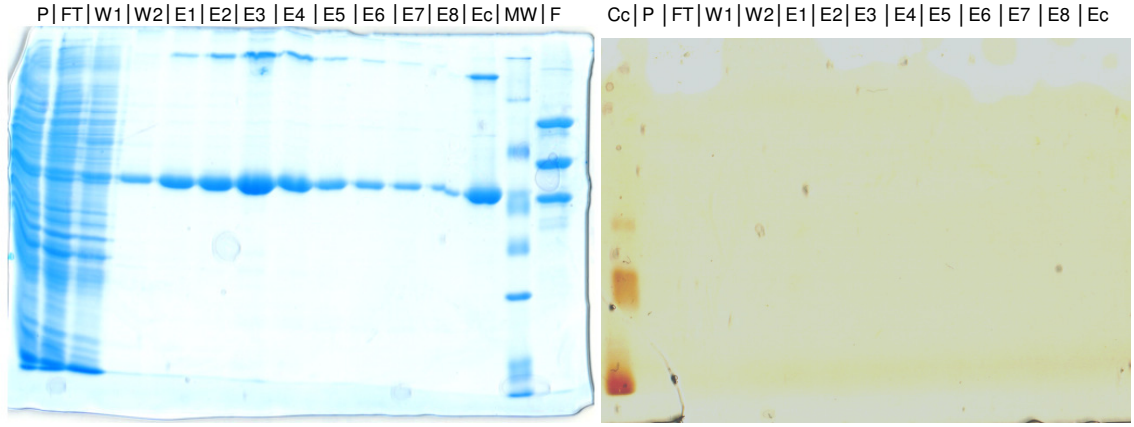

Figure 10: Total protein and heme stained gel of TSA 232-236. Left: total protein stain of TSA 232-236. Right: heme stain of ThAS. Abbreviations: P: periplasm, FT: flow through, W: wash fraction, E: elution fraction, Ec: concentrated elution fraction, MW: low molecular weight marker, F: fluorescence marker containing AldO (45.7 kDa, lower band), EUGO (59.5 kDa, middle band) and MBP-AldO (88.1 kDa, higher band), and Cc: heme containing cytochrome c.

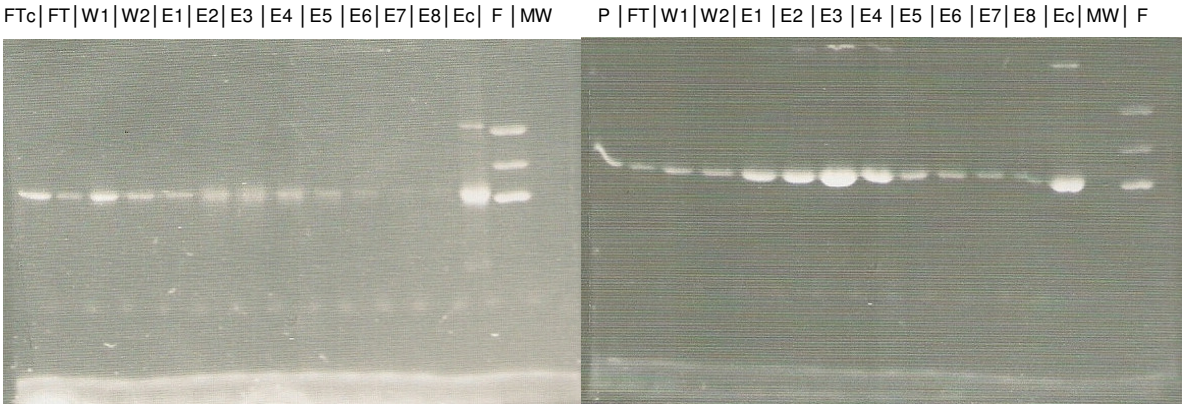

Figure 11: Fluorescence stained gel of ThAS and TSA 232-236. Left: fluorescent stain of the left gel shown in figure 9 showing ThAS: fluorescent stain of the left gel shown in figure 10 showing TSA 232-236. Abbreviations: P: periplasm, FT: flow through, W: wash fraction, E: elution fraction, Ec: concentrated elution fraction, MW: low molecular weight marker and F: fluorescence marker containing AldO (45.7 kDa, lower band), EUGO (59.5 kDa, middle band) and MBP-AldO (88.1 kDa, higher band).

The on gel analyzed fractions of both ThAS and TSA 232-236 were also analyzed by an oxidase-peroxidase activity assay, see figure 12 for ThAS and figure 13 for TSA 232-236. Both assays show oxidase activity within all fractions, as expected. ThAS shows peroxidase and coupled activity within elution fractions E2-E5 and Ec. Ec is a concentrated fraction of the combined fraction of E1-E8. The flow through and first wash fraction show both activities, which might be due to an overloaded column or maybe to the earlier mentioned other peroxidase. TSA 232-236 did not show any peroxidase activity within the elution fraction. It did however show this activity within the periplasm and the flow through. This activity might be due to the earlier mentioned other peroxidase protein. Another explanation might be that a small part of the desired protein did actually bind heme but could, because of that, no longer bind to the Strep-Tactin column. This is however unexpected because the loop which should bind heme is located in a different part of the protein than the Strep-tag.

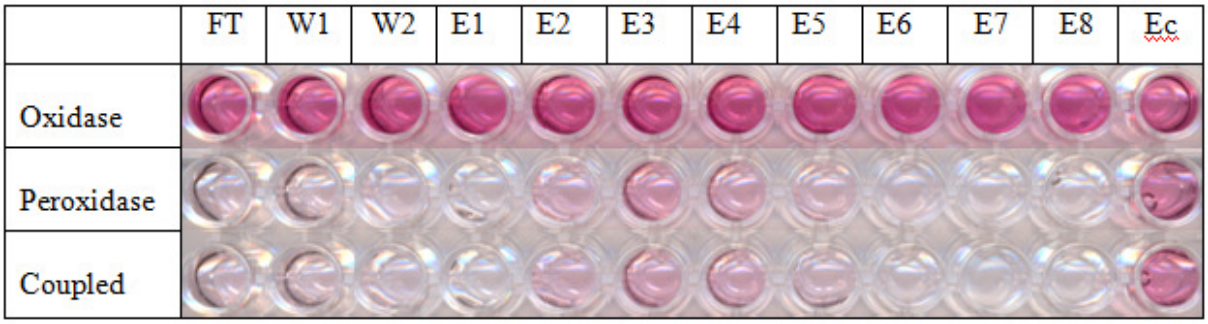

Figure 12: Oxidase-peroxidase activity assay of ThAS. Abbreviations: FT: flow through, W: wash fraction and E: elution fraction.

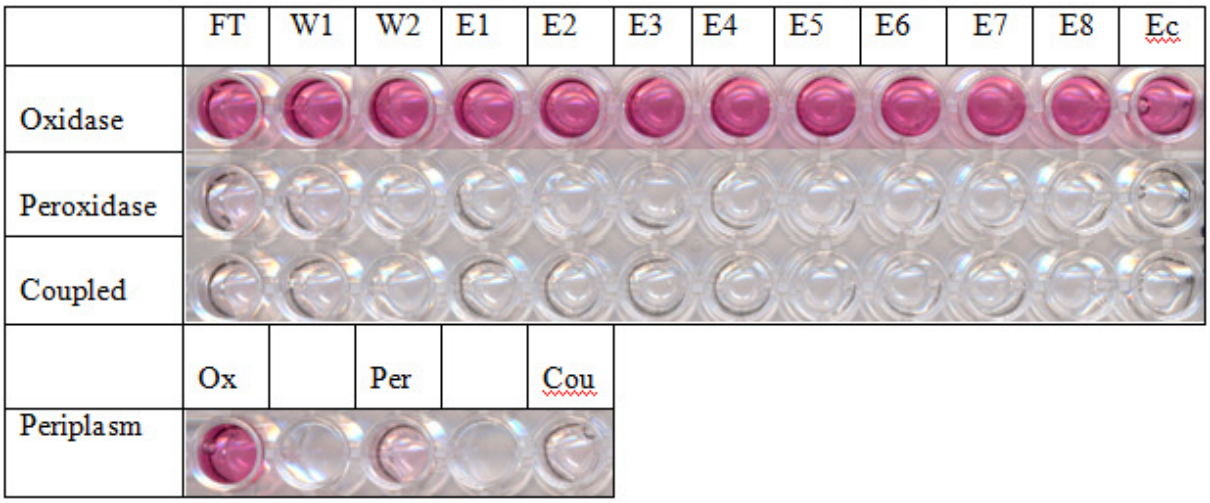

Figure 13: **Oxidase-peroxidase activity assay of TSA 232-236.** Abbreviations: FT: flow through, W: wash fraction, E: elution fraction, Ox: oxidase activity, Per: peroxidase activity and Cou: coupled activity.

Both ThAS and TSA 232-236 were analyzed by spectrophotometry between 200 and 800 nm. The absorption spectra are shown in figure 14. The spectrum of ThAS shows absorbance of both covalently bound heme and FAD. A flavin (FAD) spectrum shows a peak at 450 nm (2) and a shoulder around 475 nm while a spectrum of covalently bound heme shows a Soret band around 410 nm (17). TSA 232-236 shows in comparison to ThAS only a flavin spectrum. So ThAS did bound heme while TSA 232-236 did not, which confirms the earlier results. Both the analysis by SDS-PAGE and the oxidase-peroxidase assay showed heme and flavin presence for ThAS but showed only flavin presence for TSA 232-236. ThAS will be analyzed further by some analytical methods below.

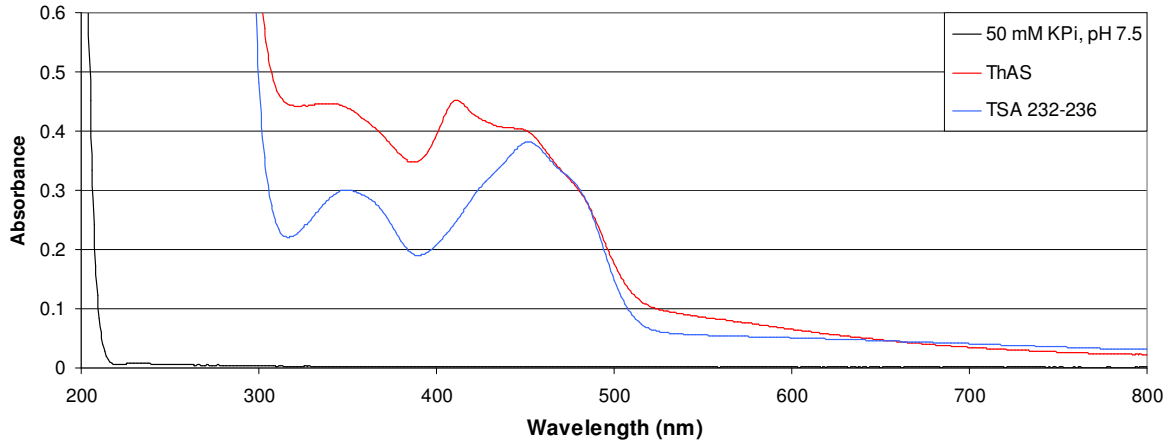

Figure 14: Spectrophotometry of ThAS and TSA 232-236

Size exclusion chromatography (SEC)

The concentrated elution fraction of the Strep-tag purified and desalted ThAS was analyzed by size exclusion chromatography in order to determine oligomeric form of the

protein. The analysis showed that ThAS is build up by monomers, no aggregates were visible. The retention volume was close to Ovalbumin (43 kDa), which was expected. ThAS (or CXXCHAldOStrep) has a molecular weight of 47 kDa in the periplasm, where the Tat-sequence has been cut off.

Protein concentration determination

The protein concentration of the concentrated elution fraction of Strep-tag purified and desalted ThAS was determined in two ways. It was determined by the Bradford method and by the Waddell method. Both methods were described in the Materials and Methods section. A concentration of 1.45 mg/mL (31 μM) protein was determined for the concentrated elution fraction of Strep-tag purified and desalted ThAS by the Bradford method. Yielding 0.51 mg ThAS in the 3L culture, which is equal to a final concentration of 0.17 mg ThAS per L grown culture. The Waddell method makes, to determine the concentration, use of the absorbances at 215 and 225 nm and equation 1 (see Materials and Methods). The absorption spectrum of ThAS between 200 and 260 nm is shown in figure 15. A concentration of 2.1 mg/mL (45 μM) was determined for the concentrated elution fraction of Strep-tag purified and desalted ThAS. This yields 0.74 mg ThAS in the 3L culture, which is equal to a final concentration of 0.25 mg ThAS per 1L grown culture.

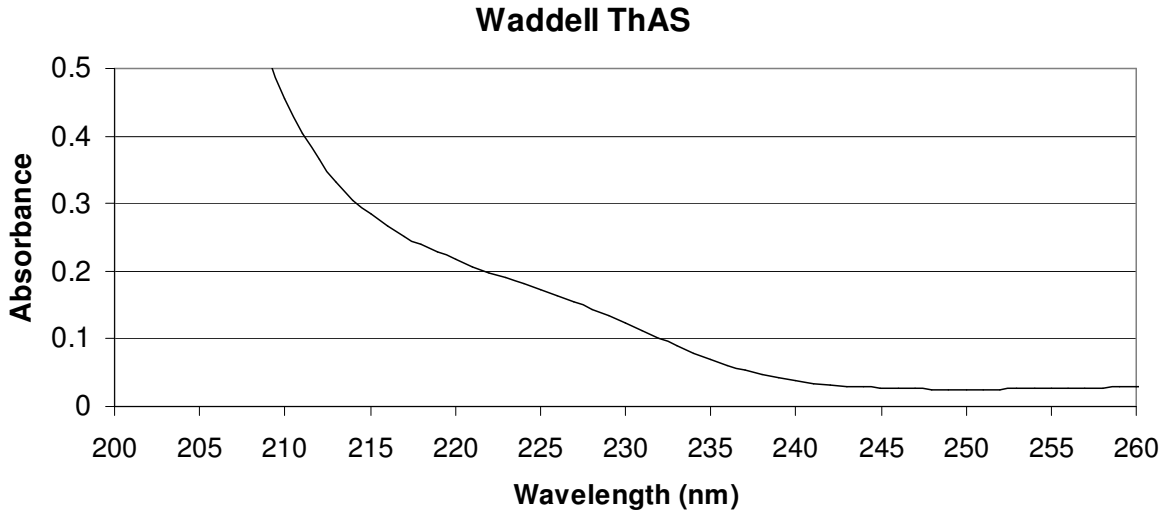

Figure 15: ThAS absorption spectrum between 200 and 260 nm.

Determination of the FAD concentration

The FAD concentration of the concentrated, Strep-tag purified and desalted elution fraction of ThAS was determined as described in the Materials and Methods part above. During this method a part of the mixture was lost through a crack in the cuvette, this crack

wasn't noticed before the pressure was raised by the nitrogen flow. The crack was closed by wrapping Parafilm around the cuvette and septum.

This method could be used to determine the ThAS concentration because every ThAS molecule should contain one molecule of FAD. Only properly folded cofactor containing protein with a Tat-sequence are by the Tat-system transported over the inner cell membrane. It is however impossible to determine the concentration in a direct way because the sorbet band of heme (410 nm) (17) absorbs near the characteristic FAD peak (451 nm) (2). The FAD content is therefore determined through the absorption difference at 451 nm between the oxidised and reduced form of ThAS. The concentration is subsequently calculated making use of Lambert-Beer law (equation 2) and the difference in the extinction coefficient, which is $\Delta\varepsilon_{451.1} = 9.43\ mM^{-1}\ cm^{-1}$. The concentration was converted to mg/mL making use of a protein mass of 47 kDa.

The absorption spectra of oxidised (red line) and reduced ThAS (light blue line) are shown in figure 16. The reduced spectrum of ThAS was corrected as evaporation due to nitrogen flushing caused the overall absorbance to increase. The spectra should overlap between 550 and 800 nm because the spectra will only be changed near the flavin peak. The reduced spectrum was corrected by moving the whole spectrum down to match the oxidised spectrum within the 550-800 nm region, dark blue line. The FAD concentration was determined from the absorption difference at 451 nm between the oxidised and the reduced form (corrected spectra) of ThAS, yielding a concentration of 0.57 mg/mL (12 µM, 350 µL) in the concentrated, Strep-tag purified and desalted elution fraction of ThAS. Yielding 0.20 mg ThAS within the 3L culture, which is equal to a final concentration of 0.067 mg ThAS per L grown culture.

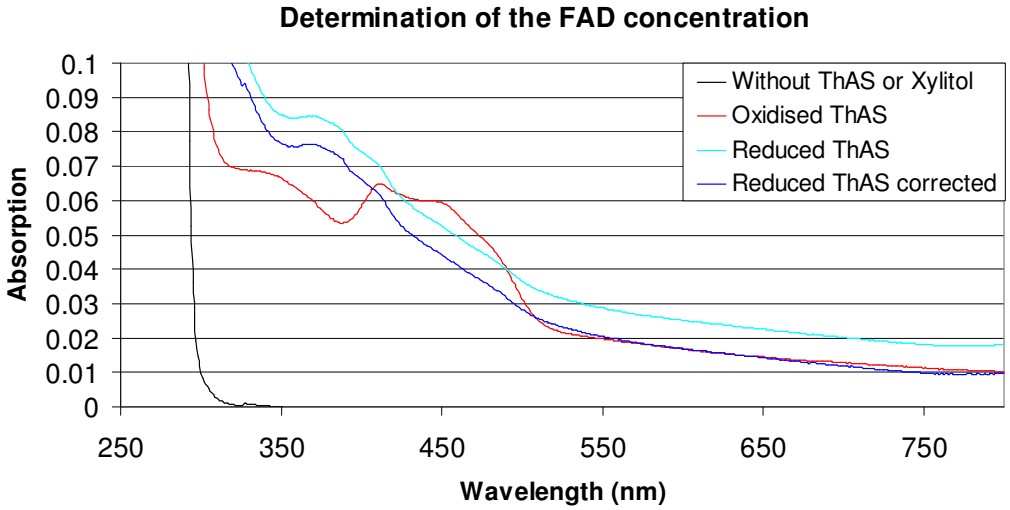

Figure 16: Determination of the FAD content of ThAS. Absorption spectrum between 250 and 800 nm.

Pyridine hemochrome assay

The heme concentration of the concentrated, Strep-tag purified and desalted elution fraction of ThAS (CXXCHAldOStrep) was determined through a hemochrome assay as described in the Materials and Methods part above. The observed absorbances between 350 and 800 nm are shown in figure 17. In this method heme binds to pyridine forming a pyridine-hemochrome complex. The high NaOH concentration hydrolyses peptide bonds, leaving only the covalently bound amino acids attached to the heme. The concentration of the pyridine-hemochrome complex will be determined making use of its absorbance at 550 nm (pyridine-hemochrome peak), the extinction coefficient of cytochrome *c* (ε_{550} = 30.27 mM^{-1} cm^{-1}) (18) and the Lambert-Beer law, equation 2. The extinction coefficient of cytochrome *c* is used because both cytochrome *c* and ThAS contain a covalently bound heme so the pyridine-hemochrome complexes give an equal pyridine hemochrome absorption spectrum. A concentration of 0.089 mg/mL (1.9 µM) was determined in the concentrated, Strep-tag purified and desalted elution fraction of ThAS after the blank spectrum was subtracted from the sample spectrum. Yielding 0.031 mg ThAS within the 3L culture, which is equal to a final concentration of 0.010 mg ThAS per L grown culture. A protein mass of 47 kDa was used to determine the protein concentration in g/mL. This concentration is less than the determined FAD concentration (0.067 mg/L) or the other earlier determined protein concentrations. In comparison to the FAD concentration heme was only attached to 15.4 % of ThAS.

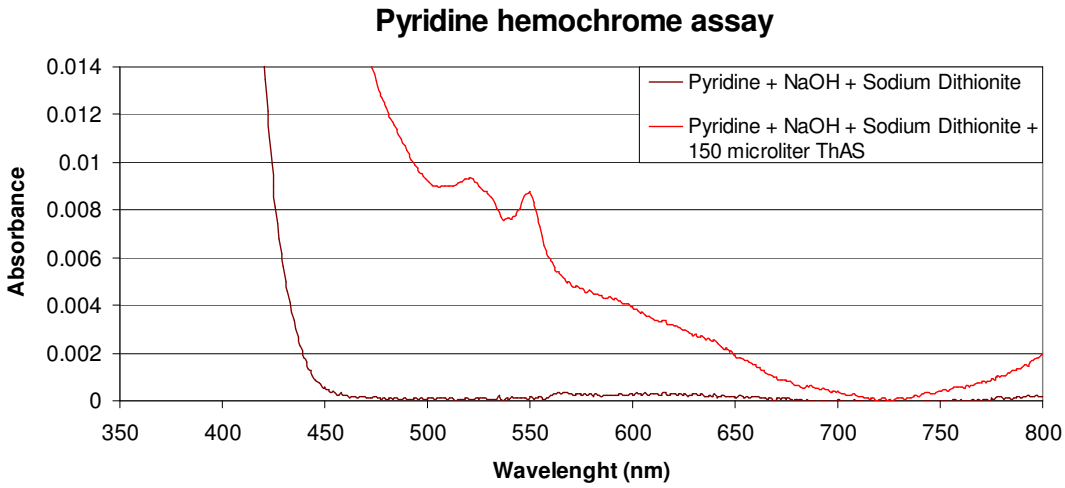

Figure 17: Pyridine hemochrome assay of ThAS. Absorption spectrum between 350 and 800 nm.

Determination of the kinetic parameters

The kinetic parameters of the oxidase activity of ThAS were determined as described in the Materials & Method section. The initial absorption rate (dA_{515nm}/dt ($min^{-1)}$) was measured and converted to k_{obs} (s^{-1}) by dividing the rate through 60 (min^{-1} to s^{-1}), the extinction coefficient (ε_{515} = 26 mM^{-1} cm^{-1}) and the concentration of the enzyme within the cuvette (12 nM). The concentration determined by the determination of the FAD concentration was used because this method is more accurate than the Waddell or Bradford method. The calculated k_{obs} values were plotted against the used substrate concentrations see figure 18. The plot was subsequently fitted to the Michaelis-Menten equation, equation 3.

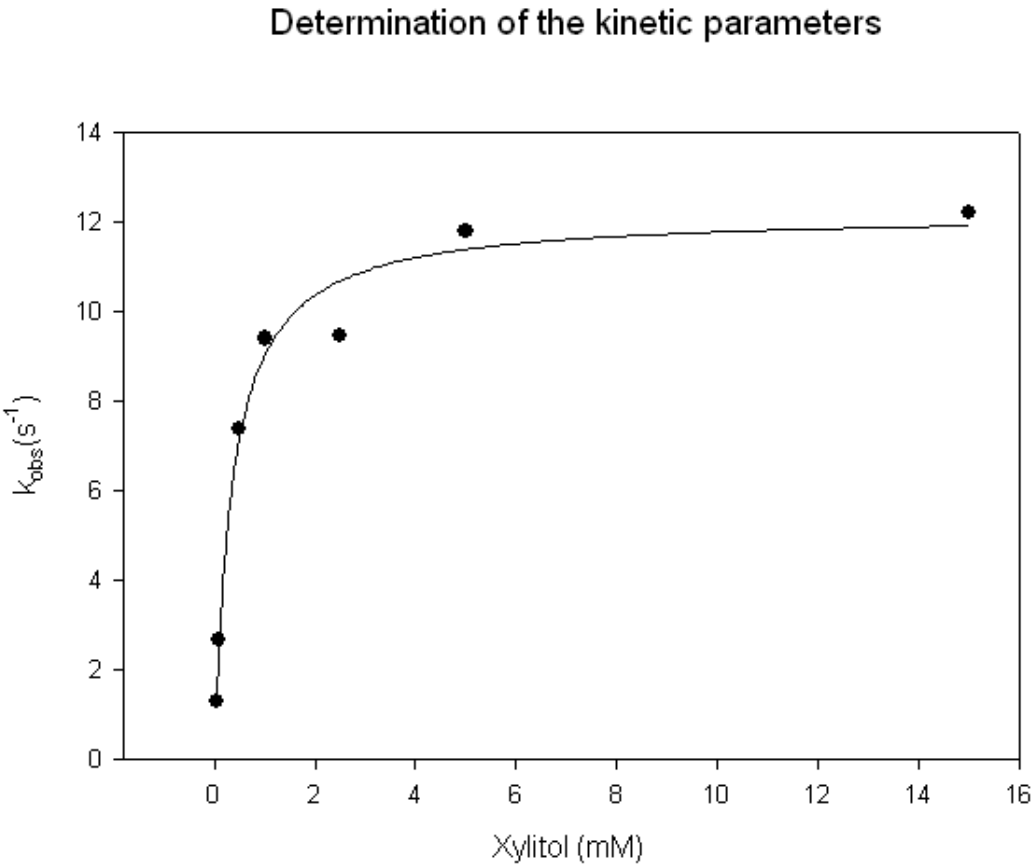

Figure 18: Michaelis-Menten plot of the steady state kinetic parameters of ThAS.

K_M and k_{cat} were determined from this fit, yielding the following values: K_M = 0.35 mM and k_{cat} = 12.2 s^{-1}. These values are close to the literature values of AldO (K_M = 0.32 mM and k_{cat} = 13 s^{-1}) (2). So the kinetic parameters of the oxidase activity of ThAS are approximately the same or equal to the kinetic parameters of original AldO. This result was expected because the heme was attached to a mutated portion of the N-terminus leaving the active site of AldO untouched.

Discussion

This project shows that it is possible to turn AldO into an oxidase-peroxidase chimera. Eight different mutants of AldO were analyzed. Six with a loop changed to the CXXCH-motif, the motif which binds heme (4) and two with 5 amino acids near the N-terminus changed to the same motif. The 6 hemeloop mutants, TatAldoStrep Hemeloop 232-236, TatStrepAldo Hemeloop 232-236, TatAldoStrep Hemeloop 235-239, TatStrepAldo Hemeloop 235-239, TatAldoStrep Hemeloop 280-284 and TatStrepAldo Hemeloop 280-284 did not show peroxidase or coupled activity within the DCHBS/AAP assay while they did show the oxidase activity of AldO. Hence these mutants did not bind heme and no chimera was formed. The DCHBS/AAP assay did however reveal something unexpected. The expression of all mutants was checked in the presence and absence of arabinose. As expected no oxidase activity was shown in the periplasm or cytoplasm of the mutants which were cultured without arabinose because the protein was not expressed. But the periplasm did however show peroxidase activity, while the same cultures grown with arabinose did not. This unexpected activity might be due to some unknown protein with peroxidise activity, a protein whose expression is suppressed in the presence of arabinose. A possibility is a peroxidase whose expression in *E. coli* is suppressed in the presence of glucose (22). Maybe arabinose suppresses this peroxidase as well because *E. coli* could use arabinose as a carbon and energy source (21).

Beside these hemeloop mutants 2 N-terminal mutants were analyzed. One of the mutants, mutant TatCXXCHAldOStrep, showed oxidase, peroxidase and coupled activity within the periplasm. This protein was further analyzed after it was expressed on a large scale, purified over a Strep-Tactin column, desalted and concentrated. The 3 L culture yielded 0.20 mg protein according to the FAD concentration determination. So 0.067 mg protein L^{-1} grown culture was yielded. This concentration is almost 40 times lower than the concentration determined by T. van den Berg and Drs. R.T. Winter in 2009 for the oxidase peroxidase fusion protein of AldO (9). The reason for this low yield is unknown. The purified protein showed on different 12 % agarose SDS-PAGE gels a fluorescent, protein or heme stained band at the same height. So a chimera was formed, TatCXXCHAldOStrep (ThAS) contains both FAD (oxidase activity) and heme (peroxidase activity).

The other N-terminus mutant, TatStrepCXXCHAldO (TShA), showed both activities within the periplasm but not within the elution fractions of the Strep-tag purification. The flow through and wash fraction of this purification did however show peroxidase activity.

From this activity assay and the analysis of the fractions on gel it was concluded that the protein to which heme was attached could not bind to the Strep-Tactin column while the protein which had not bound heme could. Hence, the functionality of the Strep-tag was influenced by the attachment of heme to the CXXCH-motif. The fraction of the protein which did bind heme could no longer be purified over a Strep-Tactin column, because it could not bind to it. So a part of the protein (the part which could not be purified) contained both FAD and heme and formed a chimera.

TatAldOStrep 232-236 and TatStrepAldO 232-236 showed higher protein expression than all of the other mutants. Although hard to estimate precisely, the protein expression appeared to be higher by a factor of 10 or more in comparison to the other mutants. These mutants did show the presence of AldO on the fluorescent stained gel were the other mutants did not. These mutants also showed a higher oxidase activity in the oxidase-peroxidase activity assay; they showed oxidase activity within a few seconds. In comparison the other mutants displayed activity after much longer time periods. The mutants did not show peroxidase activity. The reason for the high protein expression is unknown. The expression of this mutant of AldO might even be higher than the expression of wild-type Tat-AldO, but this needs to be confirmed. This mutant might be useful for whole cell biocatalysis if the expression level of this mutant is indeed higher.

The analytical analysis of Strep-tag purified, desalted and concentrated ThAS yielded two different protein concentrations of 0.17 mg/L and 0.25 mg/L grown culture for respectively the Bradford and Waddell methods. An FAD concentration of 0.067 mg/L was analyzed from the oxidised and reduced spectra of ThAS. And a heme concentration of 0.010 mg/L was determined from a pyridine hemochrome assay. The first three concentrations should be similar because only properly folded protein could be transported over the inner cell membrane to the periplasm by the Tat-sequence. AldO folds only properly if it binds FAD because FAD stabilizes the protein (7). The protein concentration determined by the FAD concentration is expected to be most accurate protein concentration because of the good results of the kinetic study, see below. The reason for the difference between the protein concentration determined by the FAD concentration determination in comparison to the Bradford and Waddell methods is unknown. The heme concentration was lower than all other concentrations which indicates that only a part of ThAS bound heme. This result agrees with the DCHBS/AAP assay which showed a lower peroxidase activity in comparison to the oxidase activity. Approximately 15.4 % of ThAS bound heme, comparing the heme

concentration with the protein concentration (determined by the FAD concentration determination).

The kinetic parameters of the oxidase activity of ThAS were determined and compared to the kinetic parameters of wild type AldO. A K_m-value of 0.35 mM and k_{cat} of 12.2 s^{-1} were measured. Both agree well with the literature values of AldO (K_m = 0.32 mM and k_{cat} = 13 s^{-1}) (2). So the kinetic parameters of the oxidase activity of ThAS are approximately the same or equal to the kinetic parameters of wild type AldO. This result was expected because the heme was attached to a mutated portion of the N-terminus leaving the active site of AldO approximately or completely untouched.

This study showed that it is possible to prepare an oxidase-peroxidase chimera of AldO if the CXXCH-motif of AldO is located within the N-terminus of AldO. Beside that it was shown that six studied mutants of AldO containing the CXXCH-motif within a loop could not bind heme or only in such a low fraction of the protein that it could not be determined. In an earlier study of T. van den Berg and Drs. R.T. Winter it was shown that a fusion protein of AldO and a microperoxidase tag could be formed (9). This suggests that heme could only be bound to the CXXCH-motif by the cytochrome *c* maturation system if the CXXCH-motif is located at one of the termini of the protein and not to a loop which is attached to the protein at both ends. This characteristic of the Ccm-system was earlier observed by Mavridou et al (2008) (4). According to Mavridou the Ccm-system needs a CXXCH-motif containing a linear peptide chain without a secondary structure to bind heme. AldO is however properly folded when it enters the periplasm, which makes it harder or even impossible for the Ccm-system to bind heme to it. Except for the region near the N- and C-terminus who are less folded into a secondary or tertiary structure because they are only attached to the protein at one end. The impossibility of the Ccm-system to bind heme to a loop of AldO might be overcome by choosing another translocation system, a system which transports the protein in an unfolded state. The used Tat-system transports only properly folded protein over the inner cell membrane (7). But this might give another problem. Van Bloois at al (2008) showed that AldO transported by the Sec-translocon, who transports protein in an unfolded state over the inner membrane, did not contain FAD after transport and lost its oxidase activity (7). So this concept might only work if AldO will be transported over the inner membrane in a less folded way but containing its FAD. But even than it might not work because Mavridou showed that the Ccm-system could only bind heme to the CXXCH-motif of linear peptide chains and not

to loops of quickly folding proteins. Quickly folding proteins are proteins which are quite stable. AldO might be a too stable protein for this purpose; it is stabilized by its FAD (7).

This project showed that it is possible to prepare an oxidase-peroxidase chimera of AldO. In the future it would be interesting and useful to prepare and investigate other types of chimera. Chimera could be applied as a biosensor, which makes compound detection within medical, pharmaceutical and environmental sciences easier. Perhaps chimeras of other enzymes could be analyzed, enzymes with non-covalently bound cofactors which could be transported over the inner membrane by the Sec-system after which they fold and bind one or more cofactors within the periplasm.

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