Towards NMR-based identification of oxygen diffusion pathways in Alditol Oxidase

Stijn Kiers (s1771159), bachelor project November 2011, Supervisors: Dr. D.W. van Bloois, Prof. Dr. Ir. M.W. Fraaije, Dr. F.A.A. Mulder.

Flavin containing proteins play an important role in many cellular processes. Recent study on oxygen utilizing flavoenzymes indicated that oxygen reaches the flavin cofactor via specific 'oxygen tunnels'. The goal of this bachelor-project is to use NMR to confirm these oxygen diffusion pathways in Alditol Oxydase (AldO). For this, triple (^{13}C, ^{15}N, D) labelled and unlabelled AldO was used. Unfortunately the purification of triple labelled AldO did not succeed, probably due to overheating during cell lysis. Therefore the NMR experiments had to be conducted with the unlabelled protein. By comparing the spectra of an atmospheric and a degassed sample, residues that were affected by the presence of oxygen could be identified. However, these residues did not match the ones found in previous research, which could not be distinguished because the spectra were too crowded. For this, triple labeled protein is needed.

Introduction

Flavin containing enzymes catalyze a wide range of reactions important in cellular processes. An example is succinate dehydrogenase (complex II), which plays a role in the citric acid cycle and the electron transport chain. Based on the different reactions they catalyze, the flavoenzymes can be divided into three major groups: monooxygenases, oxydases and dehydrogenases(1). Monooxygenases activate oxygen by forming a C4a-(hydro)peroxyflavin, which is then used to insert an oxygen atom into the substrate molecule. Oxidases and dehydrogenases oxidize the substrate molecule. For this, oxidases use oxygen (O_2) as electron acceptor, thus producing hydrogen peroxide or water, whereas dehydrogenases use other molecules such as NADH. The main reason these flavoenzymes can catalyze such a variety of reactions is because their ability to control the reactivity of their flavin cofactor with oxygen. Despite all the research done on flavins, there are still no clear rules to predict whether or how a flavoenzyme reacts with oxygen. Factors such as charge distribution, dipole-preorganization and salvation in the active site all play a role, as well as accessibility of the active site(1). It appears that nearly all oxidases of the vannilyl-alcohol oxidase (VAO) flavoprotein family(2) contain a conserved glycine or proline near the C4a atom of the flavin(3). For members of this family that do not react with oxygen contain another residue at this position. Substitution of this residue by glycine increases the reactivity with oxygen manifold, which indicates that this reside functions as a gatekeeper for oxygen. A recent study on two flavin containing enzymes, using molecular dynamics simulations, by Baron et al.(4) indicated the existence of specific oxygen diffusion pathways in the enzyme, leading to the flavin cofactor. One of the proteins studied was *Streptomyces coelicolor* A3(2) alditol oxidase (AldO), a soluble monomeric flavoprotein of 45.1 kDa(5). AldO belongs to the VAO family and, as is not uncommon for members of this family, contains an FAD cofactor covalently bound to a conserved histidine (His^{46}). In fact, sequence motif analysis on this histidine was used to identify AldO as an oxidase(5). The goal of this Bachelor-project is to confirm the existence of oxygen diffusion pathways in AldO using nuclear magnetic resonance (NMR). The residues lining these pathways can be identified using the paramagnetic line-broadening induced by their interaction with oxygen. By comparing the 2D NMR spectra, correlating the chemical shift of ^{1}H and ^{13}C spins through a ^{1}J coupling, of an atmospheric (oxygen containing) and a degassed sample, an attempt at finding these residues

could be made. Due to the lack of labelled (^{13}C, ^{15}N, D) protein, the spectra had to be recorded with unlabelled protein, which resulted in a very crowded spectrum. Therefore only two different residues could be found that seemed to play a role in oxygen diffusion; isoleucine and threonine. The residues found to play a role in oxygen diffusion by Baron et al.(4) all have resonances more to the middle of the spectrum, which was too crowded to clearly discern any separate peaks. In order to be able to say more about the behaviour of these residues a complete sequential assignment has to be conducted, for which triple labelled protein is needed.

Materials and Methods

Biochemical part

Chemicals and Enzymes

Western blotting detection reagent was from Amersham Biosciences. Horseradish peroxidase was from Fluka. All other chemicals were from Sigma-Aldrich and of analytical grade.

Strains and Plasmids

E. coli strain MC1061(6) was used as host for all plasmid constructs. The plasmids used were pBAD-MBP-AldO, which has been described earlier(5), and pBAD-AldO C-Strep and were kindly provided by Dr. D.W. van Bloois.

Evaluation of 3 different minimal media for the production of labelled protein

1. m9 minimal medium provided by Dr. D.W. van Bloois

1.1. Influence of different glycerol concentrations
Five cultures were prepared, consisting of 5 ml m9 minimal medium, according to the recipe provided by Dr. D.W. van Bloois (appendix I), with the different glycerol concentrations listed in Table 1. Ampicilin was added to a final concentration of 100 µg/ml and to the ones indicated with '+' arabinose was added to a final concentration of 0.02%. The fifth tube, the one without arabinose, functions as a blank. 50 µl cell culture was added to each of the tubes and grown as described(5).

Table 1: Glycerol concentrations per sample. '+' indicates induction with arabinose

Sample	1	2	3	4	5
Glycerol concentration	0.05%	0.1%	0.2%	0.4%	0.4%
Induction with arabinose	+	+	+	+	-

After this, the OD_{600nm} was measured to get an indication of the cell density. The cell density was used to determine the amount of cells needed to load 0.1 OD units on gel, which was used to check the protein expression using SDS-PAGE. Also, oxidase activity was checked for using a horseradish peroxidase (HRP) assay(7).

1.2. Expression in absence of glycerol
Four reaction tubes with the different combinations: glycerol and arabinose, glycerol only, arabinose only and none of both, were prepared with glycerol concentrations of 0.05% and arabinose concentrations of 0.02%. 50 µl of cell culture was added to each of these tubes and grown as described(5). Approximately 0.1 OD unit of sample was loaded on gel for analysis.

2. m9 minimal medium provided by R.T. Winter

The first experiment for the previous medium (*Influence of different glycerol concentrations)* was repeated again for the medium provided by Winter (appendix I), but this time with two blanks, one without arabinose and one without glycerol, as shown in Table 2. Another difference being, that they were grown two hours at 37°C before induction with arabinose and relocation to 17°C.

Table 2: Glycerol concentrations per sample. '+' indicates induction with arabinose

Sample	1	2	3	4	5	6
Glycerol concentration	0.4%	0.2%	0.1%	0.05%	0.05%	0%
Induction with arabinose	+	+	+	+	-	+

3. m9 minimal medium provided by N.A. Octaviani

3.1. Conditions for this medium
A total of eight tubes was prepared according to Table 3, with 5 ml medium (Octaviani, appendix I) and 100 µg/ml ampicilin. These tubes were inoculated with 100 µl cell culture and shaken 4 ½ hours at 37°C before induction with 0.02% arabinose, after which they were placed at 17°C and grown as described(5).

Table 3: the important variables per sample

sample	construct	% glycerol	solvent
1	AldO C-strep	0.2	H_2O
2	AldO C-strep	0.2	D_2O
3	AldO C-strep	0.05	H_2O
4	AldO C-strep	0.05	D_2O
5	MBP-AldO	0.2	H_2O
6	MBP-AldO	0.2	D_2O
7	MBP-AldO	0.05	H_2O
8	MBP-AldO	0.05	D_2O

3.2. Yield for AldO C-Strep
Two cultures were made, each containing 0.05% glycerol and 100 µg/ml ampicilin in 30 ml of respectively H_2O-based and D_2O-based m9 medium (Octaviani, appendix I). Both cultures were inoculated with 600 µl cell culture and shaken at 37°C. At mid-log, the cultures were induced with

0.02% arabinose and placed at 17°C to be grown and harvested as described(5). The pellet was re suspended in 100 mM Tris-HCl, pH 8 and 100 µl lysozyme was added. The cells were disrupted by sonification and centrifuged again to obtain the cell free extract (CFE). This was purified according to instructions of the supplier. All fractions, as well as the re suspended pellet were analysed on gel.

3.3. Yield for MBP-AldO

Two cultures, one with H_2O- and one with D_2O-based medium, consisting of 100 ml medium with 0.05% glycerol, 100 µg/ml ampicilin and 1 ml cell culture and were shaken at 37°C before induction with 0.2% arabinose at mid-log, after which they were placed at 17°C to be grown as described(5).

After these two nights the cells were harvested by centrifugation, the supernatant was removed and the pellet was re suspended in buffer W (50 mM KPi, pH 7.5). The cells were disrupted by sonification and centrifuged again to obtain the CFE, which was purified according to the following protocol:

~2 ml Bio-Works WorkBeads 40Q suspension was loaded into a 30 ml Bio-Rad gravity flow column, to a final volume of ~1 ml beads. The columns were first washed with demi water to remove the ethanol, and then equilibrated with minimal 5 CV of buffer W. After equilibration the column was loaded with the CFE, closed and incubated at 4°C for 1 hour under rotation. Subsequently the column was opened again and the flowtrough (FT) was collected. Then the column was washed, eluted and finally stripped in steps of 5 ml according to the scheme in Table 4. The CFE, FT, wash fractions (W1 t/m 6), elution fractions (E1 and 2) and the stripping fraction (S) as well as the re suspended pellet (I) were analysed on gel.

Table 4: purification steps for ion-exchange chromatography. Fractions are 5 ml each.

fraction		Solvent composition	
washing	W1	50 mM KPi, pH 7.5	
	W2	”	
	W3	”	+ 10 mM KCl
	W4	”	+ 50 mM KCl
	W5	”	+ 100 mM KCl
	W6	”	+ 250 mM KCl
elution	E1	”	+ 500 mM KCl
	E2	”	+ 1 M KCl
stripping	S	”	+ 2 M KCl

Final culture for analysis

1. labelled protein

1.1. Growing and expression

In order to obtain a decent amount of labelled protein, a 500 ml culture was used. In a 2½ L Erlenmeyer with 500 ml m9 medium, for which $^{15}NH_4Cl$, D_2O and 3x ^{13}C enriched glycerol were used, 100 µg/ml ampicilin and approximately 550 µl glycerol (final concentration 0.11%) was added. The culture was inoculated with 5 ml overnight culture and shaken at 37°C until mid-log, after which it was induced with 0.02% arabinose and shaken at 17°C until saturation.

After these nights the cells were harvested by centrifugation, the supernatant was removed and the pellet was re suspended in buffer W. The cells were disrupted by sonification and centrifuged again to obtain the CFE for purification.

1.2. Testing for activity
Oxidase activity of the CFE and the insoluble fraction was tested for using a HRP activity assay as described(7). However, in the case of the CFE, it was not spun down, but instead 10 µl CFE was used directly. As a positive control, 10 µl of CFE from the culture grown in TB medium was used, because this was certain to show activity.

2. Unlabelled protein

2.1. growing and expression
Three cultures, two of 0.5-liter and one of 1-liter, were prepared with 100 µg/ml ampicilin and 1% overnight culture in Terrific Broth medium. These cultures were shaken at 37°C for 3½ hours, after which they were induced with 0.02% arabinose and grown, harvested and purified as described(5, 8). However, instead of dividing the cell free extract into small aliquots, it was loaded onto the Q-Sepharose collumn for ion exchange chromatography all at once. This resulted in overloading of the collumn, therefore two portions of approximately 50 ml flowthrough was loaded on the collumn again.

2.2. Concentration determination with absorption spectroscopy
80 µl of sample was added to 800 µl of buffer (50 mM KPi, pH 7.5; 11 times dilution) and the A_{450nm} was measured. To this sample, 9 µl of 10% SDS was added to unfold the protein and the A_{450nm} was measured again. Using Beer's law and the absorption coefficient of free FAD, the concentration of protein could be calculated.

Removal of MBP by tryptic digestion

1. Finding the protein/trypsin ratio

1.1. variable incubation time
10 µl of a 0.1 µg/µl trypsin solution was added to 100 µl of protein solution. This mixture was then shaken at 37°C and samples were taken after 30, 60, 70, 80 and 90 minutes. These samples were analysed on gel using SDS-PAGE to determine the optimal incubation time

1.2. Variable concentrations (i)
Trypsine was added to 20 µl of MBP-AldO solution (0.1 mM) according to Table 5. To make sure the MBP-AldO concentration in all samples was the same, buffer (50 mM KPi, pH 7.5) was added to the mixture to a total volume of 220 µl. The experiment was conducted two times, once at room temperature and once at 4°C. After the 1 hour incubation, the reaction was quenched with SDS loading buffer and the samples were analysed on gel.

Table 5: Different trypsin concentrations per sample. Concentrations are in µg trypsin per ml MBP-AldO solution

Sample	1 (blank)	2	3	4	5	6	7	8	9	10	11	12
Trypsin concentration (µg/ml)	0.0	0.05	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0

1.3. Variable concentrations (ii)

The set-up of this experiment was the same as *(i)*, using the concentrations in Table 6. Because the trypsin stock used in this experiment was more concentrated, a total volume of 100 µl was sufficient.

Table 6: Different trypsin concentrations per sample. Concentrations are in µg trypsin per ml MBP-AldO solution

Sample	0 (blank)	1	2	3	4	5	6	7	8	9
Trypsin concentration (µg/ml)	0.0	0.5	1.0	2.0	5.0	10	15	20	30	40

2. Tryptic digestion

A total of 48 µg trypsin was added to the protein sample (13.5 ml), resulting in a trypsin concentration of 3.5 µg/ml. This was incubated at room temperature for 2 hours, and then purified as described in the next section.

2.1. Separation of MBP and AldO

Two columns with 5 ml amylose resin each were washed with demi water, equilibrated with buffer A (50 mM KPi, pH 7.5) and then loaded with approximately 7.5 ml protein solution each. The columns were then incubated at 4°C under rotation for 1 hour. After incubation the flowthrough (FT) was collected, the columns were washed two times with 5 ml buffer A and then MBP was eluted with two times 5 ml buffer B (50 mM KPi, pH 7.5 + 10 mM maltose). This procedure was repeated with the FT, after which all fractions were analysed on gel using SDS-PAGE and with Western blotting as described(9).

NMR part

Using the purified AldO solution, two samples were prepared of ~1mM AldO in 50mM KPi-buffer, pH 7.5 with ~7% D_2O + DSS. Of these two samples, one was degassed using freeze-pump-thaw cycles on a vacuum line, the other was left under atmospheric conditions. The tube containing the degassed sample was closed before it was removed from the line, thus keeping it under vacuum. For both samples an HSQC-spectrum was recorded on a Varian INOVA 600 MHz NMR spectrometer using the parameters in Table 9. This experiment correlates the chemical shift of ^{1}H and ^{13}C spins that are directly bonded and thus have a ^{1}J-coupling. The data were processed using NMRPipe and visualized with Sparky.

Table 7: the parameters used in the HSQC experiments

nt	200

dl	1 s
np	2048
at	0.128 s
ni	150
sw (^{1}H)	8000 Hz
sw (^{13}C)	12000 Hz
Carrier position (^{1}H)	4.8 ppm
Carrier position (^{13}C)	45.7 ppm
Temperature	27°C

Results and Discussion

Biochemical part

Evaluation of 3 different media for the production of labelled protein

1. m9 minimal medium provided by Dr. D.W. van Bloois

1.1. Influence of different glycerol concentrations
Because labelled carbon sources are very expensive, it is desirable to use as little as possible. Therefore it was necessary to determine the lowest glycerol concentration at which the yield of AldO is still good. To do this, five samples with different glycerol concentrations were tested for oxidase activity with a horseradish peroxidase (HRP) assay and analysed with SDS-PAGE. All samples tested positive in the activity assay, so they all contained active protein. Measurement of the OD_{600nm} (Table 7) showed an increasing cell density for lower glycerol concentrations. From SDS-PAGE (figure 1) can be concluded that protein expression was approximately equally good for all samples, including the blank. This means that the blank was probably induced with arabinose too, but can still be used to conclude that 0.05% glycerol is sufficient for good expression and cell growth.

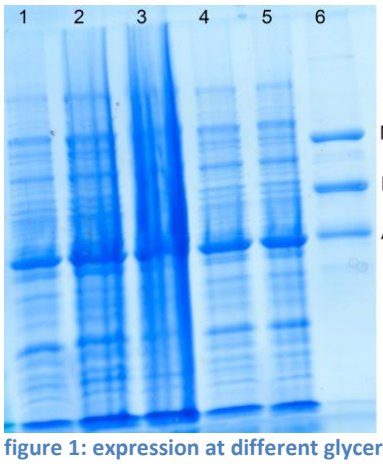

figure 1: expression at different glycerol concentrations.
The lanes are divided into the following concentrations: 1: 0.4%, 2: 0.4%, 3: 0.2%, 4: 0.1%, 5: 0.05%. Lane 1 is not induced and functions as a blank and lane 6 contains three different fluorescent proteins as a marker.

Table 8: the optical density at 600nm (OD_{600}) measured for different glycerol concentrations

Sample	0.4% (blank)	0.4%	0.2%	0.1%	0.05%
OD_{600nm}	1.56	1.58	1.78	1.87	1.99

1.2. Expression in absence of glycerol

Because the cell growth and expression on this medium were quite high, suspicion arose that other medium components in addition to glycerol and ammonium chloride (NH_4Cl) were used for protein expression. Therefore the expression was tested in presence and absence of glycerol and arabinose.
The samples were analysed using SDS-PAGE (figure 2). It is clearly visible that without induction with arabinose there is no expression. On the other hand, for the samples that were induced the glycerol concentration makes no difference for the expression of AldO C-Strep. This means that, as suspected, other medium components than glycerol and NH_4Cl were used for protein expression. This will result in mostly unlabelled protein, which is unusable for assigning the resonances in the NMR-experiment and a waste of money. Therefore it can be concluded that this medium is too rich to use for the expression of labelled protein.

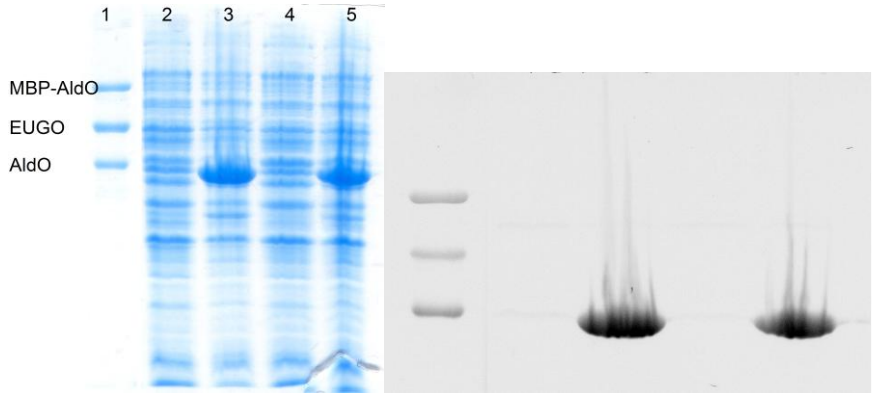

figure 2: the influence of arabinose and glycerol on expression of AldO C-Strep.
On the left gel, lane 1 contains three fluorescent proteins as a marker. The samples in lane 2 and 3 contain 0.05% glycerol and those in lane 4 and 5 0%. Of these samples, 3 and 5 were induced with glycerol, while 2 and 4 were not.
On the right, a picture of this gel is shown, showing the fluorescence when exposed to UV light.

2. m9 minimal medium provided by R.T. Winter

Because the medium provided by Dr. D.W. van Bloois was to rich, another, poorer, medium was required. A recipe for such a medium was kindly provided by R.T. Winter. For this medium the optimal glycerol concentration had to be determined again, so the same experiment as for the previous medium was repeated again, but this time with two blanks, one without arabinose and one without glycerol. However, after two days of shaking at 17°C all samples were still completely clear. Unless there was some mistake in the preparation of the medium, this means that this medium was too poor for *E. coli* to grow upon.

3. m9 minimal medium provided by N.A. Octaviani

3.1. conditions for this medium

With the second medium being too poor, another recipe had to be found. This was kindly provided by N.A. Octaviani, who had used this recipe before to make labelled protein. Again it is necessary to test the conditions for this medium. In addition to the glycerol concentration, the solvent was varied to. This is because the protein grown with H_2O-based medium could be useful in case the yield of the triple labelled

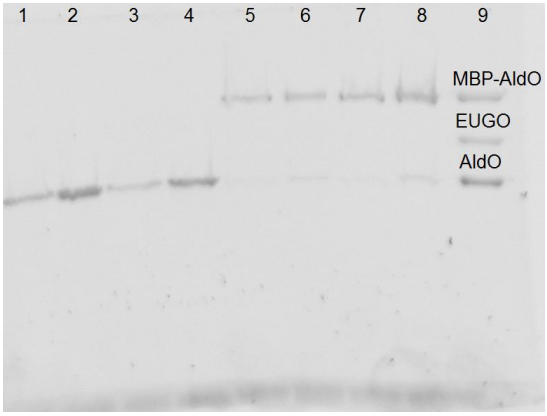

figure 3: results for different growing conditions.
This picture shows the fluorescence when exposed to UV light. Lane 9 contains three fluorescent proteins as a marker. The other lanes are divided as follows:
1: AldO C-Strep, 0.2% glycerol, H_2O based medium
2: AldO C-Strep, 0.2%, D_2O
3: AldO C-Strep, 0.05%, H_2O
4: AldO C-Strep, 0.05%, D_2O
5: MBP-AldO, 0.2%, H_2O
6: MBP-AldO, 0.2%, D_2O
7: MBP-AldO, 0.05%, H_2O
8: MBP-AldO, 0.05%, D_2O

protein would turn out to be too low. In addition to this, two different constructs, AldO C-strep and MBP-AldO, were used. All samples were analysed with SDS-PAGE to find the conditions that would give the best yield.
From the gel (figure 3) it appears that the D_2O-based medium in combination with the AldO C-strep construct gave the best yield. This is surprising, because the yield for the H_2O-based medium was suspected to be higher because of kinetic isotope effects. This result might be due to the late induction of the H_2O-based medium compared to the D_2O-based one.

3.2. Yield for AldO C-Strep
In order to find the size the culture should have to be able to obtain enough protein for NMR, a small scale purification experiment was performed, using Strep-Tactin Sepharose as column material. Unfortunately, most of the protein did not bind to the column, so somehow the Strep-tag did not bind. Therefore the experiment will be repeated with the MBP-AldO construct.

3.3. Yield for MBP-AldO
MBP-AldO was purified by ion exchange chromatography using Q-Sepharose and gravity flow. The results were analysed using SDS-PAGE, but the fractions were hardly visible on gel. What could be seen is that the flowtrough still contained quite a lot of protein, which means that the protein did not bind to the column very well. Also, the elution fractions still contained lots of impurities, so in order to get better results in the final purification experiment, the Äkta purifier will be used, with, if necessary, multiple purification steps.

Purification of AldO

The previous experiment demonstrates that, in this case, ion exchange chromatography is not very efficient. However, by using the Äkta purifier and by purifying the flowthrough and/or washing fractions again the efficiency should increase. Therefore this method was chosen for the purification of MBP-AldO. Because of limited time, it was decided that, in addition to labelled protein, also unlabelled protein would be produced, using the method described by Heuts et al.(5) If the purification of labelled protein would somehow not succeed, this unlabelled protein could be used instead.

1. labelled protein

Because the insoluble fraction was yellow coloured and the CFE missed this yellow colour, suspicion arose that the protein was in the insoluble fraction instead of in the CFE. To test whether this was true, the oxidase activity of both fractions was tested using a HRP activity assay, as described(7).
In this assay the CFE showed no oxidase activity, so the CFE contained no (active) MBP-AldO and was therefore disposed of.
The insoluble fraction also showed no activity. This means that the cells were completely lysed, but that this fraction also contained no active protein. Most probably the sample overheated during sonification and the protein was denaturated.
Because of this lack of active labelled protein, unlabelled protein had to be used for further experiments.

2. unlabelled protein

In order to be able to get any results with NMR with unlabelled protein, a 1 mM protein solution is needed. For this reason, a lot of protein had to be produced. For this, two 0.5-liter and one 1-liter culture were grown, harvested and purified as described(5).

2.1. ion exchange chromatography

MBP-AldO was purified by ion exchange chromatography as described by Heuts et al.(5), using a Q-Sepharose collumn on a Äkta purifier. Due to overloading of the collumn it was necessary to run two more purification rounds, each with 50 ml of flowthrough. From the second and third step, the fractions with absorption at 280 nm were analyzed on gel to select those that contained MBP-AldO. In figure 4, the results from the second and third step are shown. From the second step fractions 19, 20, 21 and 22 were selected for further use, together with fraction 40 from the third step and the pure fractions from the first step, which were not tested on gel.

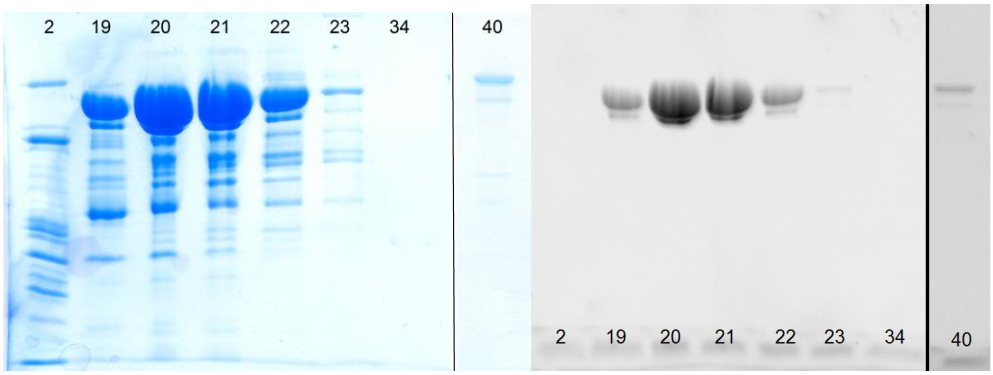

figure 4: results of the second and third purification step using ion exchange chromatography. Both were performed on combined flowthrough fractions of the first step. Fractions 2 – 34 are elution fractions of the second step and fraction 40 is an elution fraction of the third step. On the right, a picture of this gel under UV light is shown, showing the absorption of the FAD in MBP-AldO.

2.2. size exclusion chromatography

In the ion exchange chromatography purification, MBP-AldO was eluted with a high concentration KCl. Tis means that the KCl concentration in the purified protein solution is also very high. This is not good for the protein stability, therefore the sample has to be desalted.

Desalting was done as described(5), using two HiPrep 26/10 desalting collumns in series, allowing for portions of 25 ml without overloading the collumn. The protein containing fractions were selected using the absorption at 280 nm and analysed on gel. Because the gel was overloaded it is not possible to see whether the amount of eluted protein was the same as the amount of injected protein, therefore this gel is not shown. However, the conductivity meter in the FPLC machine showed that the samples were successfully desalted.

2.3 Concentration determination with absorption spectroscopy

As said before, a solution of approximately 1 mM AldO is needed for spectroscopy. In order to see whether the purified MBP-AldO sample was concentrated enough to continue with, the concentration was determined using the absorption at 450 nm. The protein concentration can then be calculated using Beer's law: $A=\varepsilon \cdot l \cdot c$, with 'A' the absorption, 'ε' the absorption coefficient, 'l' the path length of the light and 'c' the concentration of the sample.

Table 9: the absorption at 450nm (A_{450nm}) of the folded protein (sample) and the protein unfolded with 10% SDS

	A_{450nm}
Sample	0.099261
Sample + 10% SDS	0.10962

In Table 8, the A_{450nm} of the folded protein and the protein unfolded with 10% SDS is shown. For free FAD, the absorption coefficient ε is 11.3 mM cm^{-1}. This means that the concentration of the (11x diluted) sample is 9.701 µM, thus the concentration of MBP-AldO is 0.107 mM. Therefore the yield from 2 liter culture is approximately 70 mg AldO, which is much lower than the 350 mg per liter culture achieved by Forneris et al.(8). This is probably because the yield of the ion exchange chromatography steps was really low. Especially in the first step a lot of protein was lost in the flowthrough and washing fractions.

Removal of MBP by tryptic digestion

1. Finding the protein/trypsin ratio

In order for the protein to be useful in NMR spectroscopy, MBP had to be removed. This was done by tryptic digestion with sequence grade modified trypsin. Because the only part that had to be cut was the MBP-AldO linkage and not AldO itself, the right ratio of protein/trypsin had to be found.
To do this, a protein sample containing 0.01 µg/µl trypsin was incubated at 37°C. Samples taken at different incubation times were analysed on gel and showed no MBP-AldO band anymore. However, in addition to the AldO band some other bands had appeared. This means that although the MBP-AldO linkage had been completely digested, the proteins had then been digested even further. This means these conditions are not usable; therefore a new experiment was conducted varying the trypsin concentration with a set incubation time of 1 hour.

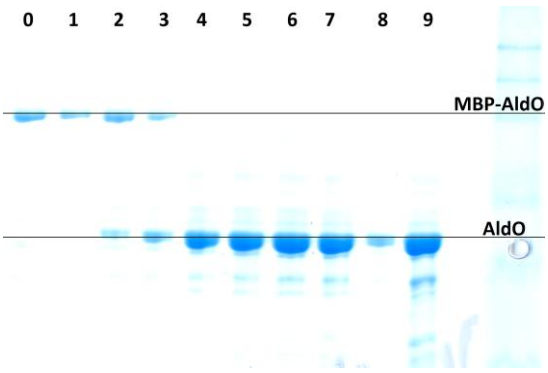

figure 5: optimisation of the trypsin concentration. 1 hour incubation at room temperature with variable trypsine concentrations: 0: no trypsin, 1: 0.5 µg/ml, 2: 1 µg/ml, 3: 2 µg/ml, 4: 5 µg/ml, 5: 10 µg/ml, 6: 15 µg/ml, 7: 20 µg/ml, 8: 30 µg/ml, 9: 40 µg/ml.

The first concentrations tested were too low, even with the highest trypsine concentration MBP-AldO was only slightly cut. Therefore this experiment was repeated with higher concentrations. The results from this experiment are shown in figure 5. This time the concentrations were high enough to cut MBP-AldO completely, but at the higher concentrations, especially 9: 40 µg/ml, digestion has gone too far and MBP or AldO is digested further. To prevent this from happening, the lowest concentration at which MBP-AldO was fully cut, 4: 5 µg/ml, was chosen for continuing the production of AldO with.

2. Tryptic digestion of MBP-AldO

Because of a shortage of trypsin, less than the optimal amount, determined in the previous experiment, was used for cutting MBP-AldO. To ensure that the protein would still be fully cut, the incubation time was doubled.
After digestion, the MBP/AldO-mixture was directly loaded on the collumns for seperation, so it has not been analysed on gel. However, after purification there is no MBP-AldO present in any of the fractions (see figure 6), so it can be concluded that the digestion was a success.

2.1. Separation of MBP and AldO
MBP and the remaining MBP-AldO was separated from AldO using a gravity flow column with amylose resin. The resulting fractions were analysed using SDS-PAGE and Western blotting.

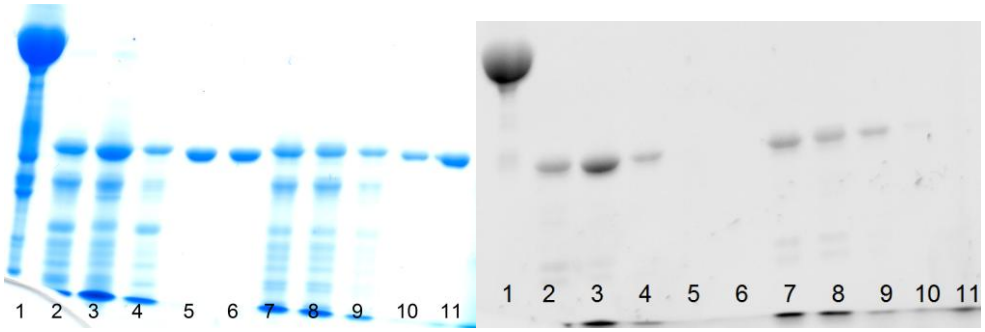

figure 6: results of tryptic digestion of MBP-AldO.
On the left the coomassie blue stained gel is shown and on the right the fluorescent picture of this gel. The lanes are divided as follows: lane 1 contains the undigested protein, lanes 2, 3, 4, 5 and 6 contain respectively the flowthrough, two washing steps and two elution steps of the first purification step, lanes 7, 8, 9, 10 and 11 contain these fractions of the second step in the same order.

The results from SDS-PAGE are shown in figure 6. In the purification fractions is no MBP-AldO present. This means that the MBP-AldO linkage was completely digested. Also, from the fluorescent picture can be seen that the elution fractions (5, 6, 10 and 11) are free of AldO, so these bands are pure MBP. For the other fractions it is not possible to tell whether the fractions contain MBP or not, because MBP and AldO are almost equally in size. In order to be able to use AldO for NMR-spectroscopy, it has to be pure. Therefore Western blotting was used as described before(9) to determine which fractions contained MBP. On this blot (figure 7) the coloured bands are those of MBP (or digestion products of MBP). Using this it can be seen that all AldO containing fractions are free of MBP. Even the first flowthrough was as good as MBP free, thus the second purification step would probably have been unnecessary. Therefore it is strange to see that the elution fractions of the second purification step contain MBP. This might be because the collumns were not completely stripped before they were reused. Using these results, fractions 1, 2, 3, 6, 7 and 8 (figure 7) were pooled and concentrated to be used for NMR.

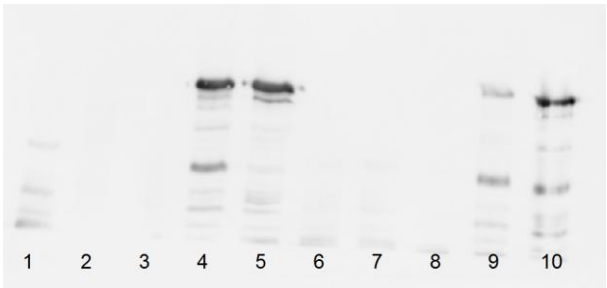

figure 7: Western blot with antibodies against MBP.
Lanes 1, 2, 3, 4 and 5 contain respectively the flowthrough, two washing steps and two elution steps of the first purification step, lanes 6, 7, 8, 9 and 10 contain these fractions of the second step in the same order.

NMR part

The HSQC spectra (appendix II) were a complete mess due to the number and width of the peaks. Still there are some areas of interest. These are shown in figure 8 and 9. In addition to the acetone peak, found only in the degassed sample, there are some other peaks that appeared only in this sample and some that are noticeably smaller. An attempt was made to assign these extra peaks to certain types of amino acid. These amino acid types were compared to the amino acids found to be involved in O_2 diffusion by Baron et al.(4)

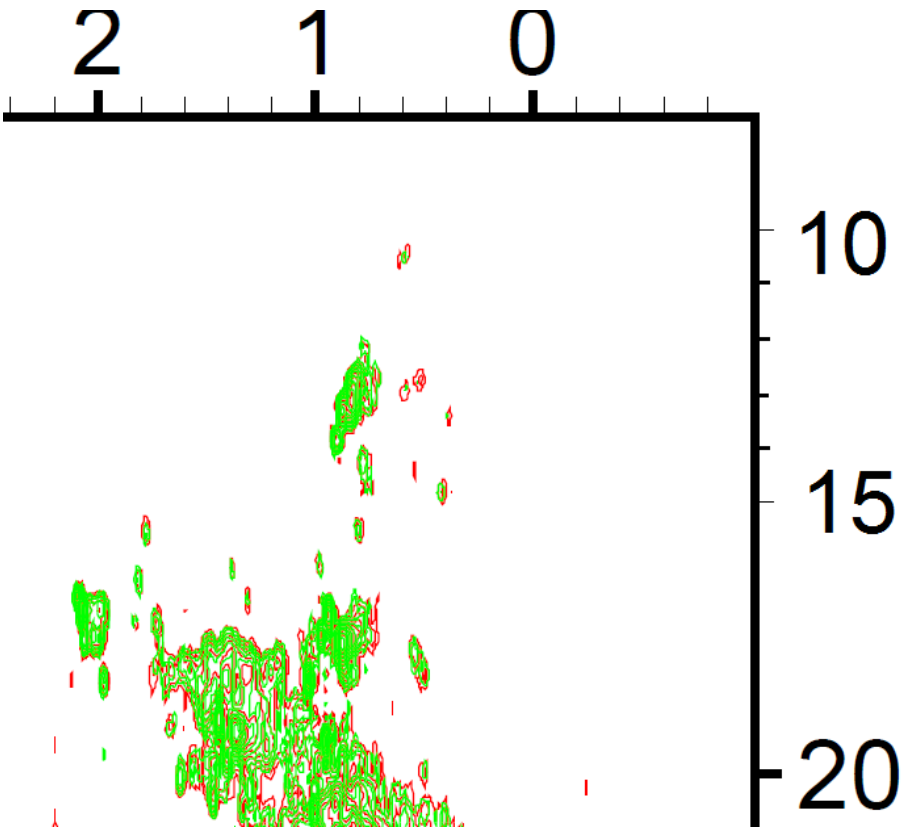

figure 9: Zoom of the top-right corner of the combined HSQC-spectra of the atmospheric (green) and the degassed (red) samples. On the horizontal axis the δ 1H (ppm) and on the vertical axis the δ 13C (ppm).

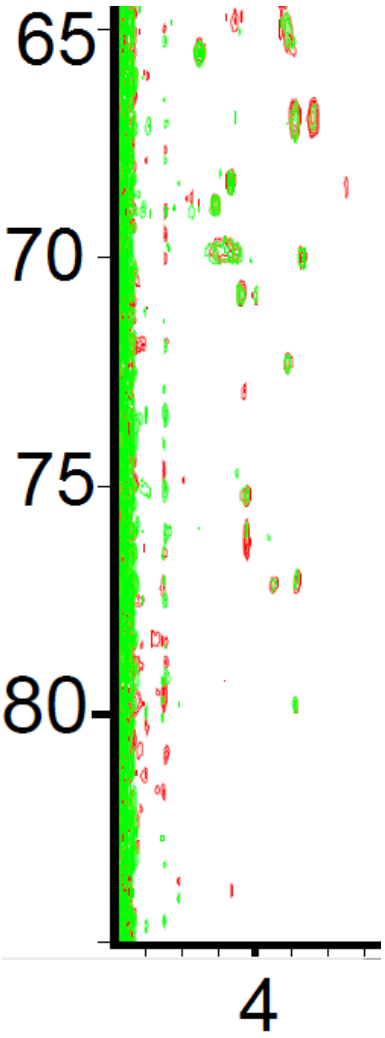

figure 8: Zoom of the bottom-left corner of the combined HSQC-spectra of the atmospheric (green) and the degassed (red) samples. On the horizontal axis the δ 1H (ppm) and on the vertical axis the δ 13C (ppm).

assignment of the extra peaks

Because the production of triply labelled AldO failed, this experiment had to be conducted using the natural abundance of NMR-active isotopes in unlabelled protein. Therefore it was impossible to perform the 3D-experiments required for assignment of all the resonances. However, some amino acids have resonances in very specific areas of a 2D ^{1}H-^{13}C correlation spectrum. An attempt to assign the extra peaks in these regions is done using the assignment charts in chapter 10 of Cavanagh(10). The extra peaks in the top-right corner (figure 9), around (0.6;13), can only be ascribed to the Cδ of an isoleucine, because this is the only methyl group with such low chemical shifts for both ^{1}H and ^{13}C. In the bottom-left corner (figure 8) there is a peak at approximately

(4;65), which could be assigned either to the Cα of a valine, threonine or isoleucine, or to the Cβ of a serine. To be sure which amino acid this is, a more complete NMR analysis of the protein is needed, which is impossible with these samples. A bit lower, at a ^{13}C chemical shift of approximately 73 ppm, there is another peak that has disappeared in the atmospheric sample. This could be the Cβ of a threonine, but actually these shifts are too high. It is impossible to assign the peaks even further downfield.

Comparison with article

In the article(4) Baron et al. mention the amino acids at the cavity: *"All protein-guided diffusion pathways converge into a site defined by Ala-105, Tyr-87, and Thr-120 at 6 Å from the re-side of FADH."* and one remarkable hydrophobic cavity on the surface of AldO: *"A remarkable example is the all-Ala pocket of Ala-88, Ala-91, and Ala-277 used to capture O_2 molecules from the bulk solvent."* In the recorded spectra it is impossible to see if the resonances change for most of these amino acids. Therefore these results cannot be compared to those found by Baron et al., except for the threonine. But, for this threonine it impossible to say if it is indeed the Thr-120 mentioned in the article. The only way to find out is to perform a complete sequential assignment using triply labelled AldO. This is also necessary for seeing if the alanine peaks and Tyr-87 disappear, because these peaks are in the most crowded part of the spectrum.
However, what can be said is that some peaks do disappear under atmospheric conditions, which is probably due to some interaction with oxygen.

Conclusion

Unfortunately purification of AldO C-Strep did not succeed for three different media tested, therefore a construct for the expression of a fusion protein consisting of maltose binding protein (MBP) and AldO, MBP-AldO, was used. Although the purification of unlabelled protein worked for this construct, the purification of triple labelled protein did not. In order to be able to produce enough protein for NMR, it was decided to produce a large amount of unlabelled protein using Terrific Broth (TB) medium as described before(5, 8).
With this protein two NMR-spectra were recorded, one being under atmospheric conditions and the other degassed, correlating the chemical shift of ^{1}H and ^{13}C spins through a ^{1}J coupling. When compared to that of the atmospheric sample, the spectrum of the degassed sample clearly shows some extra resonances. This means that a gas, probably oxygen, interacts with certain residues in protein and causes their resonances to disappear due to paramagnetic line-broadening. However, these residues are not the ones found by Baron et al.(4) to play an important role in the binding of oxygen to the flavin cofactor. The residues found by Baron et al. have resonances in the middle of the spectrum. With this sample it is impossible to study these residues, because the resonances are too broad, making the spectrum too crowded to be of use. Also, in order to be able to see whether the resonances of the residues described really change, it is necessary to know which resonances correspond to which residue. For this, a complete sequential assignment is needed, which only can be done using triple labelled protein.

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Appendix I: Recipes

Recipe for m9 minimal medium provided by Dr. D.W. van Bloois:

5x m9 salts (in 1000 ml):

- 64 g $Na_2HPO_4 \cdot 7H_2O$
- 15 g KH_2PO_4
- 25 g NaCl (here I misread the recipe, it should be 2.5 g)
- 5g NH_4Cl

1x m9 medium (total: 1000 ml):

- 200 ml 5x m9 salts
- 2 ml 1M $MgSO_4$
- 100 µl 1M $CaCl_2$
- 20 ml 1% vitamin B_1
- 50 ml 20% Hy-case Amino

Recipe for m9 minimal medium provided by R.T. Winter:

5x m9 salts (in 1000 ml):

- 33.8 g Na_2HPO_4
- 15 g KH_2PO_4
- 2.5 g NaCl
- 5 g NH_4Cl

1x m9 medium (total: 1000 ml):

- 200 ml 5x m9 salts
- 25 ml 4 mg/ml leucine
- 2 ml 1M $MgSO_4$
- 100 µl 1M $CaCl_2$
- 0.1 mg thiamine

Recipe for m9 minimal medium provided by N.A. Octaviani:

10x m9 salts (in 1000 ml):

- 70 g Na_2HPO_4
- 30 g KH_2PO_4
- 5 g NaCl

vitamin mix (in 7 ml):

- 1 mg folic acid
- 1 mg choline chloride
- 1 mg nicotinamide
- 1 mg D-pantothenic acid
- 1 mg pyridoxal·HCl
- 0.1 mg riboflavin

- 2 mg inositol

1x m9 medium (total: 1000 ml):

- 100 ml 10x m9 salts

- 20 mg thymidine (T)
- 20 mg adenosine (A)
- 20 mg guanosine (G)
- 20 mg cytidine (C)
- 20 mg thiamine
- 20 mg biotin

- 1 ml 10 mM $FeCl_3$
- 2 ml 1M $MgSO_4$
- 1 ml 50 mM $MnCl_2$
- 2 ml 50 mM $CaCl_2$

- 100 µl 20 mM ZnAc
- 100 µl 4 mM $CoCl_2$
- 100 µl 4 mM Na_2MoO_4
- 100 µl 4 mM $CuCl_2$
- 100 µl 4 mM $NiCl_2$

- 7 ml vitamin mix

- 1.0 g NH_4Cl (in case of labelled medium: $^{15}NH_4Cl$)

Terrific Broth (TB)

For 2 L Terrific Broth (TB) medium:

Nutrients:

- Yeast extract 48 g
- Tryptone 24 g
- Glycerol 85% 10 ml
- Demi water 1800 ml

Salts:

- KH_2PO_4 4.63 g
- $K_2HPO_4 \cdot 3\ H_2O$ 32.87 g
- Demi water 200 ml

Autoclave nutrients and salts separately, then add together after cooling.

Spectrum: degassed_AldOhsqcC13_obj_130711
User: alia Date: Wed Jul 20 10:49:57 2011
Positive contours: low 3.00e+04 levels 10 factor 1.40
Negative contours: low -3.00e+04 levels 10 factor 1.40