Characterization of the catalytic activity of propanediol oxidoreductase (FucO) mutants

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Abstract

FucO is an alcohol dehydrogenase class III from Escherichia coli that naturally converts (S)-lactaldehyde into (S)-1,2-propanediol with NADH as a cofactor. It is an efficient catalyst for short chained substrates, but works poorly for bulkier substrates. In an attempt to produce a biocatalyst for stereoselective conversion of aromatic diols to aldehydes, a library with FucO mutants had previously been developed and screened for activity with phenylacetaldehyde. The aim of this project was to characterize the catalytic properties of some of the hits from that screening. Steady-state kinetic parameters were determined for four different mutants ([N151G], [N151T], [T149S, N151V] and [T149S, N151W]) with 1-propanol, (S)-1,2-propanediol, (R)-1,2-propanediol and phenylacetaldehyde. The result showed that two of the mutants, [N151T] and [T149S, N151V], had a tenfold increase in the $k_{cat}/K_M$ value compared to wt FucO together with phenylacetaldehyde while the [N151G] mutant had a 100-fold increase in the $k_{cat}/K_M$ value. All mutants showed a decrease in activity with (S)-1,2-propanediol and (R)-1,2-propanediol compared to the wild-type enzyme, while the activity with 1-propanol basically remained the same for the mutants as compared to wild type enzyme.

In addition to determining steady state kinetic parameters, the purification protocol for wt FucO needed to be optimized. The His-tagged enzyme was purified through affinity chromatography on a Ni$^{2+}$ column, but unfortunately a lot of FucO was lost during one of the washing steps. By changing the composition of the washing buffer, it was possible to elute unspecifically bound proteins without losing as much FucO.

Introduction

Biocatalysis

Catalysts are important to a majority of the processes in the chemical industry. The importance of good catalysts can be illustrated by the most recent choice of Nobel prize winners in chemistry (2010), namely Akira Suzuki, Ei-ichi Negishi and Richard F. Heck, for the development of palladium catalyzed cross coupling. In addition to the traditional catalysts used in organic chemistry, biocatalysts are becoming increasingly interesting. A biocatalyst could be an isolated enzyme or a whole cell. For example, humans have been using yeast as a biocatalyst for thousands of years in the production of alcohol. Although whole cells are interesting as biocatalysts, this project is focused on isolated enzymes.

Enzymes have been developed though millions of years of evolution, and it is possible to find enzymes with a wide range of properties. What makes them interesting as biocatalysts are properties such as high catalytic efficiency, high substrate specificity, high regioselectivity and perhaps most important, high enantioselectivity. These qualities could be very helpful for organic chemists, especially in the production of enantiomerically pure chemicals. In some instances, it would also be preferable to use enzymes over traditional methods from an environmental point of view since traditional methods often require high temperatures and leave heavy metals and organic solvents as a waste. Enzymatic reactions on the other hand take place in aqueous solutions at lower temperatures.
Unfortunately, there are several problems with using enzymes as catalysts, which is why they are not very widespread on an industrial scale. A major problem is that every reaction may need a specific enzyme, and to find a naturally occurring enzyme that catalyzes a specific reaction with high efficiency and specificity could be like looking for a needle in a haystack. It might also be that such an enzyme does not exist. A second major problem is that enzymes often are too unstable to be used in industrial processes.

But there are methods to create enzymes with new properties, and the technique of directed evolution has become the most important tool in order to produce customized enzymes. Directed evolution is in many ways mimicking the principles of natural evolution, where mutations in the DNA give rise to new properties in an organism. If the new property is beneficial for the organism, the mutation is likely to be passed on to future generations. In directed evolution of enzymes, a library of mutants is generated and screened for desired properties. Interesting mutants from the first library may be mutated further; generating new libraries (the mutation is so to speak passed on to future generations). The different methods of mutagenesis were made possible by the huge development of recombinant DNA-techniques in the 1980’s, and today there are established methods both for site-directed mutagenesis and random mutagenesis.

**Enzyme kinetics**

In enzyme kinetics, the rates of chemical reactions that are catalyzed by enzymes are studied. Enzyme kinetics is important in many ways; it can for example be used as a tool to obtain information about the mechanism behind enzyme catalysis. But it can also be used in order to get an idea about the catalytic activity of an enzyme, which is the purpose of this project. The most basic way to describe the behavior of an enzyme is by means of the Michaelis-Menten equation (eq. 1), by determining the steady-state kinetic parameters $K_M$, $k_{cat}$ and also $k_{cat}/K_M$.

\[
v_0 = \frac{[E]_0[S]k_{cat}}{K_M + [S]} \quad \text{(eq. 1)}
\]

$v_0 =$ initial rate, $[E]_0 =$ total enzyme concentration, $[S] =$ substrate concentration

$K_M =$ the Michaelis constant, $k_{cat} =$ the catalytic constant

The Michaelis-Menten equation describes how the initial rate, $v_0$, of a reaction varies with substrate concentration. This requires that only the initial rate is measured, so that the change in substrate concentration is so small that $[S]$ can be considered to be constant. Also, by just measuring the initial rate of the reaction only a small amount of product will have time to form, which is preferable since the product for example might give rise to inhibition effects. The equation can be derived from the scheme in figure 1, which was proposed by Michaelis and Menten in 1913, by assuming that the ES complex is at steady state (which is why the study of initial rates as a function of substrate concentration might be referred to as steady state kinetics). This means that the rate of formation of ES approximately equals the rate of destruction of ES. To do the derivation one also needs to use the relationship $v_0 = k_2*[ES]$, which describes that the rate of the reaction is dependent of the conversion of ES into enzyme + product. A derivation will not be performed here, but it would show that $K_M$ is defined as $(k_1 + k_2)/k_{cat}$, and that $k_{cat} = k_2$. 
Many enzymes give rise to a behavior that follows the Michaelis-Menten equation although they have a completely different mechanism. Then $K_M$ and $k_{cat}$ usually do not follow the original definitions; they can for example be composed of complex combinations of rate constants from intermediate steps. Although it is impossible to know what $K_M$ and $k_{cat}$ exactly are without knowing the catalytic mechanism, $K_M$ is usually thought of as an apparent dissociation constant. In other words, $K_M$ tells us something about the affinity between the enzyme and the enzyme bound species. $k_{cat}$ describes the limiting rate at saturation. It is also called the turnover number, because it is equivalent to the maximum number of substrate molecules converted into product per active site per unit time. $k_{cat}/K_M$, or the specificity constant, is also used as a catalytic parameter. This is an apparent second order rate constant which describes the behavior and reactions of free enzyme and free substrate.

The project

The aim of the research project that I have been a part of is to develop an enzyme catalyzed synthesis pathway of hydroxyl-substituted hydrofuranes from chiral epoxides (fig. 2). In the first step of the pathway an epoxide hydrolase is used to convert an epoxide to a vicinal diol, which in the second step is converted into a 2-hydroxyaldehyde by a diol dehydrogenase. An aldolase is then used to condense the generated 2-hydroxyaldehyde with acetic aldehyde, giving a diol aldehyde derivative that in a spontaneous cyclization step will give rise to a hydrofurane product. This reaction was found to be interesting because it is provides an example of when enzymes could be used in order to synthesize an enantiomerically pure product. The development of this pathway would thus work as a proof of principle. The group is currently trying to optimize the epoxide hydrolase and the diol dehydrogenase for this reaction by protein engineering. I have been working with the optimization of the diol dehydrogenase.

Propanediol oxidoreductase from *Escherichia coli* (*E.coli*) was chosen as a starting point for the conversion of the diol to 2-hydroxyaldehyde. Propanediol oxidoreductase is also called FucO since it is encoded by the *fucO* gene. FucO is a class III alcohol dehydrogenase that is believed to be
dependent on iron for its function\textsuperscript{8,9}. It plays a part in the metabolism of L-rahmnose and L-fucose under anaerobic conditions, where it converts (S)-lactaldehyde into (S)-1,2-propanediol with NADH as a cofactor\textsuperscript{8,10,11}. It is also capable of converting (S)-1,2-propanediol to (S)-lactaldehyde in the presence of NAD\textsuperscript{+}, which makes it interesting for this project\textsuperscript{10}. The enzyme is highly stereoselective, with 320 times higher activity with (S)-1,2-propanediol than with (R)-1,2-propanediol\textsuperscript{12}. It is also regiospecific and only catalyzes the oxidation of primary alcohols\textsuperscript{12}. It efficiently catalyzes the conversion of short chained substrates, but is a poor catalyst for bulkier substrates. Since it is of interest to use aromatic diols in this reaction, protein engineering was used in order to manipulate the substrate specificity for the enzyme, hopefully giving rise to mutants with the ability to stereoselectively convert aromatic diols to aldehydes.

The crystal structure of FucO has been determined\textsuperscript{9}, which is important since the structure reveals essential information and might be a crucial help in the planning of directed-evolution experiments. The structure of FucO shows that the entrance to the active site is quite narrow, which might explain why the enzyme only can use short chained substrates but not bulkier ones (fig 3). There are six amino acids creating the narrow “waist” that is restricting the entrance to the active site, and three small libraries were created on basis of this information. In one of the libraries, named Library A, two of the six amino acids were targeted by mutagenic PCR. More specifically, Thr149 could be exchanged for Ser, Ala or Thr, while Asn151 could be exchanged for any of the 20 amino acids. The library was screened by first cultivating the transformed bacteria in 96-well plates and inducing overexpression of FucO. The cells were then lysed and the crude lysate was screened for activity with 1-propanol, propanal, (S)-1,2-propanediol, (R)-1,2-propanediol and phenylacetaldehyde. The reason for using phenylacetaldehyde in the screen, even though the aim of the project is to create an enzyme that converts vicinal diols to 2-hydroxyaldehydes, is that the conversion from aldehyde to alcohol has a higher catalytic activity and therefore it should be easier to find hits by using the aldehyde in the screen.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3.png}
\caption{FucO in complex with adenosine-5-diphosphate, (S)-1,2-propanediol and Zn\textsuperscript{2+}, which is replacing Fe\textsuperscript{2+} in this crystal structure. The pictures were created in PyMol from the pdb file 1RRM a) The native dimeric form. The zinc ion is represented by the red sphere and the ligands are represented by the blue/yellow sticks. b) A close up of the narrow waist. The light blue molecule represents (S)-1,2-propanediol, the red sphere represents the zinc ion and the red/orange molecule represents adenosine-5-diphosphate. The sticks colored in orange and green represent the amino acids that are creating the waist, where the amino acids colored in green represent T149 and N151. These are the amino acids that are mutated in Library A.}
\end{figure}
The result from the Library A screen showed three “clusters” of enzymes with distinctive catalytic activities (fig. 4). One cluster showed wild-type activity, that is, the mutants had not acquired activity with phenylacetaldehyde. Another cluster showed high activity with phenylacetaldehyde but decreased activity with (S)-1,2-propanediol and the third cluster showed high activity with both phenylacetaldehyde and (S)-1,2-propanediol. My role in this project was to characterize the hits from the cluster that showed high activity with phenylacetaldehyde but decreased activity with (S)-1,2-propanediol. This cluster is interesting for several reasons. It contains the hits that in this screen had the highest detected activities with phenylacetaldehyde. It is also interesting that the activity with (S)-1,2-propanediol has changed. This means that the targeted amino acids actually may be contributing to the substrate specificity, which is information that may be important in the design of new generations of libraries. The mutants in this cluster were [N151G], [N151T], [T149S, N151V] and [T149S, N151W]. To characterize them, the $k_{cat}$, $K_M$ and $k_{cat}/K_M$–values for 1-propanol, (S)-1,2-propanediol, (R)-1,2-propanediol and phenylacetaldehyde (fig. 5) were determined.

Figure 4 – Result from the screen with mutants in library A. Results are presented in a principal component analysis biplot displaying both the score and the loading plot. Yellow – wild type FucO. Blue – inactive control. Purple – Library A clones. The mutants that are going to be characterized in this project are shown in the blue circle.
Although the catalytic characterization of the FucO mutants is the main goal of this project I will also optimize the purification protocol of wt FucO. Currently, the His-tagged enzyme is purified through affinity chromatography with a Ni\(^{2+}\)-column. During one of the washing steps, a buffer containing 100 mM imidazole is used in order to elute loosely bound proteins. Unfortunately, a lot of FucO is lost during this washing step. By lowering the imidazole concentration to 60 mM, it is hopefully possible to wash out unwanted proteins without losing as much FucO. The optimized purification protocol for wt FucO will also be used in the purification of FucO mutants.

**Methods**

**Bacterial cultures**

*E. coli* XL1 Blue had previously been transformed with plasmids containing the genes for the desired FucO-mutants. The mutants were grown on agar plates, and over day cultures were made by inoculating 1 ml 2TY-medium containing 100 µg/ml ampicillin with a single colony from the plate. The cultures were left to grow in a shaker at 30 °C for approximately six hours. These over day cultures were then transferred to 35 ml 2TY-medium containing 100 µg/ml ampicillin. The cultures were left to grow over night in a shaker at 30 °C. The following morning, 5 ml of overnight culture was transferred to 500 ml 2TY-medium containing 50 µg/ml ampicillin. The cultures were allowed to grow until the OD\(_{600}\) had reached a value of about 0.3 and they were then induced by adding IPTG to a final concentration of 1 mM. Since FucO is an iron-dependent protein, also Fe(II)Cl\(_2\) was added to a final concentration of 100 µM. The cultures were then left over night in a shaker at 30 °C. The bacteria were harvested by centrifugation at 5000 rpm, 4 °C for 12 min. The pellets were stored at -80 °C.

**Purification of FucO**

This purification protocol was used for both wt FucO and the mutant forms of FucO. A pellet from 500 ml bacterial culture was thawed on ice and resuspended in 10 ml binding buffer (20 mM imidazol, 20 mM NaP, 0.5 M NaCl) containing complete, Mini, EDTA free (Roche). To disrupt the cells, sonication on ice with output=7, pulse =90% for 20 seconds was performed. This was repeated another three times, with a couple of minutes in between in order to keep the lysate cold. The lysate was then centrifuged at 15000 rpm, 4 °C for 35 min.

The supernatant was collected, filtered through a 0.2 µm filter and then loaded with a flow rate of 0.5 ml/min onto a 1 ml HisTrap™ HP-column (GE healthcare) equilibrated with binding buffer. When the sample had been loaded, the column was washed with binding buffer until a stable baseline had

![Figure 5 - The substrates used in the characterization of wt FucO and four FucO mutants.](image-url)
been reached. To wash out loosely bound proteins, the column was washed with washing buffer (60 mM imidazol, 20 mM NaP, 0.5 M NaCl) until a stable baseline had been reached. The his-tagged FucO was then eluted with elution buffer (300 mM imidazol, 20 mM NaP, 0.5 M NaCl). For the washing and elution steps, a flow rate of 1 ml/min was used.

To find the protein containing fractions from the elution, the absorbance at 280 nm was measured. These fractions were then pooled together and desalted using a PD-10 column equilibrated with 0.1 M NaP, pH 7.4.

To check whether or not the purification had worked out, an SDS-PAGE was performed. The stacking gel had an acrylamide concentration of 4 % and the separation gel had an acrylamide concentration of 12 %. Mini-Protean from Bio-Rad was used according to the manufacturer’s recommended protocol. Both lysate, flow through and fractions from the washing steps were checked on the SDS-PAGE as well as the desalted samples from the elution. The enzyme concentration was determined by measuring the absorbance at 280 nm, where ε = 41000 M⁻¹cm⁻¹.

**Steady-state kinetics**

All measurements were carried out at 30 °C in a Shimadzu UV-1700 spectrophotometer. The initial rate of the reaction was determined by measuring the change in concentration of NADH at 340 nm, where ε = 6220 M⁻¹cm⁻¹. To obtain a pseudo first order reaction the nucleotide was used at a saturating concentration of 0.2 mM in all experiments while the alcohol/aldehyde concentration was varied. Suitable enzyme concentrations were found by testing different concentrations to see where there would be enough activity, but where the reaction still would be at steady-state (table 1). The steady-state kinetic parameters, $K_M$, $k_{cat}$ and $k_{cat}/K_M$, were determined by using two different non-linear regression programs in SIMFIT ([http://www.simfit.man.ac.uk](http://www.simfit.man.ac.uk)). $K_M$ and $k_{cat}$ were extracted using the program MMFIT, which fits the Michaelis-Menten equation to the experimental data. $k_{cat}/K_M$ was extracted using the program RFFIT, which fits eq. 2 to the experimental data.

$$f(x) = \frac{A(0) + A(1)x}{1 + B(1)x} \quad (eq. 2)$$

A(0) is set to 0 in these experiments, $A(1) = k_{cat}/K_M$ and $B(1)=1/K_M$

**Measurements with alcohols**

A buffer with 0.1 M glycine, pH 10 was used for the measurements on alcohols. The cofactor NAD⁺ was used in a saturating concentration of 0.2 mM. The alcohols used in this experiment were 1-propanol, (S)-1,2-propanediol and (R)-1,2-propanediol. Dilution series of these were made in MilliQ-water and different substrate concentrations were used for the different enzymes (table 1).

**Measurements with aldehydes**

A buffer with 0.1 M NaP, pH 7.0 was used for the measurements on aldehydes. The cofactor NADH was used in a saturating concentration of 0.2 mM. The aldehyde used in this experiment was phenylacetaldehyde and different substrate concentrations were used for the different enzymes
Since phenylacetaldehyde is not very soluble in water, a dilution series was first made with acetonitrile. This dilution series was subsequently diluted further in buffer (10 µl phenylacetaldehyde in acetonitrile to 990 µl buffer, giving a total acetonitrile concentration of 1%). This mixture of buffer and substrate was vortexed vigorously for 30 sec, and was left to incubate for one hour in a 30 °C water bath before the measurements. It was found that measuring with only enzyme and NADH gave rise to a background activity. A background was therefore measured for each enzyme and subtracted from the measurements with substrate.

<table>
<thead>
<tr>
<th>Type of FucO</th>
<th>1-propanol</th>
<th>(S)-1,2-propanediol</th>
<th>(R)-1,2-propanediol</th>
<th>Phenylacetaldehyde</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[S] (mM)</td>
<td>[E]₀ (µM)</td>
<td>[S] (mM)</td>
<td>[E]₀ (µM)</td>
</tr>
<tr>
<td>wt FucO</td>
<td>1.25 - 40</td>
<td>0.3</td>
<td>0.313 - 20</td>
<td>0.14</td>
</tr>
<tr>
<td>N151G</td>
<td>5 - 100</td>
<td>2.4</td>
<td>2.5 - 80</td>
<td>2.1</td>
</tr>
<tr>
<td>N151T</td>
<td>1.25 - 40</td>
<td>0.35</td>
<td>0.5 - 100</td>
<td>1.1</td>
</tr>
<tr>
<td>T149S, N151V</td>
<td>1.25 - 20</td>
<td>1.1</td>
<td>5 - 60</td>
<td>1.1</td>
</tr>
<tr>
<td>T149S, N151W</td>
<td>0.05 - 5</td>
<td>4.8</td>
<td>0.5 - 10</td>
<td>7.1</td>
</tr>
</tbody>
</table>

Results

Optimizing the purification protocol for FucO

In previous purifications of wt FucO, a buffer with 100 mM imidazole was used in the second washing step to in order to elute unspecifically bound proteins from the Ni²⁺-column. An SDS-PAGE from one of those purifications showed that high amounts of FucO were lost during this step (fig. 6). To see if the purification protocol could be improved, the imidazole concentration in the second washing step was lowered to 60 mM. SDS-PAGE from a purification with 60 mM imidazole in the second washing step shows that even though a little FucO is leaking already with 60 mM imidazole the leakage is not as large as with 100 mM imidazole (fig. 7). The fractions from the elution with 300 mM imidazole are also pure, so most of the unwanted proteins are washed out with 60 mM imidazole. This purification protocol was therefore used during this whole project.

This purification protocol also seemed to be suitable for the mutant forms of FucO, since the yield was approximately the same for the mutants as for wild type enzyme (table 2). The yields for the first two purifications, where wt FucO and N151G were purified, are a bit higher than for the following purifications. This might be because when wt FucO was purified for the second time some air got into the column, and perhaps it lost some of its capacity because of that. The SDS-PAGEs from the purification of the mutants resembles the one in figure 7.
Figure 6 - SDS-PAGE showing fractions from different steps in the FucO purification. 1-2: FucO containing fractions from the elution with 300 mM imidazole. 3-8: Washing step with 100 mM imidazole, indicated by the red box. 9-12: Washing step with 20 mM imidazole. 13: Flow though. 14: Lysate: 15: Marker.

Figure 7 - SDS-PAGE showing fractions from different steps in the FucO purification. 1: Marker. 2-5: FucO-containing fractions from the elution with 300 mM imidazole. 6-10: Washing step with 60 mM imidazole, indicated by the red box. 11-14: Washing step with 20 mM imidazole. 15: Lysate

Table 2 - Yield for the purifications of the different types of FucO.

<table>
<thead>
<tr>
<th>Form of FucO</th>
<th>Yield (μg protein / liter cell culture)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt FucO</td>
<td>0.45</td>
</tr>
<tr>
<td>N151G</td>
<td>0.51</td>
</tr>
<tr>
<td>wt FucO</td>
<td>0.33</td>
</tr>
<tr>
<td>T149S, N151V</td>
<td>0.31</td>
</tr>
<tr>
<td>N151G</td>
<td>0.34</td>
</tr>
<tr>
<td>N151T</td>
<td>0.30</td>
</tr>
<tr>
<td>T149S, N151W</td>
<td>0.34</td>
</tr>
</tbody>
</table>
Steady state kinetics.

To characterize the catalytic activity of wt FucO and the four mutant forms of FucO, steady state kinetic parameters for several substrates were determined. The mutant forms have in a screening been found to have high activity with phenylacetaldehyde and it is of interest to see if they were true or false hits and to characterize the new enzymatic properties. The previous screening also indicated that the mutants would have lower activity with (S)-1,2-propanediol than wt FucO. The results are presented in table 3. To illustrate the results, the \( k_{cat}/K_M \) values for the different substrates and variants of FucO are showed in diagrams (fig. 8-11).

No measurements were performed with [T149S, N151W] and (R)-1,2-propanediol because of time deficiency. Only \( k_{cat}/K_M \)-values were obtained with (S)-1,2-propanediol+[N151G] and (R)-1,2-propanediol+[N151T] since saturation never was reached with the substrate concentrations used in the experiment. Only \( k_{cat}/K_M \) was obtained with (R)-1,2-propanediol+[T149S, N151W] because only three data points could be collected, which in not good enough to extract \( k_{cat} \) and \( K_M \).

**Phenylacetaldehyde**
The measurements showed that three out of the four mutants actually had an increased catalytic activity with phenylacetaldehyde. The \( k_{cat}/K_M \) values for the [T149S, N151V] and [N151T] mutants with phenylacetaldehyde are ten times higher than the values for wt FucO and the value for the [N151G] mutant is a 100 times higher. The [T149S, N151W] mutant turned out to be a false hit with no detectable activity with phenylacetaldehyde.

**(S)-1,2-propanediol and (R)-1,2-propanediol**
The measurements also showed that all mutants had a decreased activity with (S)-1,2-propanediol compared to wild type FucO. The decrease in \( k_{cat}/K_M \) can be explained by a drastic increase in \( K_M \) rather than a decrease in \( k_{cat} \). The enzymes that were tested with (R)-1,2-propanediol shows a decrease in activity also with this diol.

**1-propanol**
The activities of [N151T] and [T149S, N151W] with 1-propanol are approximately the same as for wt FucO. [N151G] shows a decreased \( k_{cat}/K_M \) value compared to the wild type enzyme, which is due to an increase in the \( K_M \)-value rather than a decrease in the \( k_{cat} \)-value. The [T149S, N151S] mutant shows a slight increase in the \( k_{cat}/K_M \) value compared to the wild type enzyme, which in contrast to [N151G] is due to a decrease in the \( K_M \)-value rather than an increase of the \( k_{cat} \)-value.
Table 3 – Steady state kinetic parameters for wild-type FucO and four different FucO mutants with varying substrates. In some cases it was not possible to extract $k_{cat}$ and $K_M$ and only $k_{cat}/K_M$ is given in those instances. (n.d.a. = no detected activity)

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Form of FucO</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$K_M$ (mM)</th>
<th>$k_{cat}/K_M$ (s$^{-1}$mM$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenylacetaldehyde</td>
<td>wt FucO</td>
<td>0.006 ± 0.001</td>
<td>13 ± 4</td>
<td>0.00041 ± 0.00006</td>
</tr>
<tr>
<td></td>
<td>N151G</td>
<td>1.9 ± 0.6</td>
<td>38 ± 16</td>
<td>0.049 ± 0.005</td>
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<tr>
<td></td>
<td>N151T</td>
<td>0.032 ± 0.002</td>
<td>6.3 ± 0.9</td>
<td>0.0049 ± 0.0004</td>
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<tr>
<td></td>
<td>T149S, N151V</td>
<td>0.016 ± 0.0009</td>
<td>3.2 ± 0.6</td>
<td>0.0051 ± 0.0007</td>
</tr>
<tr>
<td></td>
<td>T149S, N151W</td>
<td>n.d.a</td>
<td>n.d.a</td>
<td>n.d.a</td>
</tr>
<tr>
<td>1-propanol</td>
<td>wt FucO</td>
<td>0.95 ± 0.02</td>
<td>19 ± 2</td>
<td>0.049 ± 0.002</td>
</tr>
<tr>
<td></td>
<td>N151G</td>
<td>0.31 ± 0.02</td>
<td>92 ± 9</td>
<td>0.0033 ± 0.0001</td>
</tr>
<tr>
<td></td>
<td>N151T</td>
<td>2.6 ± 0.4</td>
<td>50 ± 10</td>
<td>0.053 ± 0.004</td>
</tr>
<tr>
<td></td>
<td>T149S, N151V</td>
<td>0.28 ± 0.01</td>
<td>3.7 ± 0.7</td>
<td>0.078 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>T149S, N151W</td>
<td>0.011 ± 0.0004</td>
<td>0.21 ± 0.03</td>
<td>0.050 ± 0.005</td>
</tr>
<tr>
<td>(S)-1,2-propanediol</td>
<td>wt FucO</td>
<td>1.6 ± 0.08</td>
<td>3.6 ± 0.5</td>
<td>0.43 ±0.04</td>
</tr>
<tr>
<td></td>
<td>N151G</td>
<td>-</td>
<td>-</td>
<td>0.00097 ±0.00003</td>
</tr>
<tr>
<td></td>
<td>N151T</td>
<td>0.50 ± 0.009</td>
<td>57 ± 2</td>
<td>0.0088 ±0.0002</td>
</tr>
<tr>
<td></td>
<td>T149S, N151V</td>
<td>0.13 ± 0.02</td>
<td>45 ± 10</td>
<td>0.0029 ±0.0003</td>
</tr>
<tr>
<td></td>
<td>T149S, N151W</td>
<td>-</td>
<td>-</td>
<td>0.018 ±0.006</td>
</tr>
<tr>
<td>(R)-1,2-propanediol</td>
<td>wt FucO</td>
<td>0.073 ± 0.006</td>
<td>105 ± 16</td>
<td>0.00071 ± 0.00005</td>
</tr>
<tr>
<td></td>
<td>N151G</td>
<td>n.d.a</td>
<td>n.d.a</td>
<td>n.d.a</td>
</tr>
<tr>
<td></td>
<td>N151T</td>
<td>-</td>
<td>-</td>
<td>0.000064 ± 0.000003</td>
</tr>
<tr>
<td></td>
<td>T149S, N151V</td>
<td>n.d.a</td>
<td>n.d.a</td>
<td>n.d.a</td>
</tr>
</tbody>
</table>
Figure 8 – The $k_{cat}/K_M$ value for wt FucO and four mutants with phenylacetaldehyde

Figure 9 – The $k_{cat}/K_M$ value for wt FucO and four mutants with 1-propanol

Figure 10 – The $k_{cat}/K_M$ value for wt FucO and four mutants with (S)-1,2-propanediol
Discussion

Some interesting results were obtained from the steady-state kinetic measurements, and perhaps the most interesting is that the [N151G] mutant had such high activity with phenylacetaldehyde. That one single mutation may give rise to 100 times higher activity is quite fascinating, and seems to support the assumption that the narrow entrance to the active site is a good target for site-directed mutagenesis. Models of the entrances to the active site for the mutants were created in PyMol (figure 12), and even though they are just computer made models and not actual crystal structures they might still be useful as a basis for this discussion. The model of [N151G] does for example show that the entrance is widened compared to the entrance in wt FucO, which could explain why phenylacetaldehyde is more readily used as a substrate in [N151G]. The aromatic molecule simply fits better in the active site since there is more space. The widened entrance could also explain other acquired properties in [N151G]. For example, that $K_M$ with 1-propanol and phenylacetaldehyde has increased, and that there were no sign of saturation with (S)-1,2-propanediol up to a substrate concentration of 80 mM. Since $K_M$ usually is considered to say something about the affinity between the enzyme and the enzyme bound species, the increase in the $K_M$ might tell us that the widened entrance causes the substrate to bind less tightly to the enzyme.

As have been mentioned earlier, the [T149S, N151W] mutant was a false hit with respect to activity with phenylacetaldehyde. Instinctively, this seems very plausible. If a tryptophan is placed in a place that is quite narrow one would not think that it would generate more space for bulky substrates, although the model in figure 12 shows an entrance that is quite similar to the one in the wild type enzyme. But the model might not represent the real situation; if the tryptophan would be pointing in a different direction the entrance could be much smaller. An interesting result is the $K_M$ value for [T149S, N151W] with 1-propanol, which is about 100 times lower than the $K_M$ value for wt FucO. This would indicate that the enzyme bound species are more tightly bound to [T149S, N151W] than the wt enzyme. Perhaps this could be because the tryptophan actually is creating an even narrower waist than in wt, and if the substrate enters the active site it would be held in place.
Fig 12 – The entrance to the active site in FucO, where the light blue molecule represents (S)-1,2-propanediol. The pictures were created in PyMol from the pdb file 1RRM a) Crystal structure of wild type FucO, the six amino acids that are creating the narrow waist are represented as sticks. The amino acids colored in green are Thr149 and Asn151, the amino acids that are mutated in library A. b) Model of [N151G], the mutated amino acid is shown in blue. c) Model of [N151T], the mutated amino acid is shown in blue. d) Model of [T149S, N151V], the mutated amino acids are shown in blue. e) Model of [T149S N151W], the mutated amino acids are shown in blue.
The [N151T] and [T149S, N151V] have quite similar $k_{\text{cat}}/K_M$ values for most substrates, although the [N151T] mutant consistently has a higher $k_{\text{cat}}$ value and higher $K_M$ value than the [T149S, N151V] mutant. In the case of 1-propanol, the [N151T] mutant even has got twice as high $k_{\text{cat}}$ than the wild type enzyme. When comparing the models of the two mutant structures in figure 12, the [T149S, N151V] mutant seem to have a slightly wider opening. Perhaps this could explain why the $K_M$ value generally is higher for the [N151T] mutant.

The kinetic parameters for the wild-type enzyme with 1-propanol, (S)-1,2-propanediol and (R)-1,2-propanediol have previously been reported\textsuperscript{12} (table 4). It is obvious that the $k_{\text{cat}}$ values obtained in this project are lower than the literature values, which could indicate that the enzyme lost activity at some point in the handling. One reason for this might be that the enzyme was not used directly after the purification, but was sometimes stored in the fridge for a week or two before the measurements. This might indicate that the obtained $k_{\text{cat}}$- and $k_{\text{cat}}/K_M$-values for the mutants in reality might be a bit higher than the ones given in table 3. To obtain better values, one could do new measurements with enzyme that had just been purified one or two days earlier.

Table 4 - Steady state kinetic parameters for wt FucO catalyzed reaction (reference 12, C. Blikstad & M. Widersten)

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$k_{\text{cat}}$ ($s^{-1}$)</th>
<th>$K_M$ (mM)</th>
<th>$k_{\text{cat}}/K_M$ ($s^{-1}$mM$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-propanol</td>
<td>2,8 ± 0,05</td>
<td>12 ± 0,6</td>
<td>0,24 ± 0,008</td>
</tr>
<tr>
<td>(S)-1,2-propanediol</td>
<td>3,8 ± 0,04</td>
<td>5,4 ± 0,1</td>
<td>0,71 ± 0,01</td>
</tr>
<tr>
<td>(R)-1,2-propanediol</td>
<td>0,16 ± 0,01</td>
<td>74 ± 7</td>
<td>0,0022 ± 0,0005</td>
</tr>
</tbody>
</table>

In conclusion, the project has been quite successful. It was a relief that the purification with 60 mM imidazole instead of 100 mM imidazole worked out so well, so that it was possible to spend more time on characterizing the mutants than on optimizing the purification protocol. The steady-state kinetic measurements also generated some interesting information that might be used in the development of new generations of libraries. It does for example seem that the [N151G] mutant would be a good starting point. So the next step would now be to determine the catalytic properties of the mutants with a bulky alcohol, since the goal is to produce an enzyme for biocatalysis of aromatic alcohols to aldehydes, and then produce a new library.

**Acknowledgements**

First and foremost, I wish to thank my supervisors Mikael Widersten and Cecilia Blikstad for all the help and guidance. I learnt a lot and had a lot of fun while doing it. I also wish to thank Åsa Janfalk Carlsson and Mikael Nilsson, who really have been like a couple of extra supervisors. I especially want to thank everyone for being so patient when I needed help, and for making me feel welcomed. Thanks!
References


