Manipulation of the pH dependence of the propanediol dehydrogenase, FucO, catalyzed alcohol oxidation

Undergraduate thesis
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Abstract

The *E. coli* Propane-1,2-diol oxidoreductase enzyme, FucO, exhibits a large degree of stereospecificity in catalysis. This property, together with mutationally induced altered characteristics, could result in a FucO variant capable of serving as part of a biocatalysis system of engineered enzymes acting in sequence, providing a stereospecific catalysis system for organic synthesis. In this context, the present study investigated the effect of the N71D mutation on FucO characteristics, with the primary purpose of lowering the pH optimum of the oxidation reactions catalyzed by this enzyme so as to optimize FucO for incorporation into a coupled enzyme catalysis system.

His-tagged, mutated, FucO protein was generated, employing standard biochemical methodology, via PCR-mediated site-directed mutagenesis, subsequent expression, and purification by affinity chromatography, before kinetic characterization proceeded over a pH interval of 8.0-10.25 in regards to the FucO-catalyzed oxidation of the model substrate 1-propanol.

The N71D mutation generated a FucO variant with subtle differences in characteristics compared to the wt. pH-dependence was noted for $k_{\text{cat}}$ and $k_{\text{cat}}/K_m$, with both parameters being overall slightly lowered by the mutation. $K_m$ exhibited weak pH dependence, with small differences seen in the mutant compared to the wt. $pK_a$ was determined in terms of $k_{\text{cat}}$ and $k_{\text{cat}}/K_m$, and in both cases the mutant displayed essentially identical values as the wt – indicating no effect on the local catalytic environment. Results hinted that the mutation might have affected the active site structure, and possibly the stability of the enzyme-substrate complex, or provided a possible conformational change, towards a lower affinity complex.
# Table of contents

Abstract........................................................................................................................................3
List of abbreviations......................................................................................................................5
Introduction..................................................................................................................................6
  Context & perspective .............................................................................................................6
  Propane-1,2-diol oxidoreductase.............................................................................................7
  Aim...............................................................................................................................................9
Materials and methods..............................................................................................................10
  Organisms and plasmids..........................................................................................................10
  Subcloning of E. Coli FucO .....................................................................................................10
  FucO mutant generation via site-directed mutagenesis .......................................................11
  Expression of FucO mutants....................................................................................................12
  Protein purification..................................................................................................................13
  Enzyme kinetics/pH dependence assays..............................................................................13
  Determination of kinetic parameters and pH dependence of catalysis.............................14
Results.........................................................................................................................................14
  Generation of FucO mutants.................................................................................................14
  Enzyme kinetics measurements..............................................................................................16
Discussion....................................................................................................................................21
  N71D mutation effect on kinetic parameters.......................................................................22
  Characteristics of mutated residues and interactions..........................................................24
Conclusions..................................................................................................................................25
  Future prospects......................................................................................................................25
References....................................................................................................................................26
List of abbreviations

ADH, alcohol dehydrogenase
FAD, flavin adenine dinucleotide
FucO, propane-1,2-diol oxidoreductase
IMAC, immobilized metal ion affinity chromatography
IPTG, isopropyl-beta-D-thiogalactopyranoside
NAD, nicotine amide dinucleotide
O/D, over day
O/N, over night
VdW, Van der Waal
Wt, wild-type
Introduction

Context & perspective
In the world of organic synthesis, the involvement of enzymes as biocatalysts in synthetic pathways represents an increasing trend. A range of reactions of diverse chemistry has been described in the literature (Urlacher & Schmid, 2006; Matsumura, et al. 2006; García-Urdiales, et al. 2005). With the advent of ever improved technologies and knowledge in biochemistry and molecular biology - in various relevant fields such as genetic engineering, directed evolution, etc. - it is likely that the range of useful enzymes for biocatalysis will expand in the not-too-distant future.

Biocatalysts possess features that allow the use of aqueous solvents and reactions run at moderate temperatures, which in turn enables more environmentally friendly synthetic production and additionally less hazards involved in the handling of the reactions.

Enzyme catalysts also exhibit a characteristic high level of specificity for reactant molecules involved in the catalyzed reactions. The production of fine chemicals and pharmaceuticals, often involving difficult organic synthesis with high demands on product purity, could greatly benefit from the use of enzymes as biocatalysts. This is particularly relevant when taking into account that, in many cases, the aforementioned high enzymatic specificity provides the possibility of conversion of only a specific isomer of a chiral molecule. Traditional routes of organic synthesis can be problematic in, for example, synthetic pathways involving chiral centers. In such situations, the use of stereospecific enzymes could relax the need for building blocks of ultra-high purity as well as allowing the generation of optically pure products from racemic reactant mixtures – a scenario made possible by the acceptance of only the correct stereoisomer as substrate for the enzyme.

The present study is set in the context of the design of enzyme biocatalysis systems for modular sequential synthesis of chemical substances, containing functional groups such as alcohols, aldehydes, and ketones, amendable to additional derivatization. These additional functionalizations could be afforded by traditional organic synthesis or by additional enzyme-catalyzed reactions.

Conceptually, the present study is part of an overall strategy aiming to produce a variety of product molecules of controlled diversity, by enzymatically catalyzed specific reaction types, via the use of native or structurally engineered enzymes acting in sequence on a set of substrate molecules structurally related. These enzymatic systems could be used for regio- and stereospecific compound production, such as is exemplified in fig. 1, illustrating an example synthesis using chiral epoxides as starting material for production of chiral hydrocarbonyl compounds. This example entails the epoxide hydrolase catalyzed conversion of chiral epoxides to vicinal diols, which in turn can be oxidized via diol dehydrogenases, forming end product molecules which may also contain prochiral centers, potentially useful for the generation of further arrays of chiral products upon additional derivatizations (M. Widersten, dept. of biochemistry and organic chemistry, Uppsala University, research program).

The present study focuses on the study of one of these aforementioned diol dehydrogenases, namely propane-1,2-diol oxidoreductase - commonly abbreviated FucO - also known as L-lactaldehyde reductase, from Escherichia coli.
Figure 1. Schematic overview of an example enzyme-catalyzed stepwise synthesis, involving the epoxide hydrolase catalyzed stereospecific hydrolytic ring opening of substituted epoxides to generate vicinal diols. Diols, which may in turn be oxidized by diol dehydrogenases – such as the one studied in the present study; propane-1,2-diol dehydrogenase, FucO, from *E.coli*. Subsequent additional derivatizations, by organic synthesis or additional enzyme catalysis, may provide for an extended range of possible hydroxyl carbonyl compound products. (Image courtesy of Mikael Widersten, Research program, appendix A).

**Propane-1,2-diol oxidoreductase**

There are three major categories of classification for the oxidoreductase enzymes, which catalyze the interconversion of alcohols, aldehydes and ketons – as described by Reid and Fewson (1994). The first of these categories consists of the NAD(P)-dependent alcohol dehydrogenases (ADHs), the NAD(P)-independent ADHs constitute the second category, and the FAD-dependent alcohol oxidases represents the third category. Further subdivisions, into three groups, can be made for the NAD(P)-dependent ADHs. Medium chain zinc-dependent dehydrogenases constitute group I, short chain zinc-independent dehydrogenases make up group II, and group III is the iron-dependent dehydrogenases (Obradors, *et al.* 1998).

1,2-propanediol oxidoreductase (henceforth referred to simply as FucO), also known by its alternative name L-lactaldehyde reductase, is a protein belonging to the group III iron-dependent alcohol dehydrogenases, and consequently also requires NAD(H) as cofactor.

The bacterial catabolism of L-fucose and L-rhamnose incorporates the propanediol oxidoreductase enzyme (Ros & Aguilar, 1985; Badia *et al.* 1985; Baldoma & Aguilar, 1988; Patel, *et al.* 2008). The best studied isoenzyme is the *E.coli* FucO protein, for which the metabolic role has been extensively studied (Baldom & Auguilar, 1988).

FucO has been reported to be encoded by the *fucO* gene, of the fucose regulon (Boronat, *et al.* 1979), and the protein has a homodimeric structure with each monomer having a molecular mass of 40644 Da and consisting of 383 amino acids (Montella, *et al.* 2005).

Physiologically, FucO serves to reduce L-lactaldehyde (also known as (S)-lactaldehyde) to L-1,2-propanediol (alternatively referred to as (2S)-1,2-propanediol) in a process requiring NADH as
cofactor (Obradors, et al. 1998). (2S)-1,2-propanediol can subsequently be excreted as a fermentation product (Cocks, et al. 1974). As depicted in fig. 2, FucO catalyzes the reversible interconversion, in both reaction directions, of (S)-lactaldehyde and (2S)-1,2-propanediol. However, studies have shown FucO to be more effective in catalyzing the direction of, NADH-mediated, reduction of (S)-lactaldehyde to (2S)-1,2-propanediol – as judged from specific activities (Boronat & Aguilar, 1979) – thus suggesting this to be the physiological role of FucO.

Additionally, the enzyme is capable of acting on alternative substrates, such as propanol, ethylene glycol, ethanol, and glycerol (Boronat, & Aguilar, 1979).

Due to the strong preference exhibited by the enzyme for the S-isomer over the R-isomer, of the (2S)-1,2-propanediol substrate, there is a certain stereospecificity in the catalytic reaction, which is also one reason for why this particular enzyme is interesting from the perspective of the wider scope of the present study – presented above. The pH optimum of the FucO-catalyzed (S)-lactaldehyde reduction reaction has previously been determined to be at pH 6.5, as opposed to the dehydrogenation of (2S)-1,2-propanediol for which the enzyme has an optimum at pH 9.5 (Boronat, 1979).

A number of homologous microbial iron-activated dehydrogenases, based on structural homology, have been identified; including ADH II from Zymomonas mobilis, the Saccharomyces cerevisiae protein ADH IV, the two Clostridium acetobutilicum butanol dehydrogenases BDH A and -B, and the Bacillus methanolicus methanol dehydrogenase (MDH) (Obradors, et al. 1998).

The exact details of the catalytic mechanism of FucO has not been elucidated at present but crystallographic structural data has been presented, exemplified by the molecular model reported by Montella, et al. 2005, of FucO in complex with NAD+ cofactor and modeled (2S)-1,2-propanediol substrate bound to the active site (pdb id 2bl4). Additionally, a crystal structure has been presented (though has yet to be published) by Kumaran, D. and Swaminathan, S., deposited at the pdb structural database by the identification 1rrm - providing the basis for the visualization of the FucO active site presented in fig. 3. Figure 3 additionally visualizes the nucleotide cofactor and the tightly coordinated iron (II) ion needed for catalysis. The channel of the active site is indicated, with the narrow “waist” that prevents larger substrates from entering. It is conceivable that mutagenesis of residues at the site of this “waist” could make the active site channel wide enough for entry of larger substrates, which could be interesting in the context of the design of the aforementioned enzyme-based biocatalysis systems. This channel-widening parameter is outside the primary scope of the present study, however.

Figure 2. The primary reaction catalyzed by FucO; the interconversion of 1,2-propanediol (left) and lactaldehyde (right), with NAD as cofactor. Picture retrieved from BRENDA (http://www.brenda-enzymes.info/) - comprehensive enzyme information system, entry for E.C 1.1.1.77.
Figure 3. The active site of the FucO enzyme, showing the tightly coordinated iron (II) ion (orange) on which catalysis is dependent. The image indicates the active site channel, into which the NAD cofactor enters from the left and diol substrate (blue) enters through the channel from the right. Entry of larger diol substrates is prevented due to a narrow "waist" in the channel. Mutagenesis of residues at the waist could conceivably widen the channel enough to allow entry of larger substrates, for example diols which have been formed by epoxide hydrolase reactions, such as phenylethyl and phenylpropyl diols. (Image courtesy of Cecilia Blikstad, adapted from atomic coordinates in IRRM (PDB identification).

Figure 4. The FucO catalyzed oxidation reaction of propanol (model substrate) to propanal (propionaldehyde). Picture retrieved from BRENDA (http://www.brenda-enzymes.info/) - comprehensive enzyme information system, entry for E.C 1.1.1.77.

**Aim**

The aim of the current study was to investigate how the pH dependence of the FucO catalyzed oxidation of alcohols can be manipulated, in an attempt to lower the pH optimum of the enzymatic reaction. 1-propanol (see fig.4) was chosen as a model substrate in the oxidation reactions studied.

The main reason for lowering the pH optimum of the oxidation reaction, as well as possibly altering the substrate specificity of the enzyme, was – in the wider, more long-term perspective – to find a manipulated FucO variant which could be incorporated as a part of a biocatalysis system, such as is described above (in the introduction, as well as in fig. 1), for stepwise enzymatically catalyzed synthesis.

A rational approach to the selection of residues to modify was employed, where the selection criteria was primarily based on the availability of corresponding residue interchanges in related proteins, in combination with the speculation that these residues might be of relevance to the proton transfer occurring during the studied oxidation reactions (personal communication, prof. Mikael Widersten, dept. of biochemistry and organic chemistry, Uppsala university, Uppsala, Sweden).

Certain mutations of active site amino acid residues were considered promising to accomplish the stated aim of this project, based on knowledge obtained in previous studies in the field (Montella, *et al.* 2005, etc.), combined with the positioning of these residues at the enzyme active site and their
possible involvement in the aforementioned proton transfer during catalysis. Proposed mutations to introduce included the residues N71-> D71 and N274->H274, both of which are substitutions that can be found in related proteins, positioned in the vicinity of the nucleotide binding site of the enzyme, as well as possibly participating in the proton transfer during catalysis. The hypothesis of the present study was that these selected mutations would result in a change in the local catalytic environment of the enzyme, in such a way as to alter the pH optimum of the studied oxidation reactions.

The current project aimed at testing these proposed mutations in the context of pH optimum manipulation, and to do so by standard biochemical methods following the route of expressing, purifying, and finally analyzing and evaluating the catalytic rates \( k_{cat} \) achieved by the mutated enzymes at varying pH to deduce the effect of the selected mutations on the pH optimum of the relevant reactions.

As a result of the stereo specific nature of FucO it is an interesting target to manipulate to, in a wider perspective, obtain specific and efficient biocatalysts capable of generating chiral molecules which can be useful in stereo specific synthetic chemistry.

Materials and methods

Organisms and plasmids

All bacterial strains used in the present study were electrocompeotent \textit{E. coli} XL-1 Blue cells, in combination with a vector – described in fig. 5 – providing ampicillin resistance as a selectivity marker and means to express His\textsubscript{6}-tagged FucO protein, cleaved into the vector at the available XhoI and SpeI restriction sites. Plasmids/vectors, XL-1 blue \textit{E. coli} strains, and FucO DNA were provided by Mikael Widersten/Cecilia Blikstad (dept. of biochemistry and organic chemistry, Uppsala University, Sweden).

![Figure 5. The pGTacFuco vector (derived from the pGTacStEH1-5H vector, described by Elfström & Widersten, 2005) into which the mutated FucO gene was inserted. Indicated are the sites for XhoI and SpeI restriction enzymes, as well as ampicillin resistance (AmpR), used as a selectivity marker, and the tag of six Histidines (Hisx6) added for IMAC protein purification.](image)

Subcloning of \textit{E. Coli} FucO

Due to technical issues (see results and discussion sections) the N274H mutant was prepared on two separate occasions. Both variants were however prepared along the same protocol (described below).

Genomic DNA corresponding to wild-type (wt) \textit{E. coli} strain XL-1 Blue, FucO was amplified by PCR (using primers Fuco-1 & -2, described in table 1) followed by ligation into a vector (pGTacFuco, see fig. 5) by XhoI and SpeI restriction enzymes, resulting in a wt FucO expression plasmid.

Preparation of the N71D mutant expression plasmid followed the same procedure of PCR amplification followed by ligation into a vector as for the N274H variant, where the previously described PCR amplified genomic FucO DNA provided the template material for the PCR-mediated mutant generation via site-directed mutagenesis.

The N71D plasmid material used in the present study had previously been prepared by Saga Runarsdottir (undergraduate student) and Cecilia Blikstad (graduate student at the dept. of biochemistry and organic chemistry, Uppsala University, Sweden), by a protocol analogous to the one described for the N274H mutant.
Table 1. Primers used in PCR mutagenesis generation of mutant (N27H & N71D) and wt FucO.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>FucO-1 (fwd)</td>
<td>5' - TTT TTT CTC GAG ATG ATG GCT AAC AGA ATG ATT -3'</td>
</tr>
<tr>
<td>FucO-2 (rev)</td>
<td>5' - TTT TTT TCT AGA TTA TTA ACT AGT CCA GGC GGT ATG GTA AAG -3'</td>
</tr>
<tr>
<td>FucO-N274H-2 (fwd)</td>
<td>5' - ACT CCA CAC GGT GTT GCG -3'</td>
</tr>
<tr>
<td>FucO-N274H (rev)</td>
<td>5' - CGC AAC ACC GTG TGG AGT GTG ATA AAA CGC GCC CAG TGG -3'</td>
</tr>
<tr>
<td>FucO-N71D-2 (fwd)</td>
<td>5' - CCA ACA ATT ACT GTC GTC AA – 3'</td>
</tr>
<tr>
<td>FucO-N71D (rev)</td>
<td>5' – TT GAC GAC AGT AAT TGT TGG GTC GGG CAC TAG GCC GTC G – 3'</td>
</tr>
</tbody>
</table>

**FucO mutant generation via site-directed mutagenesis**

Generation of the FucO mutants N274H (Asn274-> His; AAC to CAC) and N71D (Asn71-> Asp; AAC to GAC), was accomplished through PCR and the use of primers containing the target mutations (see table 1). As template material for the mutagenesis reactions, wt fucO plasmid material was used. Mutagenesis PCR was performed in two successive reactions where the first round of PCR reactions consisted of the generation of mutated FucO divided into two fragments (“A” and “B”) incorporating the desired mutations. These fragments were generated in parallel during the first round of PCR reactions. In the second round of PCR reactions the two fragments (having some overlap) were mixed and allowed to hybridize, before amplification of the full length mutated FucO construct (see figure 6). This general approach was employed for both the N274H and the N71D mutants.

All PCR reactions were performed in a final reaction volume of 50 μL, consisting of 50 pmol of each primer, 200 μM dNTP mix, 1.5 mM of MgCl$_2$ (preheated to 80 °C for 5 min. prior to usage), appropriate volume of 10x Taq buffer (+KCl -MgCl$_2$), and 2.5 U Taq polymerase. All PCR components were purchased from Fermentas.

For the initial, round one PCR - generating the two N274H fragments - 16 ng template (wt FucO plasmid material) was used. In the subsequent reaction, PCR round two, where the two fragments were combined, approx. 1.2 μg of fragment “A” (see fig. 6a & b) and 0.4 μg fragment “B”, from the first round of PCR, was used as template (the other reagents were added in the same concentrations and amounts as previously described). The relative ratio of fragment amounts in the final PCR reaction was aimed at compensating for the rather large size difference between the respective fragments (approx. 3:1) so as to maintain roughly stoichiometric proportions in the PCR reaction and thus maximize the yield. All fragment and vector concentrations were determined by a nanodrop nd-1000 spectrophotometer (Thermo scientific).

1. FucO-1
2. FucO-2
3. N274H
4. N274H

**Figure 6.** a. Schematic overview of the FucO gene divided into fragments A and B, for

```
wt FucO gene
---
PCR round 1
---
Fragment "A"
Generation of fragments, in parallel
Fragment "B"

wt FucO gene
---
PCR round 2
---
Mix of fragments
Amplification of full length mutated FucO construct

Mutated FucO construct (full length)

6a. 6b.
```
N274H mutagenesis PCR, with corresponding primers used (for seq. see table 1). Primers 3 & 4 are the primers containing the mutation. A similar subdivision of the FucO gene, in the N71D case, would be similar but with forward primer FucO-N71D-2 and reverse primer FucO-N71D.

b. Schematic overview of the two-step PCR reaction performed to obtain the full length mutated N274H FucO. In PCR round 1, fragments A and B were generated (see fig. 6a). Primers 1 & 4 (table 1) were used for the “A” fragment, while primers 2 & 3 were used for the “B” fragment. In the second round of PCR the two fragments were combined for full-length N274H mutant FucO (using primers corresponding to the full gene, nr. 1 & 2 (table 1)). Generation of N71D proceeded through an analogous procedure, though with corresponding mutation primers for that mutant.

All PCR reactions utilized the same program, in which initial denaturation was performed via 5 min. at 95 °C, followed by 40 cycles of 95 °C (denaturation), annealing for 30 sec. in a temperature gradient from 60-53 °C, and elongation for 2 min. at 72 °C. After the end of the 40 cycles, incubation at 72 °C for 7 min followed.

PCR samples containing appropriate product (as deduced by analytical gel electrophoresis), was – in all cases – pooled and subjected to DNA precipitation procedures to concentrate the sample. The DNA precipitation procedure employed constituted addition of 3 M NaOAc (1/10 of the volume of pooled PCR product) and 99.7 % EtOH (2 volumes of PCR product with NaOAc added) to the pooled PCR product, followed by approx. 30 min. at -80 °C. The next step consisted of centrifugation at 13000 rpm for 30 min., after which the supernatant was discarded and the pellet washed with 500 μL 99.7 % EtOH. Finally the pellet was dried and then dissolved in H2O.

Purification of all PCR products was subsequently accomplished via (1%) agarose gel electrophoresis, in combination with a gene clean procedure according to the Geneclean II kit (Qbiogene). Following the gene clean procedure the PCR products were subjected to restriction enzymes (XhoI/SpeI) to enable ligation of PCR product into the previously mentioned (fig. 5) vector at corresponding restriction sites. In preparation of sequencing, the prepared DNA constructs were transformed into XL1- Blue cells and colonies of transformed cells were subjected to MiniPrep procedures, according to the Quick Protocol accompanying the Wizard Plus miniprep DNA purification system, from Promega.

For verification of mutations, the prepared DNA constructs were subsequently sequenced.

Expression of FucO mutants

Expression procedures were performed by means previously described (Elfström & Widersten, 2005). FucO mutant plasmids (generated as described above) were transformed by electroporation (at 1.25V) into E. coli XL-1 Blue cells. These cells were then grown for approx. 1 h at 37 °C (on shaker) in 1 mL 2TY media consisting of trypton (1.6% w/v), yeast extract (1 % w/v), and NaCl (0.5 % w/v), supplemented with ampicillin (100 μg/mL). Incubated cells were plated on agar plates (10 μL and 100 μL of each culture, on separate plates) in the presence of ampicillin (100 μg/mL) and incubated o/n at 37 °C.

One colony from the plates was transferred to a 1 mL 2TY media solution, with ampicillin (100 μg/mL), and grown over the day (approx. 6-7 h) at 30 °C, after which the o/d cultures were used to inoculate a 35 mL volume of 2TY media (including ampicillin, 100 μg/mL) which in turn was incubated o/n at 30°C. This culture was subsequently used to inoculate 3 L of 2TY medium, fortified with 50 μg/mL ampicillin, allowed to grow at 30 °C until the optical density (OD600) of the culture reached 0.3.

Induction of protein production commenced through addition of 1 mM IPTG, together with 100 μM Fe(II)Cl2·x4H2O to take the Fe-dependence of the FucO protein into account, and incubation followed for approx. 17-20 h. Induced cells were collected via centrifugation for 12 min. at 5000 rpm,
at 4 °C, and the pellet was resuspended in buffer A (10 mM sodium phosphate (pH 7.0), 0.02 % (w/v) sodium azide, and Complete Mini EDTA-free protease inhibitor added).

The resuspended cells were stored at -80 °C.

**Protein purification**

Protein purification was performed in general accordance with the method of Elfström & Widersten (2005).

Unless otherwise stated, all purification steps were performed at 4 °C. By means of equipment from Constant cell disruption systems, run at 1.7 kPa, bacterial lysate was generated from the thawed resuspended cells. To sediment insoluble debris, the cell suspension was subjected to centrifugation at 15 000 rpm at 4 °C for 45 min. Adjustment of the salt composition of the *E. coli* bacterial lysate was accomplished via gel filtration chromatography using a buffer B (pH 7.0, 0.5 M NaCl, 20 mM imidazole and 10 mM sodium phosphate) equilibrated Sephadex G-25 column. Pooled material, containing protein, from the G-25 column was applied to a chelating Sepharose HiTrap IMAC column (GE healthcare), preloaded with Ni(II) ions, equilibrated with buffer B and connected to an Äkta prime purification system (Pharmacia Biotech/GE healthcare).

For the purpose of washing off proteins weakly adsorbed, buffer B with an increased imidazole concentration (100 mM) was used and to elute the more tightly bound proteins the imidazole concentration of buffer B was further increased to 300 mM. Gathered fractions containing protein (as deduced from spectrophotometric A$_{280}$ measurements) were pooled. Sample volume was decreased to approx. 2 mL through concentration via ultrafiltration (Amicon ultrafiltration cell, model 52).

Concentrated sample was applied to a 0.1 M sodium phosphate (pH 7.4) equilibrated HiPrep Sephacryl S-200 (16/60) gel filtration column (GE healthcare), for the final purification step.

Spectrophotometric absorbance measurements at 280 nm provided the enzyme concentration in the final purified sample. A molar absorption coefficient (ε) of 41000 M$^{-1}$cm$^{-1}$ and a molecular weight of FucO of 76000 Da (Boronat, et al. 1979) was employed.

**Enzyme kinetics/pH dependence assays**

The kinetic parameters of the FucO propanediol oxidoreductase enzyme were studied via spectrophotometric measurements of the NAD$^+$ cofactor dependent FucO catalyzed oxidation of model substrate, in which NADH is generated. The measurements were performed at 340 nm, following the increasing NADH absorbance as described previously (Boronat, et al. 1979 & Montella, et al. 2005), using a molar absorption coefficient of 6.22E-03 M$^{-1}$cm$^{-1}$.

For the purpose of studying the effect of the present mutations on the pH optimum of the FucO enzyme, the reaction assays where studied over the pH range 8-10.25 (8.0, 8.56, 9.0, 9.25, 9.5, 10.0, 10.25), using 0.1 M glycine buffers.

All reactions were consistently studied at 30 °C, and utilized 1-propanol as model substrate. The activity was measured in 300 μL assay mixture volumes, in triplicates, in a 96 well microtiter plate, analyzed by a SpectraMax 190 plate reader (Molecular Devices) set to gather data during 5-10 minutes of the studied reactions from addition of enzyme at reaction start.

The assay mixtures consisted of 1-propanol, over a concentration range of 1.76-30 mM (run in parallel on the same plate), together with 0.20 mM NAD$^+$ and approx. 0.3 μM mutated (or wt) FucO enzyme, mixed with 0.1 M glycine buffer of the appropriate pH.

For comparative purposes all reactions were additionally studied in the presence of wt FucO (courtesy of Cecilia Blikstad, prepared along the same method used for the mutated proteins) in
reactions run in parallel or directly following the reactions with mutated FucO, using the same setup and solutions.

Additionally, measurements were performed by the same kinetic assay scheme as described but with the concentration of NAD’ cofactor altered, instead of the substrate, to investigate the saturating conditions of the NAD’ system. For these measurements the NAD’ concentration was varied between 0.01 and 1 mM, whereas the enzyme concentration was maintained at 0.1 μM and the substrate 1-propanol concentration was kept at 40 mM. These measurements were, however, only performed at pH 10 (0.1 M glycine buffer), to provide values in the vicinity of the pH optimum of the enzyme. For these measurements the 96 well plate reader was not used, instead measurements were conducted using a Shimadzu spectrophotometer, in a sample volume of 1 mL using a quartz cuvette (corresponding to the use of a molar absorption coefficient of 6.22E-03 M⁻¹cm⁻¹), and an analysis time of 1 min. As in the previous case, this assay was also performed with the wt FucO, for comparative purposes.

**Determination of kinetic parameters and pH dependence of catalysis**

Extraction of the kinetic parameters, at varying pH, was accomplished via the use of the MMFIT program of the SIMFIT package (url: http://www.simfit.man.ac.uk). The gathered experimental kinetic data was fitted to the Michaelis-Menten equation, by nonlinear regression, to obtain the $k_{cat}$ and $K_m$ parameters. Additionally, for the extraction of the $k_{cat}/K_m$ specificity constants, the RFFIT program of the SIMFIT package was utilized. In this case the experimental data was fitted to equation 1. RFFIT was additionally used to extract $pK_a$-values through fitting of the previously deduced $k_{cat}/K_m$ and $k_{cat}$ parameters vs. pH, expressed in [OH⁻], to an adaptation (eq. 3 & 4) of eq. 2, so that the inflection point of the graph (fig. 11) obtained provided the $pK_a$ values (Fersht, A.).

$L_H$ is the pH dependent kinetic parameter ($k_{cat}/K_m$ or $k_{cat}$), $L_{HA}$ and $L_A^-$ represent protonated and deprotonated form of acid affecting $L_H$, and $K_s$ is the acid constant. In eq. 3 and 4, the parameters of eq. 2 have been expressed in terms of [OH⁻] and base constants ($K_B$). Consequently, $L_{OH}$ is the [OH⁻] dependent kinetic parameter ($k_{cat}/K_m$ or $k_{cat}$), $L_A$ and $L_{AH}$ represent the various forms of base affecting $L_{OH}$, and $K_B$ is the base constant.

\[
v = \left( \frac{k_{cat}}{K_m} \right) \left( 1 + \left( \frac{S}{K_m} \right) \right)
\]

\[
L_H = \left( L_{HA} \frac{[H^+]}{K_a} + L_A^- \right) \left( 1 + \frac{[H^+]}{K_a} \right)
\]

\[
L_{OH} = \left( L_A [OH^-] + L_{AH} K_B \right) \left( K_B + [OH^-] \right)
\]

\[
L_{OH} = \left( L_A [OH^-] K_B + L_{AH} \right) \left( 1 + [OH^-] / K_B \right)
\]

**Results**

**Generation of FucO mutants**

The current study encompassed the study of two *E.coli* FucO mutants; N274H (asparagine to histidine) and N71D (asparagine to aspartic acid), in relation to wild type (wt) FucO, regarding the effect of these mutations on certain catalytic properties of this enzyme. Primarily the focus was set on the obtained effects on the pH optimum of the catalyzed oxidation of 1-propanol.

To obtain FucO protein for this analysis, an expression system capable of generating C-
terminally His-tagged FucO protein was utilized, in combination with protein expression in *E. coli*. Purification proceeded primarily through Ni (II) affinity chromatography and gel filtration.

As previously mentioned, technical problems led to two separate attempts at cloning and purifying the N274H mutant. In the initial attempt, the mutant was purified successfully -though at very low quantities (data not included) – but no meaningful catalytic data could be gathered. DNA sequencing revealed that an L245P (Lysine to Proline) additional mutation had been introduced in this mutant, due to an alteration of CTC—>CCC introduced during PCR amplification (data not included). Due to the placement of this additional proline residue (see fig.7), combined with the known “helix-breaking” properties (Voet & Voet, 2004) of this amino acid, it is likely that this unwanted mutation caused the production of a mis-folded or otherwise non-functional protein, thus negatively affecting catalysis.

In the second attempt at N274H mutant generation, problematic and time-consuming issues at the PCR amplification stage caused the work on this mutant to end after transformation and DNA sequencing, as time limitations did not allow for protein purification and subsequent catalytic characterization to proceed. Consequently, contrary to the aim of this study, no catalytic data was generated for any of the N274H mutants.

The N71D mutant was efficiently expressed and purified, and could subsequently be studied by the described kinetic assay. The final concentration of purified N71D mutant FucO was determined, via $A_{280}$ measurements, to 42 µM. For comparative purposes, wt FucO – prepared by Cecilia Blikstad along the same expression and purification scheme – was also included in the kinetic analysis.

![Figure 7](image_url)

*Figure 7. The placement of the unwanted L245P additional mutation, likely causing the problematic purification and characterization experienced during the initial attempts at generating the N274H FucO mutant. The image shows the L245 residue, at the central cleft between the monomers of the homodimeric FucO protein, as part of an alpha-helix. Proline*
generally considered to have structural properties, causing strain and destabilization, when in an alpha-helix. The additional residues and molecules shown, depicted as sticks, represent the nucleotide binding site. These residues have been included as a reference point for the L245 residue location in relation to the enzyme active site. The image also reflects the overall tertiary structure of FucO, and the dimeric fold. Image created using the pymol molecular graphics system (Delano scientific), using the coordinates from 1rrm (PDB).

**Enzyme kinetics measurements**

For the purpose of determining the effect of the present mutations on the pH optimum of the alcohol (1-propanol) oxidation reactions of FucO, a study by pH titration curves of corresponding kinetic parameters ($k_{\text{cat}}$ and $k_{\text{cat}}/K_m$) was conducted where the values obtained were compared to those of the wt FucO. Deduced kinetic parameters have been collected in table 3. As has already been mentioned, kinetic measurements were only successfully gathered for the N71D mutant, and consequently only that mutant could be analyzed and compared to the wild-type.

As can be seen in table 3 and figures 9 and 10, the kinetic parameters deduced form the aforementioned assay indicated a certain level of pH-dependence of the studied oxidation reactions, most clearly seen in the $k_{\text{cat}}/K_m$ and $k_{\text{cat}}$ values. $k_{\text{cat}}$ increases more than tenfold from the lowest pH measured (pH 8; 0.16 s$^{-1}$ (wt), 0.10 s$^{-1}$ (N71D)) up to approx. pH 9.5 (1.72 s$^{-1}$ (wt), 1.49 s$^{-1}$ (N71D)). After the pH 9.5 point the $k_{\text{cat}}$ values for the wt appear to level out whereas the mutant exhibits a more scattered pattern. The $k_{\text{cat}}$ increase starts from a rather low level, though, which indicates that the observed $k_{\text{cat}}$ increase can not be considered overly dramatic in neither wt nor mutant FucO. Both wt and N71D mutant FucO follow the same general pattern in this regard, though with N71D mostly exhibiting slightly lower $k_{\text{cat}}$ values throughout the pH interval. Exceptions in this pattern can be noted at pH 9.25 (0.50 s$^{-1}$ (wt), 0.54 s$^{-1}$ (N71D)) and 10.25 (1.62 s$^{-1}$ (wt), 1.89 s$^{-1}$ (N71D)), both at which the mutant has slightly higher $k_{\text{cat}}$ values – though hardly significantly so. The overall differences between the N71D mutant and the wt are comparably small.

As opposed to the $k_{\text{cat}}$ values, the $K_m$ values appear largely unaffected by the pH change, or at most showing weak pH dependence, in wt and mutant alike. However, the N71D mutant exhibit slightly higher values than the wt, particularly at higher pH, though the overall pattern is similar and the differences seen over the pH interval have to be considered small and subtle at most – particularly considering that the $K_m$ parameter apparently is only weakly pH-dependent. However, something of a peak $K_m$ value – however small - can be discerned at approximately pH 9 (32.9 mM (wt), 35.9 mM (N71D)) for both wt and mutant. The weak pH dependence of $K_m$ suggests that the binding of the substrate is largely unaffected by the ionization of the catalytically important groups of the enzyme, in wt as well as N71D mutant FucO.

The catalytic efficiency, $k_{\text{cat}}/K_m$, exhibits notable pH dependence in both wt and N71D FucO, as illustrated in fig.10. Again, the mutant consistently exhibits a lower value compared to the wt. Generally the wt values are roughly twice those of the mutant, but both share a similar overall pattern of increasing $k_{\text{cat}}/K_m$ with increasing pH with no apparent peak being reached. The values cover a range from 4.1 M$^{-1}$s$^{-1}$ (N71D) and 6.7 M$^{-1}$s$^{-1}$ (wt) to 85.6 M$^{-1}$s$^{-1}$ (N71D) and 152.9 M$^{-1}$s$^{-1}$ (wt).

In this context it should be noted that the conditions in which the kinetic parameters were gathered, particularly regarding the NAD$^+$ cofactor concentration, might not have been entirely saturating conditions for the N71D mutant measurements. From fig. 8 it can be noted that saturation appears to have been reached for the wt at the 0.2 mM NAD$^+$ used in the kinetic assay, but that this might not be the case for the N71D mutant. These measurements were conducted for diagnostic purposes, and the values gathered only represent one measurement per concentration, at one select pH, and are thus not statistically reliable, but still indicates that the $k_{\text{cat}}$ and $K_m$ values deduced might in fact be slightly too low and could be considered apparent values.

pH titrations of $k_{\text{cat}}$ and of $k_{\text{cat}}/K_m$ were also analyzed, resulting in p$K_a$ determinations gathered
in table 2.

Given that the data, upon which the pKₐ determination was based, could not be ideally fitted to the model used (fig. 11) the pKₐ values calculated have a rather high standard error. The poor fit was most likely a result of too few data points gathered. As a consequence of the high standard error there is an overlap in the possible pKₐ range determined for the N71D mutant and the wt FucO, making absolute interpretations somewhat difficult. It can, however, be noted that the N71D mutant – however subtle the difference may be – exhibits a lower pKₐ value compared to the wt, regarding the kₐ/⁰ₚ⁻¹ based determinations of the pKₐ (9.87 (N71D), 9.92 (wt)) and the situation is reversed for the kₐ⁻¹-based pKₐ (9.49 (N71D), 9.19 (wt)). However, in the kₐ⁻¹/Km⁻¹-based determination, it can be argued that the obtained value is practically the same for both the wt and the N71D mutant. The kₐ⁻¹-based pKₐ differs slightly more between wt and mutant, but the rather wide pKₐ range permitted via the standard error implies that this pKₐ determination too might essentially be the same between wt and mutant. The slightly lower pKₐ value deduced from the kₐ⁻¹ plot could be indicative of some effect on the enzyme-substrate complex.

Table 2. pH dependence of kₐ and kₐ⁻¹/Km. The pKₐ values for wt and N71D mutant FucO, based on kₐ or kₐ⁻¹/Km as parameters, using 1-propanol as substrate.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>N71D FucO</th>
<th></th>
<th>wt-FucO</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pKₐ</td>
<td>std. err.</td>
<td>pKₐ</td>
<td>std. err.</td>
</tr>
<tr>
<td>kₐ</td>
<td>9.49</td>
<td>0.23</td>
<td>9.19</td>
<td>0.32</td>
</tr>
<tr>
<td>kₐ⁻¹/Km</td>
<td>9.87</td>
<td>0.26</td>
<td>9.92</td>
<td>0.16</td>
</tr>
</tbody>
</table>

Table 3. Kinetic parameters of FucO (wt and N71D mutant) at varying pH, substrate (1-propanol), and constant concentration of NAD⁺ cofactor.
Figure 8. Graphical representation of the NAD$^+$ saturation curve performed as a diagnostic to deduce saturating conditions of the cofactor. Kinetic data was gathered at pH 10 (0.1 M glycine buffer), in the presence of 40 mM substrate (1-propanol), 0.1 µM (wt or N71D) FucO, and NAD$^+$ in a concentration range between 0.01-1 mM, at 340 nm.

Table 4. Kinetic parameters, for overview of saturating conditions of cofactor, deduced from the NAD$^+$ saturation curve ($[\text{NAD}^+] = 0.01-1$ mM), with $[E] = 0.1$ µM (wt or N71D FucO) and 1-propanol as substrate (at 40 mM), at pH 10.

<table>
<thead>
<tr>
<th>FucO variant</th>
<th>pH</th>
<th>$k_{\text{cat}}$ (s$^{-1}$)</th>
<th>std err</th>
<th>$K_m$ (mM)</th>
<th>std err</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
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<td>1.6130</td>
<td>0.0592</td>
<td>2.23E-02</td>
<td>3.75E-03</td>
</tr>
<tr>
<td>N71D</td>
<td>10</td>
<td>2.4240</td>
<td>0.0699</td>
<td>5.98E-02</td>
<td>6.40E-03</td>
</tr>
<tr>
<td>N71D</td>
<td>18</td>
<td>2.0540</td>
<td>0.0678</td>
<td>8.48E-02</td>
<td>6.20E-03</td>
</tr>
</tbody>
</table>

Figure 8. A. $K_m$ (M) vs. pH. B. $k_{\text{cat}}$ vs. pH.
Figure 9. The pH dependence of (a) $K_m$ (M) and (b) $k_{cat}$ (s$^{-1}$) for wt and N71D FucO.

A. Determination of $pK_a$, ionization constant, for wt FucO; $k_{cat}$ (s$^{-1}$) vs. $[\text{OH}^-]$ (M, normalized)

B. Determination of $pK_a$, ionization constant, for wt FucO; $k_{cat} / K_m$ (M$^{-1}$ s$^{-1}$) vs. $[\text{OH}^-]$ (M, normalized)
Figure 11. Graphs, from which the pKₐ values have been determined from the inflection point of the curves. The upper row (a and b) of graphs represents the pKₐ determination for the wt FucO, using $k_{\text{cat}}$ (a) and $k_{\text{cat}}/K_m$ (b) as parameters vs. [OH⁻], respectively. [OH⁻] has been normalized by a factor of $10^7$ for computational purposes.

The lower row (c and d) represents the pKₐ determination for the N71D mutant FucO, using $k_{\text{cat}}$ (c) and $k_{\text{cat}}/K_m$ (d) as parameters vs. [OH⁻], respectively. The graphs were constructed in SIMFIT.
Discussion

The main purpose of the study at hand was the investigation of whether a few rationally selected amino acid mutations in the *E. coli* FucO enzyme could alter the pH dependence of the enzymatically catalyzed oxidation of alcohols – more specifically in this case, using propanol as model substrate as opposed to the natural substrate 1,2-propanediol.

The main basis for the selection of residues to modify was the location of the residues in relation to the enzyme active site (fig. 12), and the observation that the suggested residue interchanges can be found in related proteins – such as, for example, alcohol dehydrogenase IV from *S. cerevisiae* (Montella, *et al.* 2005).

An additional consideration was the possible involvement of these residues in the proton transfer during the enzymatic catalysis. As the catalytic mechanism of FucO has not been elucidated, a proposed proton transfer pathway (fig. 12) could be constructed based on what is known from similar proteins. In this scheme the proton is transferred from the substrate (alcohol or diol) hydroxyl, via a molecule of water, to the 3'-OH of the cofactor ribose moiety, further on to the NH$_2$-group of the highly conserved K162, followed by the amide of N71, before finally being passed on to the amide of N274 (Mikael Widersten, personal communication). By this model, alterations of the proposed residues N71 and N274 could be expected to result in a detectable effect on the catalytic characteristics of the enzyme, thereby shedding further light on the possible mechanisms involved in catalysis.

As already stated, only the N71D mutant was successfully expressed, purified and characterized and consequently only results pertaining to that mutant – in relation to the wt FucO also characterized - will be discussed.
Figure 12. The nucleotide binding site of FucO, based on the coordinates of 1RRM (PDB). The proposed proton transfer pathway is suggested. In this scheme the proton is transferred from the substrate (alcohol or diol) hydroxyl, via a molecule of water, to the 3'-OH of the cofactor ribose moiety, further on to the NH$_2$-group of the highly conserved K162, followed by the amide of N71, before finally being passed on to the amide of N274 (Mikael Widersten, personal communication). Image created using the pymol molecular graphics system (Delano scientific).

**N71D mutation effect on kinetic parameters**

Overall, the effect of the N71D mutation on the pH-dependence of the oxidation reaction appears to have been subtle at most. Consequently, the interpretations of the results will by necessity have to be somewhat cautious and conservative in nature. Nevertheless, from the data collected – regarding 1-propanol as substrate - it was noted that the N71D mutation resulted in a small decrease of $k_{cat}$ over the pH range. From the $K_m$ perspective the pattern showed a concomitant slight (possibly negligible) increase – at the higher pH values - of $K_m$, compared to the wt FucO situation over the pH range. Taken together, the catalytic efficiency – $k_{cat}/K_m$ – of the mutant was overall reduced to roughly half that of the wt over the studied pH range.

Both the $k_{cat}$ values, and the $K_m$ values, did however not differ dramatically between wt and mutant. Particularly $K_m$, in relation to the substrate, varied over a very small range over the pH range and suggests that at most weak, if any, pH dependence for this parameter can be inferred. A slight “peak” of $K_m$ values may be hinted at around pH 9 for both wt and mutant but this was considered to be
too subtle to infer any relevance to. Similarly, the range over which $k_{\text{cat}}$ changed with pH was not by any means dramatic, though a certain pH dependence of this parameter was concluded. Again, a small peak was suggested at approx. pH 9.5 for wt and mutant alike, but the results were inconclusive.

At the highest pH (10.25), additional parameters such as enzyme stability at elevated pH which affect data reliability, might be reflected in the higher $k_{\text{cat}}$ observed for the mutant at this pH. This represented something of a deviation from the general pattern of overall lower $k_{\text{cat}}$ for the mutant over the pH range. If it is assumed that the values gathered at pH 10.25 may be somewhat unreliable, fig. 9 tentatively suggests an estimated $k_{\text{cat}}$ peak value at roughly pH 9.5 (1.72 s$^{-1}$ (wt), 1.49 s$^{-1}$ (N71D)) in both wt and N71D mutant. The value for the mutant is somewhat lower, but these differences cannot be considered conclusive for any extended interpretation. Nevertheless the mutant hasn’t accomplished any real improvement over the wt.

Given that the turnover number ($k_{\text{cat}}$) was not dramatically affected by the N71D mutation, it can be interpreted to suggest that the mutation did not alter a rate determining step in the catalysis but rather some aspect of the catalytic mechanism of more peripheral importance.

Considering that $k_{\text{cat}}$ describes the breakdown of the enzyme-substrate complex into product, it seems that the present mutation (N71D) has a small – if not negligible – effect on this parameter.

To interpret the significance of the N71D mutation regarding the  – however slightly - increased $K_m$, for the substrate, it should also be noted that a similar $K_m$ increase was noted for the NAD$^+$ cofactor as well. These $K_m$ changes, for substrate as well as for cofactor, could be indicative of that the mutation has to some degree affected the structure of the FucO active center (Obradors N. et al. 1998), and possibly affected the stability of the enzyme-substrate complex (Whitford, 2005). However, as previously stated, the $K_m$ values appeared largely pH independent in wt and mutant alike, thus promoting the conclusion that the ionization of the groups important for catalysis had little or no actual effect on substrate binding.

On a mechanistic note, the noted kinetic parameter changes could possibly reflect additional intermediate steps in the enzymatic reaction analogous to what has been described for the related protein alcohol dehydrogenase (Sekhar & Plapp, 1990). This related enzyme has been shown to change conformation upon cofactor binding (Eklund & Brändén, 1987) and that there is a possible rate limitation on the enzymatic oxidation of alcohols due to the rate of this conformational change. Additionally, an enzyme-NADH-aldehyde complex isomerization is postulated. While the present study did not unveil conclusive evidence of a similar effect in the present FucO system, the similarities between these two enzymes - combined with the data presented – suggests that intermediate interconversion steps and possible rate limitations due to conformational change, like those in the alcohol dehydrogenase alcohol oxidations, could have some equivalent in the present FucO system. The studied mutation might have subtly enhanced these effects. Additional studies would, however, need to be performed to investigate the true extent of such similarities.

Keeping in mind that the deduced $K_m$ for the cofactor, was roughly three times higher in the N71D mutant (59.8 μM) compared to the wt (22.3 μM) case, it is possible that the alleged structural or conformational change has primarily affected cofactor binding, possibly causing a somewhat lowered affinity compared to the wt. Alternatively, as hinted, it could also be speculated that this conformational change is somehow cofactor dependent. These interpretations, however, have to be considered with the caveat that the cofactor $K_m$ determination was not based on data from at least three measurements – as was the case for the substrate $K_m$ determination – meaning that the cofactor $K_m$ value is less statistically reliable. Additionally, the cofactor $K_m$ value was only determined at pH 10. Another caveat refers to the possibility that the $k_{\text{cat}}$ and $K_m$ values relating to the substrate might in fact be apparent values since the cofactor concentration used during the kinetics assay for the N71D mutant might not have been sufficient to provide saturating conditions. Regardless, a pH dependent
conformational change of the enzyme can likely not be conclusively ruled out.

**Characteristics of mutated residues and interactions**

The exact catalytic mechanisms involved in the FucO system are yet to be elucidated, and consequently in-depth mechanistic interpretations of the presented data is at present not possible. However, the data provide some basis for a certain tentative level of mechanistic speculation, as already hinted, though the hypothetical nature of these interpretations must be stressed.

The subtle effect on catalysis and pH dependence, resulting from the N71D mutation, suggests either that this residue is not of central importance for the catalytic activity of the enzyme or that the aspartic acid residue introduced can somehow maintain much of the functionality – if indeed it serves an important mechanistic function - of the previously available asparagine residue. Asparagine has a polar, but uncharged, side-chain with a relatively small Van der Waals (VdW) volume of approx. 148 Å$^3$, a surface area of 160 Å$^2$, and is expected to participate in hydrogen bonds due to the polar side-chain (Whitford, 2005).

Asparatate, on the other hand has a negatively charged side-chain in physiological conditions, with a p$K_a$ of approx. 4.0, a VdW volume of 67 Å$^3$, and a surface area of 150 Å$^2$. Due to the charged side-chain, the residue is expected to exhibit electrostatic interactions with positively charged groups. Keeping in mind that the discussed residue appears to be in close proximity (fig. 12) to a highly conserved Lysine residue (K162), it seems likely that the introduced negative charge, via the aspartate, could affect the charge balance in the area via increased electrostatic interactions and possibly ion pair with this Lysine. This in turn could push equilibrium of the catalytic reaction towards a complex that binds substrate and/or cofactor less well, again providing some hypothetical support for the aforementioned hypothesized conformational change of the mutant. Another parameter to consider in this context is if the addition of substrate in itself – or in combination with the cofactor - contributes to the change in equilibrium or conformation, towards a lower affinity complex.

An extended interpretation of the data, however, suggests that the local catalytic environment did not change to any appreciable extent, which is somewhat evident from the deduced ionization constants (p$K_a$) which are practically unaltered by the N71D mutation. This change, or lack thereof, seems to indicate that the N71 residue does not serve a crucial role in the FucO catalysis.

The lack of a detectable p$K_a$ effect might however not contradict an effect on the proton transfer during catalysis, or a change in the local electrostatic environment in the active site, considering that the rate of proton transfer likely is much faster than the rate limiting step -reflected in the $k_{cat}$ and p$K_a$ determinations - of the reaction. As described by Sekhar and Plapp (1990), proton transfer is usually a very fast process and a similarly rapid proton relay system is not unlikely in the present FucO system as well, though the mechanistic details might differ. Consequently, even if the proton transfer potentially was severely affected by the N71D mutation, the proton transfer rate might still have been too fast to be detected as rate limiting with the presently applied methodology.

Analysis of the pH dependence of the studied reaction revealed ionization constants, p$K_a$. This titration possibly relates to enzyme-contributed groups involved in the rate-determining steps of catalysis. Theoretically the origin of this titratable group might relate to, for example, a lysine (p$K_a$ 10.5) residue or possibly a tyrosine (p$K_a$ approx. 10), both with p$K_a$ values near 10 (Whitford, 2005) approximating the p$K_a$ values experimentally determined. Considering that there is a conspicuously located conserved lysine (K162) residue at the active site of the enzyme, it is possible that this lysine is the microscopic origin of this p$K_a$, though alternative interpretations would be possible.

The deduced p$K_a$ values (p$K_a$ ($k_{cat}$) = 9.49; p$K_a$ ($k_{cat} / K_m$) = 9.87), for the mutant, were in close proximity with p$K_a$ determinations for the wt (p$K_a$ ($k_{cat}$) = 9.3; p$K_a$ ($k_{cat} / K_m$) = 10.1) performed by
Cecilia Blikstad (unpublished observations). The present study wt data was; pKₐ (kₗ) = 9.19 and pKₐ (kₗ /Kₘ) = 9.92. Consequently, the data seems comparable, and further emphasizes that the N71D mutation effect was very small in regards to the pH dependence of kₗ and kₗ /Kₘ.

Finally, the N71D mutation appears to not have provided the pH optimum change aimed for, but rather resulted in more subtle effects on the enzyme studied.

**Conclusions**

The combined results from the data gathered for the studied N71D mutant can in conclusion only be interpreted as having, at most, resulted in subtle effects on the studied characteristics. In many regards the differences observed in relation to the wt FucO were small and possibly in some cases not significant.

While the studied amino acid interchange (N71D) did not result in a constructive alteration of the pH dependence of the enzymatic alcohol oxidation, as was the aim, it did provide data which could be of value in future studies regarding FucO catalysis and pH dependence, to for example elucidate the enzymatic mechanism involved as well as the mechanistic functionality of the active site residues.

**Future prospects**

Given that the N274H mutant was unsuccessfully purified and therefore was not characterized, this mutation is still interesting in the context of studies of the present type. Attempts to repeat characterization of the N274H mutant would be needed to deduce if this mutation actually is able to alter the pH dependence of the alcohol oxidation of FucO, as was speculated in the current study.

Another FucO variant, potentially interesting to characterize in terms of pH dependence, would be a combined, double mutant FucO, incorporating both the N274H and the N71D mutations in the same variant.

Additional studies would also be needed to elucidate the precise role of the N71D and N274H residues, if any, and overall to deduce a detailed catalytic mechanism of the FucO enzyme.

On a more general note, when it comes to the study of the FucO enzyme, having access to more crystallographic structures of higher resolution than what is presently available, as well as having structures with more well defined coordinates for substrates and cofactor, would greatly help in enabling better rationale for future attempts to manipulate the properties of FucO by rational mutagenesis.
References


