Bio-catalytic cascades and molecular oxygen-accessing amines and nitriles

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Chapter 5

Crystallographic study on the engineered amine dehydrogenase LE-AmDH-v1 originated from *Geobacillus stearothermophilus*
5.1 Introduction

Protein crystallography allows the determination of the 3D structure of proteins. Structural information plays a key role in modern bio-sciences, since it provides the crucial understanding of the interactions between either receptors and ligands, or proteins and ligands. In general, this is invaluable from the standpoint of biocatalysis. Throughout this thesis, the topic of amine synthesis by amine dehydrogenases (AmDHs) was discussed from the methodological point of view. However, the issues lying in the somewhat limited substrate scope of AmDHs holds a great potential for protein engineering approaches. The limitation itself stems from the fact that the initially reported AmDHs have mainly been engineered from L-amino acid dehydrogenases (L-AADHs). Only few native AmDHs were recently discovered through bioinformatics approaches, and possess stereocomplementary selectivity compared with the engineered enzymes. Since all of the L-AADHs share a very similar architecture of

\[ \text{Scheme 5.1. Comparison of: a) natural reaction of LysEDH and b) substrate scope of engineered LE-AmDH-v1 variant.} \]
the active site, the engineering of novel AmDHs with diverse substrate scope and enhanced catalytic performance is still of great relevance. Therefore, our group rationally engineered the ε-(deaminating)-L-lysine dehydrogenase from Geobacillus stearothermophilus (LysEDH)\textsuperscript{15}—whose natural reaction is depicted in Scheme 5.1a—to produce a wide array of optically active aromatic amines (Scheme 5.1b) with excellent enantioselectivity.\textsuperscript{16} The engineering efforts were guided by a homology model that was based on three templates (further referred to as composite homology

Figure 5.1. Active sites of LysEDH and LE-AmDH-v1 (backbone and identical amino acid residues are depicted in cyan, the single point mutation F173A is highlighted in orange) with docked L-lysine (magenta) and α-methylbenzylamine. The mutation of F173A present in LE-AmDH-v1 clearly alleviates steric hindrance, thus enabling the binding of B inside the active site of LysEDH.
model). The model revealed that the remarkable broadening of the substrate specificity of the best variant (LE-AmDH-v1) compared with the wild-type enzyme was caused by the increase of cavity size in the active site (Figure 5.1). Indeed, we were intrigued by the in silico generated explanation and we decided to perform a crystallographic study on LE-AmDH-v1 in order to verify the structure experimentally. It is important to mention that the structure of the first native amine dehydrogenases have been reported early 2019 in concomitance to our work.\textsuperscript{13}

5.2 Results and discussion

5.2.1 LE-AmDH-v1

The initial biochemical characterization of LE-AmDH-v1, performed in the original publication, revealed high stability of the enzyme. In fact, the variant was able to perform reductive amination in a wide range of both temperatures (20–60 °C) and pH (7.5–9.5), respectively. This remarkable operational stability was further highlighted by the observation of exceptional long-term stability without extensive engineering. The enzyme was fully active after seven days at 4 °C and it also retained above 80% residual activity after seven days at 40 °C and more than 60% residual activity after seven days at 50 °C.\textsuperscript{16} Additionally, we determined the LE-AmDH-v1 to be a tetramer using size exclusion chromatography. Armed with the initial data describing the behavior of the variant, we started the initial screening of the crystallographic conditions.

5.2.2 Quantification of bound cofactor

In general, rational design relies on the knowledge of reaction mechanisms. Therefore, it is beneficial to have reaction components (ligands) present inside the structure. Thus, we wanted to perform co-crystallization of our protein with the coenzyme NAD(H) bound in the active site. In order to know whether we actually have to add cofactor or it is already present in the protein after purification, we developed a HPLC-based quantification method based on a previously reported method thereby allowing the quantification of NAD and NADH in \textit{Saccharomyces cerevisiae}.\textsuperscript{17} We decided to focus
on NAD(H) instead of NADP(H), since we use NAD(H) as cofactor of choice in biotransformations. Moreover, according to the original publication about the wild-type,\textsuperscript{15} the catalytic activity of WT enzyme with NADPH is 22\% of that one with NADH. The analytical method itself entails thermal denaturation of the protein of interest, centrifugation of the sample and subsequent analysis of the sample by RP-HPLC using gradient of MeOH in Milli-Q water and 0.1\% of TFA, and quantification by UV detection at 260 nm. The method is quite sensitive, thus allowing us to work at concentration levels of protein (and cofactor) ranging from 0.01–0.25 \( \mu \text{M} \). Quantification was performed using a calibration curve, and the standard solutions were treated in the same way as the protein solution. Interestingly, we did not detect NAD\(^+\) and NADH, respectively, when we applied the method to the sample containing LE-AmDH-v1. Therefore, we performed control experiments using FDH as a standard protein in order to validate that the method is able to actually detect a cofactor present in the protein sample method. Control experiments revealed that FDH is approximately 30\% loaded after purification. This value was determined also after spiking the protein with NAD(H), albeit it appears that in the presence of protein, there was an oxidation of NADH to NAD\(^+\). We attribute this slight discrepancy to the presence of small amounts of CO\(_2\) in the reaction buffer (Table 5.1).

Table 5.1. Quantification of bound NAD(H).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Protein (( \mu \text{M} ))</th>
<th>Cofactor (( \mu \text{M} ))</th>
<th>NAD(^+)</th>
<th>NADH</th>
<th>NAD(^+)</th>
<th>NADH</th>
<th>Total</th>
<th>Without added cofactor</th>
</tr>
</thead>
<tbody>
<tr>
<td>LE-AmDH-v1</td>
<td>250</td>
<td>-</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Control (FDH)</td>
<td>250</td>
<td>-</td>
<td>75.6</td>
<td>6.0</td>
<td>29.8</td>
<td>2.4</td>
<td>32.2</td>
<td>-</td>
</tr>
<tr>
<td>Control (FDH+NAD(^+))</td>
<td>250</td>
<td>160</td>
<td>218.2</td>
<td>25.1</td>
<td>86.0</td>
<td>10.1</td>
<td>96.1</td>
<td>32.1</td>
</tr>
<tr>
<td>Control (FDH+NADH)</td>
<td>250</td>
<td>160</td>
<td>203.1</td>
<td>57.5</td>
<td>80.1</td>
<td>23.0</td>
<td>103.1</td>
<td>39.1</td>
</tr>
</tbody>
</table>
5.2.3 Initial screening

The initial screening of the crystallization conditions was performed using four crystallography cores from Qiagen (Cryos Suite, JCSG Core II-IV) with three different protein concentrations (56, 28 and 14 mg ml\(^{-1}\)) and at two different temperatures (4 °C and 20 °C). The screening was set up in 96-well TTP iQ-plates using the sitting-drop method by mixing 100 nl of reservoir solution with 100 nl protein sample (Mosquito liquid handling robot, TTP Labtech). From all of the test conditions, two yielded crystals (Figure 5.2) – condition C10 at 20 °C from Cryos Suite (56 mg ml\(^{-1}\) protein; 0.085 M tri-sodium citrate pH 5.6, 0.17 M Na/K tartrate, 1.7 M ammonium sulfate, 15% (v/v) glycerol; further referred to as condition A) and condition G4 from the same screening kit (28 mg ml\(^{-1}\) protein; 0.09 M HEPES sodium salt pH 7.5, 0.18 M magnesium chloride, 27% (v/v) PEG 400, 10% (v/v) glycerol; further referred to as condition B). Condition B

Figure 5.2. Comparison of crystals observed in a) crystallographic condition A (0.085 M tri-sodium citrate pH 5.6, 0.17 M Na/K tartrate, 1.7 M ammonium sulfate, 15% (v/v) glycerol) and b) crystallographic condition B (0.09 M HEPES sodium salt pH 7.5, 0.18 M magnesium chloride, 27% (v/v) PEG 400, 10% (v/v) glycerol) during initial round of condition screening
yielded crystals one week after application of condition A. Interestingly, none of the observed crystals were well-defined and, therefore, we have decided to further optimize the conditions in order to obtain better quality crystals.

5.2.4 Optimization of crystallographic condition

Optimization of condition A was performed in a 6x4 condition grid (total of 24 conditions) exploring the effect of changes in pH (4.5–6.1) and increasing concentration of ammonium sulfate (1.1–1.9 M). Additionally, we also prepared the same condition, but

Figure 5.3. Influence of increasing pH and increasing concentration of ammonium sulfate on formation of LE-AmDH-v1 crystals.
omitting Na/K-tartrate in order to have a better idea about the influence of tartrate on crystal formation. Fortunately, three conditions yielded crystals after a week, allowing us to observe the effect of both increasing pH and increasing concentration of ammonium sulfate. Increase in both parameters influenced precipitation of the protein, thereby yielding nicely defined crystals at condition C4 (0.085 M tri-sodium citrate pH 5.6, 0.17 M Na/K tartrate, 1.7 M ammonium sulfate, 15% (v/v) glycerol) and large amount of microcrystals in corresponding conditions with one step higher pH as well as one step higher concentration of ammonium sulfate (Figure 5.3). Further refinement of the crystallization conditions derived from condition C5, in which investigating the influence of varying concentrations of tartrate, glycerol and again ammonium sulfate yielded crystals in almost all cases. Concerning the specific influence of the additives, it was fairly straightforward to see that the earliest crystals appeared at highest concentration of tartrate, ammonium sulfate and glycerol (after 2 days). Moreover, decreasing concentrations of compounds of interest yielded crystals with delay. Interestingly, it appeared that a certain concentration range promoted the formation of well-defined single crystals.

Condition B was optimized in similar way. The 24-well grid was set in twenty-four conditions detailing the effect of pH (7–8.5, changing in rows) and growing concentration of PEG 400 (21–31%, changing in columns), which did not yield any crystals up to date (18 months). A precipitate was observed only in several drops with the highest concentration of PEG obtained after 90 days, which shows again the exceptional stability of LysEDH. This suggests also that preparation of LysEDH in HEPES buffer in more alkaline pH with MgCl₂ and certain percentage of PEG 400 might be highly useful for achieving stabilization of the protein. We have overcome the challenges linked to the solving of the structure, described in following sections, and obtained the 3D structure of the apo-enzyme. The next step was focused on the co-crystallization with the cofactor.

5.2.5 Search model based on available PDB structures

The molecular replacement generally relies on the similarity in the tertiary structure of two homologous proteins. However, the sequence identity between LE-AmDH-v1 and
closest homologous structures, found in PDB database using Phyre2 tool\textsuperscript{19} and re-examined with LALIGN server,\textsuperscript{20} was not sufficient—below or around 30\% (Table 5.2). Therefore, we attempted to generate the model based on the sequence alignment and the lowest B-factor of the structure. We decided to use entry 5 (PDB: 5L78) to start the first round of molecular replacement trials. Since the 5L78 contains three extra domain compared to the LE-AmDH-v1 (Figure 5.4a), we initially truncated the extra region as well as most of the flexible loops. However, we did not obtained any correct solution. Moreover, we were not sure about the composition of the asymmetric unit, since it was most likely for the asymmetric unit to contain three or four monomers corresponding to the Matthews coefficient of 2.64 \textsuperscript{3} Å\textsuperscript{3} Da\textsuperscript{-1} (53.4\% of solvent) and 1.98 \textsuperscript{3} Å\textsuperscript{3} Da\textsuperscript{-1} (37.9\% of solvent), respectively.

Table 5.2. List of LE-AmDH-v1 homologues available in PDB with the sequence identity and sequence similarity. TBP – to be published.

<table>
<thead>
<tr>
<th>Entry</th>
<th>PDB</th>
<th>Seq. Id. [%]\textsuperscript{19}</th>
<th>Seq. Id. [%]\textsuperscript{20} (overlapping AAs)</th>
<th>Homology model template\textsuperscript{16}</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>4rl6</td>
<td>20</td>
<td>23 (416)</td>
<td>-</td>
<td>TBP</td>
</tr>
<tr>
<td>2</td>
<td>4ina</td>
<td>22</td>
<td>25 (408)</td>
<td>-</td>
<td>TBP</td>
</tr>
<tr>
<td>3</td>
<td>1e5l</td>
<td>25</td>
<td>27 (269)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>2axq</td>
<td>25</td>
<td>28 (276)</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>5l78</td>
<td>23</td>
<td>27 (271)</td>
<td>-</td>
<td>TBP</td>
</tr>
<tr>
<td>6</td>
<td>3abi</td>
<td>33</td>
<td>33.1 (381)</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>4pip</td>
<td>13</td>
<td>n. d.</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>2ph5</td>
<td>14</td>
<td>n. d.</td>
<td>-</td>
<td>TBP</td>
</tr>
<tr>
<td>9</td>
<td>3ic5</td>
<td>33</td>
<td>33 (117)</td>
<td>-</td>
<td>TBP</td>
</tr>
<tr>
<td>10</td>
<td>2ixa</td>
<td>15</td>
<td>n. d.</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>1e5q</td>
<td>25</td>
<td>same as 2</td>
<td>Yes</td>
<td></td>
</tr>
</tbody>
</table>
5.2.6 Search model based on composite homology model

Molecular replacement using the 5L78 model failed. However, we decided to use the homology model of LE-AmDH-v1 as a search template. Again, we removed the flexible loop region (Figure 5.4b) and subjected the datasets to rounds of molecular replacement. Interestingly, we observed a better quality of density maps for monomer A and B; however, any additional monomer did not really correspond to the density map. Guided by intuition, we decided to run a round of molecular replacement by searching only for two monomers in the asymmetric unit (Matthews coefficients 3.96 Å³ Da⁻¹, 69.0% of solvent) and, to our surprise, we observed a sudden improvement in the quality of the density map along with the drop of R-factor from the range of > 0.5 to ~ 0.4. In the end, we built a model of the apo-enzyme exhibiting a satisfactory R-factor (~ 0.2). We decided to continue the project with further co-crystallization of the LE-AmDH with additional ligands (cofactor, substrate/product analogues, etc.).

5.2.7 Co-crystallization of LE-AmDH-v1 with NAD⁺

Based on the previously obtained data on the influence of buffer components, a new twenty-four condition grid was designed. The grid was based on modified optimal condition A: 0.085 M tri-sodium citrate pH 5.5, 0.16 M Na/K tartrate, 1.8 M ammonium sulfate, 15% (v v⁻¹) glycerol, thus having lowered amount of Na/K tartrate and increased...
concentration of ammonium sulfate. The latter was achieved by a 0.1 M increment in order to moderately promote precipitation and increase the final size of the crystals at the same time. Two different protein concentrations were used (21 and 28 mg ml\(^{-1}\)) as well as a control grid without the addition of cofactor in order to confirm the difference. Interestingly, a grid where 1.2 molar equivalent of NAD\(^+\) was present afforded crystals of sufficient size in two weeks (condition A5 in Figure 5.5). On the other hand, crystals started appearing only after one month when the cofactor was not present. Moreover, we also observed a phase separation between the mother liquor and the protein without the subsequent formation of crystals when the NAD\(^+\) was not present. Interestingly, this often happened in the conditions that yielded crystals slowly in presence of NAD\(^+\) (condition B3 in Figure 5.5). Interestingly, prolonged residence time of crystals resulted in their additional growth (condition D1 compared with the same condition but that did not contain the NAD\(^+\), shown in Figure 5.5). It appears that the presence of cofactor helps to induce and maintain a stable conformation of LysEDH. This hypothesis is supported by the thermostability data, since the melting temperature increased in presence of cofactor by 2 °C.\(^{16}\) The initial solving of the structure at ~ 2.4 Å demonstrated that we can observe the cofactor in the active site. Consequently, we
decided to focus the crystallization campaign on the co-crystallization with cofactor and additional ligand in order to elucidate the binding of the substrate in the active site.

Figure 5.5. Influence of NAD$^+$ presence on the formation of LE-AmDH-v1 crystals under several different conditions.
5.2.8 Co-crystallization of LE-AmDH-v1 with NADH and benzylamine

Co-crystallization of ε-LysDH with benzylamine was performed using the same grid as described in previous section (5.2.1.5). It is noteworthy that the initial target for co-crystallization was α-methyl styrene, since it is a non-reactive structural analog of the imine intermediate formed during the reaction. However, due to the insolubility of α-methyl styrene in any available solvent (e.g., water, ethanol or DMSO, at last at 10 mM concentration), it was decided to use benzylamine solubilized in DMSO together with NADH. NADH was chosen to substitute NAD⁺, since in presence of NAD⁺ the enzyme would most likely induce oxidative deamination of benzylamine to benzaldehyde. Two

Figure 5.6. Comparison of electron density corresponding to NAD⁺ in: a) dataset from crystal grown only in presence of NAD⁺ and b) the crystal grown in presence of NADH, DMSO and benzylamine, respectively.
drops were set at 28 mg ml\(^{-1}\) of protein: one of the drops contained 1.2 molar equivalent of NADH, 1.9 molar equivalent of benzylamine and 0.5% of DMSO. The second drop contained only 1.2 molar equivalent of NADH and 0.5% DMSO to investigate the effect of DMSO on the crystallization process. In general, the observed trends matched the observations from the experiment with 1.2 molar equivalent of NAD\(^+\), thus suggesting that addition of DMSO did not cause a disruption of the crystallization process. However, we have not observed the presence of the benzylamine in the active site. Moreover, we observed the difference in the quality of the electron density corresponding to the cofactor. The electron density of the NAD\(^+\) was significantly better than the one of NADH in the region corresponding to the nicotine amide section of the cofactor (Figure 5.6). At this point, we can suspect the issue to be related either to the DMSO or the benzylamine itself. However, there was no proof of any additional ligand in the vicinity of the cofactor-binding site that could explain the lower occupation of the binding site in the presence of benzylamine and DMSO.

5.2.9 Co-crystallization of LE-AmDH-v1 with NADH and L-lysine, \((R)-1\)-phenylethylamine and \((R)-1\)-phenylpropylamine

Co-crystallization of LysEDH with benzylamine did not yield the expected results. Therefore, we decided to try to co-crystallize LE-AmDH-v1 with a set of different ligands. We chose L-lysine since it is the natural substrate (\(K_M\) for the wild-type enzyme was 0.73 mM at 50 °C) as well as \((R)-1\)-phenylethylamine and \((R)-1\)-phenylpropylamine (Figure 5.7a). The experiment was performed in a modified grid utilizing only the best conditions and elevated concentrations of ligands compared with the benzylamine containing grid. Three drops were set for each condition, each containing 2 molar equivalents of NADH and 5, 10 or 15 molar equivalents of the ligand. Together, the setup added six conditions for each ligand with 18 different drops. Ultimately, each condition afforded crystals which suggests that the crystallization conditions are really well optimized. However, molecular replacement revealed that none of the ligands were bound in the active site. In fact, the solution contained the molecule of the cofactor. However, the corresponding electron density was substantially worse than the electron
density of the cofactor obtained from the conditions without the amine-containing ligands. Moreover, we observed a sulfate ion coordinated to R242, Y169 and G158 (Figure 5.7b). The sulfate ion might be effectively blocking the active site for the substrates to enter, since already the $K_m$ reported for the lysine is not very high and the $K_M$ values reported for (R)-1-phenylethylamine are almost an order of magnitude higher (7.2 mM at 50 °C and 9.6 mM at 30°C). Moreover, there is no report of $K_M$ values for (R)-1-phenylpropylamine. However, we can speculate that the affinity of the ligand to

Figure 5.7. Co-crystallization of LE-AmDH-v1 with NADH and ligands. a) Influence of ligands on crystal growth. b) Sulfate ion (yellow) blocking the active site of LE-AmDH-v1 (cyan) by forming the H-bonds with R242, Y169 and G158.
the LE-AmDH-v1 is even lower considering the lower reported conversions. Hence, we could reason that the two aromatic ligands simply do not have enough affinity towards the enzyme to occupy the active site enough to manifest their presence in the electron density and that the binding of L-lysine in the active site is prevented by the large excess of sulfate ions (1.8 M of SO$_4^{2-}$ compared to the 0.012–0.038 M of L-lysine).

### 5.2.10 Solved structures

Over the course of the project, we solved structure of LE-AmDH-v1 in the apo-enzyme form as well as with additional coenzyme bound in the active site (i.e., holo-form). The protein crystallized by applying a single condition, however, the range of the concentration of the components could be varied. In the end, we devised a quick screening setup allowing controlled growth of large crystals (> 200 μm in diameter). The general condition for growing of the LE-AmDH-v1 crystals contained 0.085 M trisodium citrate pH 5.5, 1.8 M ammonium sulfate, 0.08–0.16 M sodium-potassium tartrate and 12.5–17.5% (v/v) glycerol (for more details, see section 5.4.7). LE-AmDH-v1 crystallized in an orthorhombic space group with one-fold screw symmetry (C222$_1$). Both of the structures crystallized in the same space group and with very similar dimensions of the unit cell (a = 77 Å, b = 166–177 Å, c = 216–218 Å, α = β = γ = 90°). The details about the solutions can be found in Table 5.3. At this point in time, we are still in the process of refining building the structure, however, with ~5% of sidechains and most of the waters, ligands and ions missing, preliminary structures exhibit satisfactory R-factors ~ 0.20 and resolution around 2.2 Å. However, we can already conclude that the homology model predicted the actual structure of the enzyme monomer in a highly reliable manner. The main differences between the homology model and the X-ray structure lie in a number of loop-containing regions as well as in the position of three helices in substrate binding domain. Interestingly, we also observed a slight decrease of the distance between
the subunits when the cofactor is bound in the active site. The differences in the loop regions are understandable due to their general flexibility.

Regarding the protein itself, it can be divided into two domains, namely the cofactor binding domain and a substrate binding domain. The cofactor binding domain consists of 9 helices and 6 sheets and contains a classical Rossmann fold that allows for binding the cofactor. The nicotine amide ring of the cofactor is hidden deep inside the protein. Furthermore, the substrate binding domain consists of 4 helices and 9 sheets. Interestingly, comparison between the archaeal L-lysine dehydrogenase from
*Pyrococcus horikoshii* (Ph-LEDH)\(^{23}\), and the fungal saccharopine reductase from *Magnaporthe grisea* (Mg-ScRed)\(^{21}\) revealed that Ph-LEDH and LE-AmDH-v1 lack the third domain of the archetypal saccharopine reductase fold. Instead, both contain disordered loop-region (L258 - K273 in LE-AmDH-v1 numbering and L240 - K244 in Ph-LEDH numbering). In Mg-ScRed, domain III (I261 - N335 in LE-AmDH-v1 numbering) undergoes significant conformational change upon binding of the substrate. However, its residues appear not to be directly interacting with the substrate. In contrast, the conformational change (also described as transition between an “open” and “closed” conformation in Mg-ScRed) increases the hydrophobicity of the active site by limiting its accessibility to the solvent.\(^{21}\) The presence of domain III in Mg-ScRed is most likely necessary to facilitate the conversion of glutamate and α-aminoadipate semialdehyde into saccharopine. This particular reaction is essentially a reductive amination, thus affording a secondary amine. Therefore, insertion of domain III into LE-AmDH-v1 or direct utilization of Mr-ScRed and further engineering of the active site might be an attractive route on the quest for amine dehydrogenases enabling the synthesis of bulky-bulky secondary amines.
5.3 Conclusion

In conclusion, we have successfully solved the crystal structure of LE-AmDH-v1 using molecular replacement and the homology model of the LysEDH as the template. Albeit the comparison of the in silico model and the experimental structure of LE-AmDH-v1 revealed a slight difference between the two, the active-site shared the same spatial arrangement. Therefore, we can conclude that the homology model predicted the structure extremely well in contrast to the low sequence homology of the available templates in the PDB database. Hopefully, this achievement can stimulate more work and efforts into the further development of homology modelling tools in future. This is particularly valid for the homology model based on multiple templates.\(^{26}\) Furthermore, we were able to solve the structure of LE-AmDH-v1 as an apo-enzyme as well as with NAD\(^+\) bound (Figure 5.8). Sadly, we were not able to obtain the structure with both cofactor and ligand in the active site. The crystal structure will be finalized and submitted to the PDB database in short time and it will further serve as a template for

Figure 5.8. Crystal structure of LE-AmDH-v1 containing bound NAD\(^+\) displayed with co-crystallized sulfate ions, glycerol and tartrate.
additional engineering campaigns involving LE-AmDH-v1. Moreover, the structural similarities between the LE-AmDH-v1 and saccharopine reductase family suggest possible additional engineering targets, which may lead to the enzyme capable of synthesis of secondary amines using large amine donors.
5.4 Experimental

5.4.1 Quantification of bound cofactor

Quantification of bound NAD(H) was performed as follows. Protein was diluted with the buffer (20 mM HEPES pH 7.5, buffer of protein stock solution) to desired concentration (ideal 0.1–0.25 mM). Protein solution was subsequently heated for 10 min at 70 °C, 600 rpm in thermos shaker (Eppendorf). Subsequently, the protein sample was cooled down on ice, centrifuged (4 °C, 7 min, ca. 17000 g) and the aliquot was pipetted into the vial and subjected to analysis. Analysis was performed using RP-HPLC system (Prominence-i LC 2030 3D, Shimadzu) equipped with Shim-Pack GIST GIST 5 μm C18 AQ (150 mm length, 4.6 mm inner diameter) column.

Method: Flow: 1 ml min⁻¹; oven temperature: 30°C; gradient: 90% B in 15 min, 5 min hold; gradient: 100% B in 5 min, 15 min hold. Analysis at λ 260 nm.

Quantification was performed using calibration curve. Set of standard solution of both NAD⁺ and NADH was prepared into the buffer (20 mM HEPES pH 7.5) and treated in the same way as the protein sample. Control reactions with formate dehydrogenase (FDH) containing 0.25 mM enzyme spiked with either 0 or 0.16 mM of NAD⁺ or NADH were also treated in the same way as mentioned previously.

5.4.2 Initial screening

Initial screening of conditions was performed in 96 well plates using four crystallography cores from Qiagen (Cryos Suite, JCSG Core Suite II-IV) with three different protein concentrations (56, 28 and 14 mg ml⁻¹) and at two different temperatures (4 °C and 20 °C). The screening was performed in a 96-well iQ plate (TTP Labtech) using a siting drop – vapor diffusion technique. The 75 μl of solution from the Core was pipetted using 12-channel pipette to the 96-well plate into the reservoir spot. Protein solutions (56, 28 and 14 mg ml⁻¹) were prepare into different 96-well plate. From there, 0.2 μl drops (0.1 μl of protein solution and 0.1 μl of Core solution) were pipetted using Mosquito pipetting robot (TTP Labtech). Finally, the plates were covered with clear air-tight seal and incubated at 4 or 20°C. The drops were monitored for 1.5 month for the crystal growth.
5.4.3 Optimization of conditions A and B

General procedure for preparation of a grid for optimization of crystallographic condition A and B. The conditions were chosen to investigate the influence of the pH, and concentration of ammonium sulfate on the crystal formation in condition A (grid no. 1A), and pH and PEG 400 loading on the crystal formation in condition B (grid no. 1B). 2 ml stock solutions were prepared for each condition and two different grids were prepared for the initial optimization of conditions A and B (Figure 5.9 and Figure 5.10). Contrary to the previous section, these experiments were performed on a larger scale thus using 24-well plate and the so called “hanging drop” method. 800 μl of the solution were pipetted into corresponding well. The cover slip was laid next to the well and 1 μl drop of the mother liquor was pipetted onto the slip as a base for a single drop (3 drops of different concentration in each grid – no. 1A and 1B - grid following - drops – 1 – 56 mg ml⁻¹; 2 – 28 mg ml⁻¹; 3 – 14 mg ml⁻¹). Formation of a drop was finished by pipetting a 1 μl protein solution of aforementioned concentration into the base of a drop and proper mixing using automatic pipette. When all of the three drops were successfully formed, the cover slip was put on the top of the well (i.e., top of the well was prepared by applying a vacuum grease) and gently pressed down to ensure that the well is closed in an air-tight fashion. Process was repeated until all desired well were closed and the grids were incubated at 20 °C and monitored attentively.

<table>
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<td>A</td>
<td>pH 4.5 1.1 M (NH₄)₂SO₄</td>
<td>pH 4.5 1.3 M (NH₄)₂SO₄</td>
<td>pH 4.5 1.5 M (NH₄)₂SO₄</td>
<td>pH 4.5 1.7 M (NH₄)₂SO₄</td>
<td>pH 4.5 1.9 M (NH₄)₂SO₄</td>
<td>pH 4.5 1.7 M (NH₄)₂SO₄</td>
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<tr>
<td>B</td>
<td>pH 5 1.1 M (NH₄)₂SO₄</td>
<td>pH 5 1.3 M (NH₄)₂SO₄</td>
<td>pH 5 1.5 M (NH₄)₂SO₄</td>
<td>pH 5 1.7 M (NH₄)₂SO₄</td>
<td>pH 5 1.9 M (NH₄)₂SO₄</td>
<td>pH 5 1.7 M (NH₄)₂SO₄</td>
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<td>pH 5.5 1.1 M (NH₄)₂SO₄</td>
<td>pH 5.5 1.3 M (NH₄)₂SO₄</td>
<td>pH 5.5 1.5 M (NH₄)₂SO₄</td>
<td>pH 5.5 1.7 M (NH₄)₂SO₄</td>
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<tr>
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<td>pH 6.1 1.1 M (NH₄)₂SO₄</td>
<td>pH 6.1 1.3 M (NH₄)₂SO₄</td>
<td>pH 6.1 1.5 M (NH₄)₂SO₄</td>
<td>pH 6.1 1.7 M (NH₄)₂SO₄</td>
<td>pH 6.1 1.9 M (NH₄)₂SO₄</td>
<td>pH 6.1 1.7 M (NH₄)₂SO₄</td>
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Figure 5.9. Optimization of crystallographic condition A – grid 1A.
Further optimization of crystallographic condition A

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<td>pH 7 23% PEG</td>
<td>pH 7 25% PEG</td>
<td>pH 7 27% PEG</td>
<td>pH 7 29% PEG</td>
<td>pH 7 31% PEG</td>
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<tr>
<td>B</td>
<td>pH 7.5 25% PEG</td>
<td>pH 7.5 23% PEG</td>
<td>pH 7.5 25% PEG</td>
<td>pH 7.5 27% PEG</td>
<td>pH 7.5 29% PEG</td>
<td>pH 7.5 31% PEG</td>
</tr>
<tr>
<td>C</td>
<td>pH 8 21% PEG</td>
<td>pH 8 23% PEG</td>
<td>pH 8 25% PEG</td>
<td>pH 8 27% PEG</td>
<td>pH 8 29% PEG</td>
<td>pH 8 31% PEG</td>
</tr>
<tr>
<td>D</td>
<td>pH 8.5 21% PEG</td>
<td>pH 8.5 23% PEG</td>
<td>pH 8.5 25% PEG</td>
<td>pH 8.5 27% PEG</td>
<td>pH 8.5 29% PEG</td>
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Figure 5.10. Optimization of crystallographic condition B – grid 1B.

5.4.4 Further optimization of crystallographic condition A

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<td>pH 5.5 0.13 M Na/K tartrate</td>
<td>pH 5.5 0.15 M Na/K tartrate</td>
<td>pH 5.5 0.17 M Na/K tartrate</td>
<td>pH 5.5 0.19 M Na/K tartrate</td>
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<tr>
<td>B</td>
<td>pH 5.5 1.6 M (NH₄)₂SO₄</td>
<td>pH 5.5 1.7 M (NH₄)₂SO₄</td>
<td>pH 5.5 1.8 M (NH₄)₂SO₄</td>
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<td>x</td>
<td>x</td>
</tr>
<tr>
<td>C</td>
<td>pH 5.5 11% glycerol</td>
<td>pH 5.5 13.5% glycerol</td>
<td>pH 5.5 15% glycerol</td>
<td>pH 5.5 16.5% glycerol</td>
<td>pH 5.5 18% glycerol</td>
<td>x</td>
</tr>
<tr>
<td>D</td>
<td>x</td>
<td>pH 5.5 1.7 M (NH₄)₂SO₄ Old grid solution</td>
<td>pH 5.5 1.7 M (NH₄)₂SO₄ Old grid solution</td>
<td>x</td>
<td>x</td>
<td>x</td>
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</table>

Figure 5.11. Further optimization of crystallographic condition A – grid 2.

Following the observations based on grid no. 1A, grid no. 2 was setup to further elucidate the influence of glycerol, Na/K tartrate and further refinement of ammonium sulfate (Figure 5.11). The grid was set with inverted concentration of the drops – 1 – 21 mg ml⁻¹; 2 – 28 mg ml⁻¹; 3 – 38 mg ml⁻¹. Formation of a drop was finished by pipetting a 1 μl protein solution of aforementioned concentration into the base of a drop and proper mixing using automatic pipette. When all of the three drops were successfully formed, the cover slip was put on the top of the well (i.e., top of the well was prepared by applying a vacuum grease) and gently pressed down to ensure that the well is closed
in an air-tight fashion. Process was repeated until all desired well were closed and the grids were incubated at 20 °C and monitored attentively.

### 5.4.5 Co-crystallization of LE-AmDH-v1 with NAD⁺

Grids for co-crystallization with NAD⁺ were treated as reported in previous section. The protein concentration of the drops in experiments grid no. 3 and grid no. 4 was 21 and 28 mg ml⁻¹, respectively. Based on the same grid configuration, experiments differed in presence of NAD⁺, concentration of which was 1.2 molar eq. in experiment grid no. 3 and 0 mol eq. in experiment grid no. 4. The exact configuration of the grids is depicted in Figure 5.12.

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<tr>
<td>A</td>
<td>0 M Na/K tartrate</td>
<td>0.04 M Na/K tartrate</td>
<td>0.08 M Na/K tartrate</td>
<td>0.12 M Na/K tartrate</td>
<td>0.16 M Na/K tartrate</td>
<td>0.20 M Na/K tartrate</td>
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<tr>
<td>B</td>
<td>7.5% glycerol</td>
<td>10% glycerol</td>
<td>12.5% glycerol</td>
<td>15% glycerol</td>
<td>17.5% glycerol</td>
<td>20% glycerol</td>
</tr>
<tr>
<td>C</td>
<td>1.4 M (NH₄)₂SO₄</td>
<td>1.5 M (NH₄)₂SO₄</td>
<td>1.6 M (NH₄)₂SO₄</td>
<td>1.7 M (NH₄)₂SO₄</td>
<td>1.8 M (NH₄)₂SO₄</td>
<td>1.9 M (NH₄)₂SO₄</td>
</tr>
<tr>
<td>D</td>
<td>pH 5.1</td>
<td>pH 5.3</td>
<td>pH 5.5</td>
<td>pH 5.7</td>
<td>pH 5.9</td>
<td>pH 6.1</td>
</tr>
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</table>

Figure 5.12. Co-crystallization of LE-AmDH-v1 and NAD⁺ - configuration of grids 3, 4 and 5.

### 5.4.6 Co-crystallization of LE-AmDH-v1 with NADH and benzylamine

Grid no. 5 was setup slightly different; however, the conditions were the same as for the grids no. 3 and no. 4 (Figure 5.12). Protein concentration in both drops was the same (28 mg ml⁻¹) as well as the concentration of the cofactor (NADH, 1.5 mol eq.). However in the first drop there was benzylamine present (1.9 mol eq.) together with DMSO (0.5% v v⁻¹, final concentration) and in second drop there was only DMSO (0.5% v v⁻¹, final concentration). The drops were prepared as described in previous sections. Again, experiments were incubated at 20 °C and monitored attentively.
5.4.7 Co-crystallization of LE-AmDH-v1 with NADH and L-lysine, (R)-1-phenylethylamine and (R)-1-phenylpropylamine

Grid for experiments no. 6 was treated up as reported in previous sections. The drops were prepared as described previously and there were three different drops prepared for each condition (six best conditions for each ligand, the location of conditions is depicted in Figure 5.13). The protein concentration of all wells was 28 mg ml\(^{-1}\) and the drops differed by the concentration of the ligand – drop 1 – 6.4 mM ligand (5 mol eq.), drop 2 – 12.8 mM (10 mol eq.) and drop 3 - 19.2 mM (15 mol eq). L-lysine was added from water stock, whereas (R)-1-phenylethylamine and (R)-1-phenylpropylamine were added as DMSO stocks, thus resulting in final DMSO concentration in the drops 0.6%, 1.3% and 1.9%, respectively. Finally, all of the drops contained 2.55 mM NADH (2 mol eq.). The grid was incubated at 20 °C and monitored attentively.

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<tr>
<td>A</td>
<td>0.08 M Na/K tartrate substrates 1</td>
<td>0.12 M Na/K tartrate substrates 1</td>
<td>0.16 M Na/K tartrate substrates 1</td>
<td>0.08 M Na/K tartrate substrates 2</td>
<td>0.12 M Na/K tartrate substrates 2</td>
<td>0.16 M Na/K tartrate substrates 2</td>
</tr>
<tr>
<td>B</td>
<td>12.5% glycerol substrate 1</td>
<td>15% glycerol substrate 1</td>
<td>17.5% glycerol substrate 1</td>
<td>12.5% glycerol substrate 2</td>
<td>15% glycerol substrate 2</td>
<td>17.5% glycerol substrate 2</td>
</tr>
<tr>
<td>C</td>
<td>0.08 M Na/K tartrate substrate 3</td>
<td>0.12 M Na/K tartrate substrate 3</td>
<td>0.16 M Na/K tartrate substrate 3</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>D</td>
<td>12.5% glycerol substrate 3</td>
<td>15% glycerol substrate 3</td>
<td>17.5% glycerol substrate 3</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
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</table>

Figure 5.13. Co-crystallization of LE-AmDH-v1, NADH and L-lysine, (R)-1-phenylethylamine or (R)-1-phenylpropylamine - grid 6.

5.4.8 Data collection and processing

Diffraction images collected at synchrotron (SLS or DLS, usually 360° sweep, resulting in 3600 images around 1 Å wavelength – apo, 1.000002; apo + NAD\(^+\), 0.9787) were processed using XDS. The integration step was performed twice. In the first step, the integration was performed with non-specified space group. The aimless function was used to further verify C222\(_1\) space group and specify the unit cell dimensions. The second integration was subsequently performed with the C222\(_1\) space group and the
unit cell dimensions resulting from the aimless analysis. The .mtz file resulting from XDS analysis was further submitted to molecular replacement campaign. Determination of sequence homology was performed using LALIGN web server.\textsuperscript{20} The molecular replacement was performed using PhaserMR\textsuperscript{28} and the results were refined using refmac5.\textsuperscript{29} Both PhaserMR and remac5 are a part of CCP4 suite.\textsuperscript{30} Model building was performed in Coot\textsuperscript{31} and the refinement in later stage of the model building was performed using Phenix refine function.\textsuperscript{32,33}
5.5 References

(3) Dalby, P. A. *Recent patents on biotechnology* **2007**, 1, 1.
(18) Evans, P.; McCoy, A. *Acta crystallographica. Section D, Biological crystallography* **2008**, 64, 1.


