Structure-Based Mechanism of Oleate Hydratase from Elizabethkingia meningoseptica

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Hydratases provide access to secondary and tertiary alcohols by regio- and/or stereospecifically adding water to carbon-double bonds. Thereby, hydration groups are introduced without the need for costly cofactor recycling, and that makes this approach highly interesting on an industrial scale. Here we present the first crystal structure of a recombinant oleate hydratase originating from Elizabethkingia meningoseptica in the presence of flavin adenine dinucleotide (FAD). A structure-based mutagenesis study targeting active site residues identified E122 and Y241 as crucial for the activation of a water molecule and for protonation of the double bond, respectively. Moreover, we also observed that two-electron reduction of FAD results in a sevenfold increase in the substrate hydration rate. We propose the first reaction mechanism for this enzyme class that explains the requirement for the flavin cofactor and the involvement of conserved amino acid residues in this regio- and stereoselective hydration.

Oleate hydratases (EC 4.2.1.53) convert oleic acid (OA) into (R)-10-hydroxystearic acid (10-HSA),[1–4] a product of increasing commercial interest because of its potential application as a surfactant, a lubricant, an additive in the food or cosmetics industries, and as a starting material in polymer chemistry (Scheme 1).[5, 6] Currently, the applicability of hydratases on an industrial scale is limited primarily by their narrow substrate scope and by restricted information on protein structure and mechanism.[7] To overcome these limitations, we expressed N-terminally hexahistidine-tagged oleate hydratase from Elizabethkingia meningoseptica (OhyA; ACT54545.1) in Escherichia coli BL21 Star(DE3) (see the Supporting Information, especially Table S1, for details). The recombinant protein was purified by Ni²⁺-affinity chromatography (Figure S2A) and size-exclusion chromatography and was used for biochemical characterization and crystal structure determination.

In vitro activity assays were performed in the presence of an internal standard. Conversion was stopped by acidification, fatty acids were extracted with ethyl acetate, and silylated derivatives of fatty acids were analyzed by GC-MS (for details see Figure S1B–E). The product, (R)-10-HSA, was obtained with an...
enantiomeric excess of ≥ 98%, as confirmed by 1H NMR analysis (Figure S2). Previous studies on OhyA have reported an apparent \( v_{\text{max}} \) of \( (0.34 \pm 0.04) \, \text{mmol min}^{-1} \, \text{mg}^{-1} \) and an apparent \( K_m \) of \( (0.08 \pm 2.22) \, \text{mm} \) (sic!) at 22°C. In this work, OhyA had its maximum activity at pH 6 and 25°C with an apparent \( v_{\text{max}} \) of \( (1.0 \pm 0.1) \, \text{mmol min}^{-1} \, \text{mg}^{-1} \), an apparent \( K_m \) of \( (0.11 \pm 0.06) \, \text{mm} \), and a \( k_{\text{cat}} \) of \( (1.2 \pm 0.2) \, \text{s}^{-1} \) (Table 1 and Figure S3), in line with the published values.

### Table 1. Oleate hydratase activities determined for cell-free extracts (CFEs) of *E. coli* cells expressing OhyA, as well as for purified OhyA, apo-OhyA, reduced OhyA and OhyA variants. Apparent \( v_{\text{max}} \) values and specific activities were determined after 2 and 10 min of conversion, respectively. Data are presented as means and standard deviations of two biological and three technical replicates.

| Condition | Specific activity [mmolL\(^{-1}\) s\(^{-1}\)] | Cofactor load [\%] | Appt \( v_{\text{max}} \) [mmol min\(^{-1}\) mg\(^{-1}\)] | Appt \( K_m \) [mmolL\(^{-1}\)] | \( k_{\text{cat}} \) [s\(^{-1}\)] |
|-----------|---------------------------------|----------------|---------------------------------|----------------|----------------|
| Shake tower standard conditions (25°C, 150 rpm) | | | | | |
| OhyA (CFE) | 0.12 ± 0.01 | – | | | |
| OhyA | 0.21 ± 0.02 | 86 | 1.0 ± 0.1 | 0.11 ± 0.06 | 1.2 ± 0.2 |
| apo-OhyA | 0.06 ± 0.00 | 8.5 | | | |
| apo-OhyA + FAD | 0.16 ± 0.01 | 49 | | | |
| OhyA E699A | 0.22 ± 0.00 | 65 | | | |
| OhyA G71A | n.c.\(^{[a]}\) | – | | | |

| Manual shaking at 21°C in a glovebox | | | | | |
| OhyA (FAD\(_2\), photoreduced) | 1.23 ± 0.03 | 84 | 2.1 ± 0.3 | 0.07 ± 0.05 | 2.6 ± 0.4 |
| OhyA (FAD, reoxidized) | 0.11 ± 0.01 | 84 | | | |
| OhyA (FAD\(_2\), re-reduced) | 0.84 ± 0.16 | 84 | | | |
| OhyA (FAD\(_2\), DTT reduced) | 1.04 ± 0.11 | 84 | | | |
| OhyA (FAD, control, 0 h) | 0.14 ± 0.06 | 84 | 0.3 ± 0.1 | 0.7 ± 0.4 | 0.4 ± 0.1 |
| OhyA (FAD, control, 24 h) | 0.03 ± 0.02 | 84 | | | |
| OhyA E122A (FAD\(_2\), photoreduced) | n.c.\(^{[a]}\) | 66 | | | |
| OhyA E122A (FAD, control) | n.c.\(^{[a]}\) | 66 | | | |
| OhyA Y241F (FAD\(_2\), photoreduced) | 0.03 ± 0.00 | 76 | | | |
| OhyA Y241F (FAD, control) | n.c.\(^{[a]}\) | 76 | | | |
| OhyA Y456F (FAD\(_2\), photoreduced) | 1.05 ± 0.10 | 76 | | | |
| OhyA Y456F (FAD, control) | 0.28 ± 0.02 | 73 | | | |

[a] n.c.: no conversion. [b] Cofactor load was calculated as described in the Materials and Methods Section in the Supporting Information.

All known oleate hydratases are, like OhyA, flavoproteins and each display an N-terminal Rossmann-type domain containing the typical recognition sequence GXGXXGX\(_{12}\)/E/D (Figure S4).\(^{[9-11]}\) Hydratases belong to the 10% of flavoproteins that are classified as non-oxidoreductases.\(^{[8, 12, 13]}\) However, flavin cofactor binding was found to be essential for substrate conversion, although the exact role of the cofactor remained to be clarified.\(^{[7, 10]}\) Volkov et al. have recently determined the first and so far only crystal structure of a fatty acid double bond hydratase, the enzyme from *Lactobacillus acidophilus*.\(^{[14]}\) This structure, however, did not contain the flavin cofactor bound to the protein.

In order to maximize the possibility of crystallizing OhyA complexed with the cofactor, the protein was incubated with flavin adenine dinucleotide (FAD) prior to the last step of the purification procedure. The solution of the purified protein after size exclusion chromatography was yellow, as were the obtained crystals used for structure determination, thus indicating the presence of FAD in its oxidized state (Figure S7A). The structure of OhyA was solved at 2.75 Å resolution by molecular replacement with use of the structure of the hydratase from *L. acidophilus* (PDB ID: 4I5A) as search template (Figure 1).\(^{[14]}\)

OhyA crystallized as a homodimer with two molecules in the asymmetric unit. Well-defined electron density for the FAD cofactor was indeed observed in chain A, whereas only poorly defined electron density was observed in the corresponding region in chain B, indicating at best a low FAD occupancy. In chain A, the FAD is noncovalently bound (Figure S6) at the interface of a Rossmann-type domain and the substrate-binding domain. The two chains in the asymmetric unit are structurally very similar to each other. A major difference was observed for a loop region (R\(_{168}\)GGREM\(_{173}\)) in the FAD-binding pocket, which is well ordered in the presence of the cofactor and adopts a different, less ordered conformation in its absence, thus indicating a structural role of cofactor binding.

A narrow, elongated, predominantly hydrophilic cavity is present close to the cofactor (Figure 2A). This cavity has a V-shape, with one end being more hydrophilic (due to the presence of the residues Q265, T436, N438 and H442). At the bend of the cavity, and in close vicinity to the isoalloxazine ring, three additional polar residues (E122, G71, and...
Y241 and Y456) are located. Electron density observed in this cavity was interpreted as a PEG fragment (modelled as hexaethylene glycol); this mimics to some extent the hydrophobic character of fatty acid chain. Docking calculations (Supporting Information) produced a binding mode of the substrate OA in which the carboxylate is bound close to amino acids Q265, T436, N438 and H442. The cis double bond of the fatty acid chain, on the other hand, is located at the bend of the cavity in the vicinity of residues E122, Y241 and Y456, as well as the isoalloxazine ring (Figure 2C). On the basis of the obtained crystal structure, the docking results and the analysis of a multiple sequence alignment of oleate hydratases (Figure S4), these polar residues were chosen for site-directed mutagenesis (Figure 2A–C). In vitro activity assays were performed by using cell-free extracts (CFEs) containing 0.05 mg of total protein (Supporting Information). Protein variants T436A, N438A and H442A showed drastically reduced levels of conversion of OA, presumably because of substantially diminished binding of the carboxylate group of OA (Figure 2D). Of the protein variants generated to probe the reaction mechanism, E122A and Y241F, in contrast to Y456F, had completely lost their oleate hydratase activity. We suggest that—in a more or less concerted reaction—Y241 protonates the double bond, and E122 activates a water molecule for the re-side attack on the partially charged double bond (Scheme 2).

Up to this stage, our results suggest a structural role for FAD, because the key residue E122 is located on a flexible loop that becomes more ordered upon cofactor binding. Wondering whether FAD has further roles in this enzyme class, we investigated the cofactor dependence of OhyA function by biochemical means. Firstly, OhyA was depleted of its flavin cofactor either by chemical withdrawal (Supporting Information) or by exchanging the conserved nucleotide binding site residues, G69A and G71A. In contrast to previous studies on other hydratases, only the G71A variant lost the ability to bind FAD. Cofactor deficiency was confirmed spectrophotometrically by loss of the characteristic flavoprotein absorption spectrum (Figure S7A).

Thermodynamic parameters of FAD binding to apo-OhyA were determined by isothermal titration calorimetry (ITC). Our data confirmed that FAD was noncovalently linked to OhyA with a $K_d$ of $1.8 \times 10^{-10}$ M. Moreover, chemically prepared apo-OhyA and the G71A variant displayed $\approx 70\%$ reduced and completely abrogated enzymatic activities, respectively.

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**Figure 2.** Identification of amino acids essential for OhyA activity. A) Active site of OhyA, and B) hydrophobicity of the active-site cavity (blue (hydrophilic) to red (hydrophobic)). C) Active site of OhyA showing the best docking mode for oleic acid. FAD (yellow) and oleic acid (grey) molecules are shown in stick representation. D) Activities of OhyA variants per total protein in cell-free extracts (CFEs). Means and standard deviations of two biological and three technical replicates each are shown. CFEs and insoluble fractions (Ins. Fr.; 2 μL of each) were separated by SDS-PAGE as described in the Materials and Methods Section in the Supporting Information.
or directly in the reaction mechanism. In order to assess 
whether deflavination affects protein stability, we conducted 
differential scanning fluorimetry (ThermoFluor) measurements 
with holo-, apo- and reflavinated OhyA. Melting temperatures 

ture-based reaction mechanism for this class of flavin-depen-
without intervention as a control experiment, enzymatic assays with chemically or photoreduced 
OhyA were conducted under oxygen-free conditions in a glove-
box with manual shaking of the samples. Reduction of OhyA-
bound FAD was monitored spectrophotometrically (Figure S9).
OhyA harboring two-electron-reduced FAD displayed a \( k_{cat} \) of 
\((2.6 \pm 0.4) \text{s}^{-1}\) \( [\text{appt } v_{\text{max}} \text{ of } (2.1 \pm 0.3) \text{ mmol min}^{-1} \text{mg}^{-1}] \), which 
turned out to be six to seven times higher than in the oxidized 
control \( [k_{\text{cat, control}}=(0.4 \pm 0.1) \text{s}^{-1}\text{, appt } v_{\text{max}} \text{ of } (0.3 \pm 0.1) \text{ mmol min}^{-1} \text{mg}^{-1}] \) under inert atmosphere (Table 1). It has to be 
stressed that these reaction conditions cannot be directly com-
pared with the standard conditions and FAD-loaded OhyA pro-
tein because of the varying mixing efficiencies during execution 
of the experiments (Supporting Information). Remarkably, 
single aliquots of purified OhyA could be repeatedly photo-
reduced and auto-oxidized to deliver consistently higher and 
lower enzyme activities, respectively (Table 1, Figure S9). DTT 
treatment had effects similar to those of photoreduction. We 
assume that reduced FAD rather than oxidized FAD is in-
volved in OhyA catalysis in vivo, because the bacterial cyto-
plasm possesses a redox potential sufficient for the reduction 
of oxidized to reduced flavin.\(^{[15,16]}\) In \( E. \text{coli} \), glutathione is 
mostly present in its reduced form and features a lower redox 
potential than protein-bound flavin cofactor.\(^{[17]}\) Upon purification 
from cells, the flavin cofactor oxidizes instantly, and this 
results in the observation of significantly lower activities in 
aerobic assays. These findings are strongly consistent with a re-
cently published study by Takeuchi et al. in which the activity 
of a linoleic acid \( \Delta 9 \) hydratase from \( Lactobacillus \text{plantarum} \) 
was increased up to tenfold upon reduction of the FAD cofac-
tor by NADH under anaerobic conditions.\(^{[18]}\)

Our reaction mechanism proposes a partial positive charge 
owing to protonation of the double bond by Y241 (Scheme 2). We 
suggest that reduced FAD stabilizes this charge because the 
reduced isoalloxazine is slightly acidic and very likely nega-
tively charged under our reaction conditions.\(^{[19]}\) In addition, 
the C-terminal end of an \( \alpha \)-helix (residues N218 to F227) 
points towards the double bond and, thus, might further stabil-
ize a partial positive charge in this area. Interestingly, the role of 
the isoalloxazine ring might go beyond an electrostatic effect, as indicated by the finding that reduction of FAD in the 
Y241F variant rescues some of the activity (Table 1). This effect 
might arise from the ability of the reduced isoalloxazine ring to 
protonate the C=C double bond.\(^{[19]}\)

In summary, the first X-ray crystal structure of a hydratase in 
complex with FAD enabled us to identify the composition of 
the active site: that is, the structural arrangement of the flavin 
cofactor and amino acid residues. This insight paved the way 
to address mechanistic questions revolving around catalysis 
and the observed regio- and stereospecificity of the enzyme. As a result, we are now in a position to propose the first struc-
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tur of OhyA remained unchanged over 60 s of incubation 
with OA (Figure S8 A and B), whereas, as revealed by a control 
experiment, almost 30% conversion of OA was achieved under 
the same conditions (Figure S8C). Thus, this result confirms 
that FAD does not undergo changes in reduction/oxidation 
state during substrate turnover. Originally intended as a control 
experiment, enzymatic assays with chemically or photoreduced 
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OhyA was presumably the result of remaining traces of FAD, as 
evident from the UV–visible absorption spectrum (Figure S7 A).
The loss of FAD correlated with a loss of OhyA functionality, 
because enzymatic activity was restored to 80% upon incuba-
tion of apo-OhyA with FAD (Table 1).

Therefore, we speculated that FAD is either involved in 
enzyme stabilization, in correct localization of the substrate in 
the active site, in positioning the amino acids involved in catal-
ysis, or directly in the reaction mechanism. In order to assess 
whether deflavination affects protein stability, we conducted 
differential scanning fluorimetry (ThermoFluor) measurements 
with holo-, apo- and reflavinated OhyA. Melting temperatures 
\( (T_m) \) were consistently \((52 \pm 2) \text{°C}\) (data not shown), thus sug-
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destabilization of OhyA. Circular dichroism (CD) spectroscopy 
did not reveal any overall alterations of secondary structures 
that would account for the diminished activity (Figure S7B and 
C).

To confirm that the redox state of the flavin cofactor remains 
unaffected during substrate turnover by OhyA, stopped-flow 
measurements were conducted. The UV/Vis absorption spec-

Scheme 2. Proposed reaction mechanism for the hydration of OA.

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dent hydratases (Scheme 2). The structure–function analyses reported here show that amino acids E122 and Y241 act as acid–base catalysts in the hydration of the C=C double bond, with the FAD playing a dual role in the correct organization of the active site and, probably in concert with a helix dipole, the stabilization of the partial positive charge in the putative transition state (Scheme 2). Thus, we describe a major advance in understanding of this largely uncharacterized class of enzymes. Owing to the sequence conservation of oleate hydratases (Figure S4), we are confident that our findings are applicable to other members of the hydratase family and will help in the development of hydratases for industrial applications in the near future.

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