A SINGLE INTERSUBUNIT SALT-BRIDGE AFFECTS OLIGOMERISATION AND CATALYTIC ACTIVITY IN A BACTERIAL QUINONE REDUCTASE

Alexandra Binter¹, Nicole Staunig², Ilian Jelesarov³, Karl Lohner⁴, Bruce A. Palfey⁵, Sigrid Deller¹, Karl Gruber²*, Peter Macheroux¹*

¹Institute of Biochemistry, Graz University of Technology, Austria, ²Institute of Molecular Biosciences, University of Graz, Austria, ³Institute of Biochemistry, University of Zürich, Switzerland, ⁴Institute of Biophysics and Nanosystems Research, Austrian Academy of Sciences, Graz, Austria, and ⁵Department of Biological Chemistry, University of Michigan, Ann Arbor, MI, U.S.A.

Address correspondence to: Prof. Dr. Karl Gruber, University of Graz, Humboldtstrasse 50/III, A-8010 Graz, Austria, Tel.:+43-316-380-5483, Email:karl.gruber@uni-graz.at or Prof. Dr. Peter Macheroux, Graz University of Technology, Institute of Biochemistry, Petersgasse 12/II, A-8010 Graz, Austria, Tel.: +43-316-873-6450, Fax: +43-316-873-6952, Email: peter.macheroux@tugraz.at

Running title: Effect of oligomerisation in a quinone reductase
Summary

YhdA, a thermostable NADPH:FMN oxidoreductase from *Bacillus subtilis*, reduces quinones via a ping pong bi-bi mechanism with a pronounced preference for NADPH. The enzyme occurs as a stable tetramer in solution. The two extended dimer surfaces are packed against each other by a 90° rotation of one dimer with respect to the other. This assembly is stabilized by the formation of four salt bridges between K109 and D137 of neighbouring protomers. To investigate the importance of the ion pair contacts, the K109L and D137L single replacement variants as well as the K109L/D137L and K109D/D137K double replacement variants were generated, expressed, purified, crystallized and biochemically characterized. The K109L and D137L variants form dimers instead of tetramers, whereas the K109L/D137L and K109D/D137K variants appear to exist in a dimer-tetramer equilibrium in solution. The crystal structures of the K109L and D137L variants confirm the dimeric state, with the K109L/D137L and K109D/D137K variants adopting a tetrameric assembly. Interestingly, all protein variants show a drastically reduced quinone reductase activity in steady-state kinetics. Detailed analysis of the two half reactions revealed that the oxidative half reaction is not affected whereas reduction of the bound FMN cofactor by NADPH is virtually abolished. Inspection of the crystal structures indicates that the side chain of K109 plays a dual role by forming a salt bridge to D137 as well as stabilizing a glycine-rich loop in the vicinity of the FMN cofactor. In all protein variants this glycine-rich loop exhibits a much higher mobility as in the wild type. This appears to be incompatible with NADPH binding and thus leads to abrogation of flavin reduction.
Introduction

The search for enzymes with catalytic properties of potential application in biocatalysis and biotechnology has led to the discovery of bacterial enzymes known as azoreductases. These enzymes were found in several diverse bacterial species catalysing the reductive cleavage of azo-dyes containing one or more azo-bonds (R₁-N=N-R₂) to their corresponding amines [1-7]. Aromatic azo-dyes are artificial chemicals with potentially harmful properties causing health and environmental concerns. Recently, we have described a FMN-containing flavoenzyme from *Bacillus subtilis*, termed YhdA, capable of cleaving azo-dyes such as Cibachron Marine at the expense of NADPH [8]. YhdA shares sequence similarity with a family of flavin-(FMN or FAD) dependent quinone reductases such as mammalian NQO1 and yeast Lot6p [9]. Moreover, YhdA and these eukaryotic quinone reductases possess a similar protein topology, a so-called flavodoxin fold consisting of 5 α-helices sandwiching a 5-stranded parallel β-sheet in the centre [10, 11]. Due to these similarities, we were interested to analyze whether YhdA accepts quinones as substrates. In the present study, it is demonstrated that the enzyme indeed reduces a variety of quinones by a ping-pong bi bi kinetic mechanism with a clear preference for NADPH as reducing agent. As could be expected, the turnover rates for quinones are much higher than those obtained for artificial azo-compounds in line with the assumption that quinones, unlike azo-dyes, are cognate enzyme substrates.

Although bacterial and eukaryotic quinone reductases possess a similar protein topology, they diverge with respect to their oligomeric structure: while eukaryotic proteins form dimers, YhdA and Azo1 (from *Staphylococcus aureus*, [4]) form tetramers [8, 9]. In the case of YhdA, the tetramer is formed by two dimers, which interact through an extended concave surface. The interface between dimers is stabilized by four salt bridges formed by the side chains of residues K109 and D137 of structural neighbours (see Scheme 1). This higher oligomerisation state was held responsible for the increased thermal stability of YhdA (Tₘ = 87 °C), as compared to the dimeric yeast homolog Lot6p (Tₘ = 60.2 °C) [12]. To test this hypothesis and to obtain more insight into the importance of these salt bridges for tetramer assembly, we have created four YhdA protein variants: K109L, D137L, K109L/D137L and K109D/D137K. Characterisation of the variants showed that single replacement of K109 or D137 disrupts the tetramer whereas the two double replacement protein variants appear to have
conserved some tendency to form tetramers. Interestingly, all of the variants have a similar melting point as wild type protein suggesting that the high thermostability is an intrinsic property of the dimer. Surprisingly, the protein variants showed dramatically reduced enzymatic activity, which is due to the break-down of the reductive half reaction, suggesting that structural changes impede docking of NADPH to the active site. Crystallisation and concomitant X-ray crystallographic determination of variant structures indicate that increased mobility of a highly conserved glycine-rich loop in the vicinity of the isoalloxazine ring system might be responsible for the loss of enzyme activity.

Results

YhdA has recently been classified as an NADPH:FMN oxidoreductase with the ability to reductively cleave azo dyes [8]. Lot6p, the YhdA homolog in yeast, was shown to reduce quinones to their hydroquinone state [12], hence we have tested YhdA for its quinone reductase activity. Steady state measurements using several quinone substrates as electron acceptors resulted in much higher turnover rates than reported for the reduction of azo dyes (data not shown). For a more detailed characterisation 2-hydroxy-p-naphthoquinone was chosen as a representative substrate. Figure 1 shows the double reciprocal plot of initial velocity measurements in the presence of NADPH as the electron donor and 2-hydroxy-p-naphthoquinone as electron acceptor. The family of parallel lines obtained from data analysis, indicates a ping-pong bi-bi mechanism where both substrates consecutively bind to the catalytic site, i.e. the electron donor NADPH binds first, then dissociates to vacate the active site for binding of the electron accepting quinone substrate. The same mechanism was proposed for the homologous yeast enzyme Lot6p and other quinone reductases [12].

Using [S-^{2}H]-NADPH and [R-^{2}H]-NADPH as reducing agents and subsequent analysis of NADP^{+} by ^{1}H-NMR spectroscopy revealed that the proS-hydride of NADPH is preferentially transferred to FMN.

Heterologous expression of the four generated protein variants was performed in the same way as described for wild type protein resulting in similar amounts of soluble protein. All hexahistidine-tagged protein variants were purified by Ni-NTA chromatography according to the protocol established for wild type YhdA. YhdA possesses a non-covalently bound FMN cofactor,
which is also present in the protein variants, but showed some minor changes in their UV/Vis-absorbance spectra (Figure 2). The extinction coefficients of enzyme-bound FMN were determined using an extinction coefficient of $\varepsilon_{450} = 12,400 \text{ M}^{-1} \text{ cm}^{-1}$ for free FMN and are summarized in Table 1.

The native molecular mass was estimated by molecular sieve chromatography. Each protein variant eluted as a single species, however, all protein variants exhibited larger elution volumes indicating a lower apparent mass (Table 2). These data suggest that the two single protein variants and the K109L/D137L double protein variant form dimers in solution. On the other hand, the K109D/D137K variant showed a native molecular mass of 61 kDa, suggesting that the protein may exist in a dimer/tetramer equilibrium. This result was qualitatively confirmed by dynamic light scattering experiments (Table 2), demonstrating that the single replacement variants form dimers rather than tetramers. On the other hand, both double replacement protein variants show a tendency to form a tetramer similar to wild-type protein.

To further characterize the oligomerisation of the protein variants native PAGE was employed. As shown in Figure 3 (panel A), both single protein variants have a higher mobility as compared to wild type protein; this can be interpreted in terms of formation of dimers rather than tetramers. The different isoelectric points resulting from the aspartate to leucine (pI = 6.92) and the lysine to leucine (pI = 6.09) replacements, respectively, account for the mobility shift between the two single protein variants. The two double protein variants give rise to bands positioned between the K109L and D137L variant. Taking into account that the two double protein variants have an intermediate isoelectric point of 6.43 (the same as wild type protein), this result also suggests that both of these protein variants occur as dimers. At higher protein concentrations (Figure 3, panel B and barely visible in panel A), however, the K109L/D137L protein variant exhibits an additional band at lower mobility indicating that this protein variant may form tetramers under these conditions. Interestingly, the “inverse” K109D/D137K variant produces only a single band at high mobility (no change between panel A and B in Figure 3) in contrast to the dimer-tetramer equilibrium suggested by molecular sieve chromatography. Obviously both double replacement variants have some tendency to form tetramers, albeit much weaker as wild type protein.
Next, we characterized the protein variants with respect to their quinone reductase activity. Initial rate measurements show that all protein variants retain less than 1% of wild type activity, using molecular oxygen as well as various quinones as final electron acceptors (Table 3). Stopped flow measurements were performed to determine whether the reductive or the oxidative half reaction is impaired in the protein variants. The reductive half reaction of wild type, the D137L and the K109L/D137L protein variant was investigated in more detail. With both protein variants the rate of reduction of the FMN cofactor was very small amounting to 0.6% and 3% of the wild type rate for the D137L and K109L/D137L protein variant, respectively. The rate of reduction for the other two protein variants was much smaller and could not be determined accurately in the stopped-flow instrument. On the other hand, the oxidative half reaction using 2-hydroxy-p-naphthoquinone as a substrate was not affected in any of the protein variants yielding comparable rates for wild type and all protein variants (Table 3). Hence it can be concluded that the loss of enzymatic activity in the four protein variants observed in steady-state measurements is due to the collapse of the reduction step, i.e. the transfer of electrons from NADPH to the flavin cofactor.

YhdA has been described as an enzyme exhibiting high thermostability, with a melting temperature of 86.5 °C, determined by following thermal unfolding of the protein by CD spectroscopy [8]. The high thermostability of the tetrameric YhdA in comparison to its dimeric yeast homologue Lot6p (T_m = 60 °C) gave rise to the assumption that the tetrameric state stabilizes the protein toward thermal unfolding [13]. Both proteins possess a common structural topology, the so-called flavodoxin-like fold. They exhibit the same dimer architecture, forming a large, slightly concave surface, characterized by four α helices spanning its entire width [11]. In the case of Lot6p, several charged residues in the central part of this surface appear to interfere with the tetrameric assembly. These residues are replaced by hydrophobic or uncharged residues in YhdA, which allows tetramer formation by rotating the two dimers against each other by 90° and packing of the two dimers. To test the hypothesis that tetramerisation is responsible for increased thermostability, the apparent melting temperatures of the protein variants were determined by CD spectrometry and differential scanning calorimetry. Surprisingly, no significant changes in the thermostability of the protein variants were observed (Table 2). Thus it can be concluded that the higher oligomerisation state of YhdA compared to...
Lot6p is not the governing factor for achieving higher thermostability, as all four predominantly dimeric protein variants show similar unfolding temperatures as the tetrameric wild type protein.

All four YhdA variants were crystallized. Three structures – of the K109L, the D137L and K109D/D137K variant – were determined and refined to varying crystallographic resolution (Table 4). The crystals obtained for the K109L/D137L variant were isomorphous to the hexagonal crystal form of wild type YhdA (PDB entry: 1NNI) but diffracted to lower resolution and therefore this structure was not further refined.

The YhdA protomer belongs to the SCOP family [14] of “NADPH dependent FMN reductases”. The closest structural neighbours - according to a SSM analysis [15]– are a NAD(P)H-dependent FMN reductase from *Pseudomonas aeruginosa* (PDB entry 1x77) [16], ArsH from *Sinorhizobium meliloti* (2q62) [17], a NADH-dependent FMN reductase from the EDTA-degrading bacterium *bnc1* (2vzf, 2vzh, 2vzj) [18] and ArsH from *Shigella flexneri* (2fzv) [19]. The rms deviations are between 1.6 and 2.0 Å for 150 to 160 aligned C-α-atoms.

The oligomeric states of the different variants in the crystalline state were analyzed using the MSD-PISA server [20] by taking into account all interactions of protein chains within the asymmetric unit as well as with symmetry equivalent molecules. The crystal structure of wild type YhdA contains only one protein chain in the asymmetric unit, but a tetramer is formed by two crystallographic diads (space group *P*6$_2$22), which is predicted to be stable also in solution. This prediction was confirmed experimentally by molecular sieve chromatography and native PAGE (see Table 1 and Figure 2). This tetramer exhibits 222 symmetry and can be considered as a dimer of dimers (Figure 4) with a significantly larger interaction surface within the dimers (~1100 Å$^2$ buried surface area per chain) than between them (~660 Å$^2$). Four individual salt bridges involving K109 and D137 are formed across the dimer-dimer interface (Scheme 1). Lys-109 also forms hydrogen bonds to three carbonyl groups in the glycine rich loop (G$^{106}$-GG-K$^{109}$-GG$^{111}$) of the neighbouring subunit thereby stabilizing this loop, which is in close proximity of the N(1)-C(2=O) locus of the flavin (Figure 7). Based on the observed isomorphicity of crystals obtained for the K109L/D137L variant, the same oligomeric state can safely be assumed to be present, although the salt bridge between Lys-109 and Asp-137 cannot be formed in this case. Most of the closest structural neighbours mentioned above also form tetramers in the crystal
and the mode of oligomerisation is the same as in YhdA. The only exception is the NAD(P)H-dependent FMN reductase from *Pseudomonas aeruginosa*, which forms a dimer again equivalent to YhdA. In this context it is noteworthy that K109 and D137 are only conserved among putative oxidoreductases in the genera *Bacillus* and are not found in any of the other structurally related proteins. This clearly indicates that tetramer formation is not solely dependent on the presence of the salt-bridges formed between these residues.

The asymmetric unit of crystals of the K109D/D137K variant contains 12 protein chains forming three tetramers, which are each very similar to the wild type tetramer. Root-mean-square deviations were in the range of 0.3 to 0.4 Å after superposition of 664 to 669 C-α-atoms (>90% of the total number of Cα-atoms in the tetramers). Although the “inverse” amino acid exchange should in principle allow the formation of an inter-dimer salt bridge, this interaction is not observed in the crystal structure. In addition, the stabilizing interactions of the lysine with the carbonyl groups in the glycine rich loop in the neighbouring protomer are not formed (Figure 7). Accordingly, PISA analysis predicts a lower stability for this tetramer (calculated $\Delta G_{\text{diss}}=4.5$–6.2 kcal/mol compared to 9.8 kcal/mol for native YhdA) and thus could more easily dissociate into dimers in solution.

The remaining two variants (K109L and D137L) show different oligomeric states in each case with 4 protomers in the asymmetric unit, which form two dimers identical to the dimers found in the other YhdA structures. In the crystal, these two dimers also form tetramers which – according to the PISA analysis – should only be marginally stable in solution ($\Delta G_{\text{diss}}$ of -0.1 and 1.4 kcal/mol). These tetramer arrangements are very similar in the two structures (rms deviation of 0.6 Å for 626 superimposed Cα-atoms). Compared to the wild type tetramer the two dimers interact differently with each other. While in wild type YhdA (as well as in the studied double mutant proteins) the two dimers are aligned almost perpendicular to each other, they are essentially parallel in the single mutant proteins (Figure 5).

The isolated protomers of the YhdA variant structures show only small structural changes compared to the wild type (rms-deviations ranging from 0.3 to 0.6 Å for 166 to 168 superimposed Cα-atoms). The largest changes are observed in the region around residue 109 which is in the centre of a
the above mentioned glycine-rich loop region (Figure 6) This loop also becomes more flexible upon amino acid exchange, which is clearly indicated by the lesser quality of the electron density and the significantly higher B-factors in this region (Figure 8).

Discussion

Quinone reductases are present in many different organisms in the eubacterial, fungal, plant and animal kingdom. YhdA, previously described as an azoreductase [8], clearly possesses quinone reductase activity. Considering the similarity of YhdA both in sequence and structure with confirmed quinone reductases such as mammalian NQO1 and yeast Lot6p, this finding is not unexpected. On the other hand, YhdA differs with regard to its quaternary structure. While quinone reductases of eukaryotic origin form dimers, YhdA assembles into a tetramer, made up by a dimer of dimers (Scheme 1 and Figure 4). The reasons for adopting higher quaternary protein structures are still elusive and appear to be case-dependent. Comparisons of the quaternary structure of proteins from thermophilic organisms with their mesophilic counterparts has indicated that higher oligomeric structures provide increased thermal stability required for adaptation to elevated temperatures [13, 21, 22]. Although *B. subtilis* is not a thermophilic organism, YhdA possesses a surprisingly high thermal stability with an apparent melting temperature of $T_m = 86.5 \, ^{\circ}C$. In contrast to YhdA, its ortholog from *S. cerevisiae* has a much lower apparent melting temperature ($T_m = 60.2 \, ^{\circ}C$) as could be expected due to its lower quaternary structure. Inspection of the tetrameric structure of YhdA revealed that the main contacts between the two dimers are set up by four reciprocal salt bridges between the side chains of K109 and D137 (Scheme 1), and therefore we hypothesized that these interactions are responsible for tetramer stabilisation and this in turn will lead to increased thermostability. Based on this hypothesis we generated two variants with either K109 or D137 replaced by leucine and a third variant with both residues exchanged to leucine. In a fourth variant we have swapped the interacting residues in an attempt to restore the salt bridge and thus redesign an intact dimer-dimer interaction. The role of the salt bridges for tetramer assembly was confirmed by our experimental results as the two single replacement variants were exclusively found as dimers both in solution and in the crystal. The two double variants predominantly exist as dimers in solution although both showed some tendency to form tetramers in
solution (Table 2 and Figure 3). In the crystal, both of them were clearly present as tetramers showing similar packing as wild type protein (Figure 5). Interestingly, inverting the position of the interaction partners in the K109D/D137K protein variant does not rebuild the salt bridge as is clearly seen in the crystal structure (Figure 7, panel B). Instead, K137 forms a hydrogen bond to the backbone C=O of G108 and not to the carboxyl group of D109.

However, none of the protein variants exhibited decreased thermostability (Table 2), clearly contradicting our initial hypothesis that tetramer assembly is responsible for higher thermostability. Obviously, thermostability in this case is not a function of quaternary structure but an intrinsic property of YhdA protomers and/or the dimer.

Surprisingly, quinone reductase activity was severely compromised in all variants due to a lack of reduction of the FMN cofactor by NADPH. This was clearly unexpected since all variants appear to have similar active sites as judged by their UV/Vis-absorbance spectrum. Moreover, at a first glance, the determined structures of the variants showed no conspicuous differences that would have predicted altered enzymatic properties. Closer inspection, however, revealed that a glycine-rich loop in the vicinity of the isoalloxazine ring system with glycine 106 directly interacting with C(2=O) of the pyrimidine moiety shows substantially altered mobility (Figure 8). In wild type YhdA this loop is stabilized by K109 of a neighbouring subunit (Scheme 1 and Figure 7), in the variants this interaction is lost either due to replacement of the lysine residue or, in the case of the D137L variant by abrogated tetramer formation. It appears that the higher mobility of the glycine-rich loop is incompatible with binding of NADPH and/or delivery of a hydride to the flavin cofactor and hence K109 plays a dual role by supporting tetramer assembly through its interaction with D137 and stabilisation of the glycine-rich loop necessary to enable flavin reduction by NADPH. Unfortunately, attempts to obtain a crystal structure with NADPH or NADP+ bound to the active site have so far been unsuccessful (for a model see supplementary Figure S1).

Our findings suggest that tetramer assembly of YhdA is not responsible for the unusual thermostability, however, the quaternary structure appears to be required for catalytic activity. Although wild type enzyme clearly exists as a tetramer in solution, it is conceivable that extreme environmental conditions (e. g. high temperature) may cause dissociation of the tetramer into dimers and hence result
in deactivation of quinone reductase activity. Since YhdA dimers exhibit the same thermal stability as
tetramers, this mode of regulation is reversible, i.e. tetramers can reform once conditions favouring
tetramer assembly are restored. At this point it is not clear whether regulation of quinone reductase
activity through reversible dimer-tetramer equilibrium is relevant for the bacterium and to which end it
serves in adaptation to environmental challenges.

**Experimental Procedures**

**Reagents.** The nickel-nitrilotriacetic acid agarose (Ni-NTA) was from Qiagen. All chemicals were of
the highest grade commercially available and obtained from Sigma-Aldrich, Fluka, Merck, or Roth.

**Cloning, Recombinant Expression and Purification.** The cloning of *yhdA* from *Bacillus subtilis* into
the pET21a vector, the recombinant expression of YhdA using the host expression strain *E. coli* BL21
(DE3) and the protein purification procedure is described in [8]. The Sephadex Desalting Column PD-
10 from GE Healthcare was used for buffer exchange.

**Site-directed mutagenesis.** Site-directed mutagenesis was carried out as specified in the QuikChange®
XL Site-Directed Mutagenesis Kit from Stratagene. The pETyhdA plasmid described in [8] served as
template, performing the polymerase chain reaction-based mutagenesis. In order to obtain the two
single muteins YhdA K109L and YhdA D137L, as well as the two double muteins YhdA
K109L/D137L and YhdA K109D/D137K, the following primers and their complementary counterparts
were used: K109L: 5’-GGG CGG CGG ACTT GGC GGC ATC AAT G-3’ (sense), D137L: 5’-GCA
GCT GGT GCT TCT TCC GGT GCA TAT TG-3’ (sense), K109D: 5’-GGG CGG CGG AGA T
CGG CAT CAA TG-3’ (sense), D137K: 5’-GCA GCT GGT GCT TAA ACC GGT GCA TAT TG-3’
(sense). The underlined nucleotides represent the mutated codon. After the mutagenesis protocol the
sequences of the transformation constructs were verified by sequencing analysis. The generation of the
double mutations was achieved using pETyhdA(K109L) and pETyhdA(D137L), respectively as
templates and the relating primer pairs for the polymerase chain reaction. The mutated plasmids were
purified according to the Plasmid DNA Purification Kit from Macherey-Nagel and transformed into the
host expression strain *E. coli* BL21 (DE3). Recombinant expression of the protein variants and the purification procedure by Ni-NTA chromatography were performed as described for the wild type enzyme [8].

**Molecular weight determination.** For the native molecular-weight determination of the variants molecular sieve chromatography was used [8]. The obtained results from the molecular sieve chromatography were verified by native polyacrylamide gel electrophoresis (PAGE), following the standard procedures for SDS-PAGE as described by Laemmli, using 12.5 % separating gels and 5 % stacking gels. The gels and the running buffer, respectively lacked SDS or DTT to maintain the native state of proteins. Native PAGE was performed for 4 hours at 90 V and 4°C. In addition, dynamic light scattering of wild-type YhdA and the four protein variants was carried out with a DynaPro from ProteinSolutions.

**Spectrophotometric methods.** To determine the extinction coefficient of enzyme-bound FMN 0.2 % SDS was used to release the co-factor. UV-vis absorbance spectra were recorded before and after denaturation of the enzyme with a photometer (model specord 205) from Analytik Jena, Germany. All measurements were performed in 100 mM Tris/HCl, pH 7.5 using 1-cm quartz cuvettes unless stated otherwise.

**Steady state kinetics.** To determine the reaction mechanism, steady-state turnover of wild-type YhdA was measured by following the oxidation of NADPH spectrophotometrically in the presence of 2-hydroxy-p-naphthoquinone. Steady-state turnover of the protein variants was determined by monitoring the oxidation of NADPH in the presence of molecular dioxygen as substrate. Initial velocities were measured by monitoring the decrease in absorbance at 340 nm. All reactions were carried out in 100 mM Tris-HCl, pH 7.5 at 37 °C. The reaction mixture contained 4 µM enzyme, 10 µM FMN, and NADPH in a concentration range from 25 to 275 µM. The enzyme activity was calculated by using a molar absorption coefficient of 6220 M⁻¹cm⁻¹ for NADPH.
**Determination of the stereospecificity of YhdA.** YhdA was exchanged into appropriate buffer (30 mM Tris-HCl, pH 8.0, in D$_2$O) using Econo-Pac 10DG desalting columns (Bio-Rad). A solution (1 mL) containing the buffer mentioned above, 10 µM YhdA, and 3 mg of either [4R-2H]-NADPH or [4S-2H]-NADPH was left to react for 2 hours at 37 °C. Enzyme was removed using size-exclusion chromatography, the remaining solution was lyophilized and the product analyzed by $^1$H-NMR [23]. All listed signals are given relative to TMS as an internal standard.

**Stopped flow kinetics.** Stopped flow measurements were carried out with a stopped-flow device from Hi-Tech (SF-61DX2) positioned in a glove box from Belle Technology at 25 °C. Two reactant solutions were joined in single mixing mode, using a 0.5 mL stopping syringe. FMN oxidation and reduction were measured respectively, by monitoring changes in absorbance at 453 nm with a KinetaScanT diode array detector (MG-6560). Initial rates were calculated by fitting the curves with SpecFit 32 (Spectrum Software Associates) using a function of two exponentials.

Performing reductive half reaction 40 µM enzyme and NADPH in a concentration range from 0.5 to 8 mM in 100 mM Tris-HCl, pH 7.5 were mixed by the stopped flow device. The absorbance decrease at 453 nm was monitored spectrophotometrically.

To determine rate constants for the oxidative half reaction, 40 µM enzyme in 100 mM Tris-HCl, pH 8.4 was first reduced chemically by titration of 14 mM sodium dithionite. After mixing with 2-hydroxy-p-naphthoquinone in concentrations from 25 to 500 µM, the reoxidation of the FMN cofactor was monitored at 453 nm. For the preparation of the quinone solution, a 10 mM stock solution of 2-hydroxy-p-naphthoquinone in ethanol was diluted with 100 mM Tris-HCl, pH 8.4 to the final concentrations. All samples were prepared by flushing with nitrogen followed by incubation in the glove box environment.

**Thermal unfolding experiments.** CD Spectroscopy: Thermal unfolding of the muteins was monitored in 0.1 cm cuvettes using a Jasco J-500 spectropolarimeter at 225 nm. The cuvette was placed in a thermostated cell holder. The temperature was raised continuously from 5 to 95 °C at a heating rate of 1 °C/min. The enzyme concentration was 50 µM, in 100 mM Tris-HCl at pH 7.5. Differential scanning
calorimetry (DSC) was performed with a VP-DSC, MicroCal calorimeter. After scanning a buffer-buffer baseline of 100 mM Tris-HCl at pH 7.5, 600 µL samples containing 1-3 mg/mL protein were scanned at a heating rate of 1 °C/min in a temperature range from 5 to 110 °C.

**X-ray crystal structures**

The YhdA variants were crystallized at room temperature using the batch crystallization method with drops of 1 µL protein solution (c=10-18 mg/ml) plus 1 µL reservoir solution. Diffraction quality crystals were obtained under the following conditions: 0.1 M HEPES pH 7.5, 0.2 M (NH₄)₂SO₄, 25% w/v PEG 3350 (K109L variant); 0.1 M Bis-Tris pH 6.5, 20% w/v PEG MME 5000 (D137L variant); 0.1M Tris-HCl pH 8.5, 2.0 M (NH₄)₂SO₄ (K109D/D137K variant); 0.1 M HEPES pH 7.0, 0.2 M (NH₄)₂SO₄, 0.5% w/v PEG 8000 (K109L/D137L variant). For cryoprotection the crystals were transferred to corresponding solutions containing 25% glycerol before flash-cooling in liquid nitrogen. Diffraction datasets were collected at beamlines X13 (λ=0.8148 Å) and X11 (λ=0.8010 Å) at the EMBL/DESY Hamburg. In all cases, data reduction involved the HKL package [24] as well as software from the CCP4 suite [25].

The structures were solved by molecular replacement with the program PHASER [26] using the structure of wild type YhdA (PDB entry: 1NNI) as search model and were further refined using the program PHENIX [27]. Model building and fitting steps involved the graphics program Coot [28] using σÅ-weighted 2Fo-Fc and Fo-Fc electron density maps [29]. Rfree-values [30] were computed from 5% randomly chosen reflections not used throughout the refinement. In the higher resolution structures, water molecules were placed automatically into difference electron density maps and were retained or rejected based on geometric criteria as well as on their refined B-factors. NCS restraints were applied during all refinement steps. Details of the data collection, processing and structure refinement are summarized in Table 5. Molprobity Ramachandran plots [31] showed no outliers in the higher resolution structures (D137L and K109D/D137K) and 0.6% outliers in the lower resolution structure (K109L). Coordinates and structure factors have been deposited with the Protein Data Bank under the accession numbers 3GFQ (K109L), 3GFR (D137L) and 3GFS (K109D/D137K).
References


Footnotes

We appreciate the support of staff scientists at the synchrotron beamlines at DESY/EMBL-Hamburg during diffraction data collection. The support by the Austrian Science Fund (FWF) through Doktoratskolleg “Molecular Enzymology” (W901-B05) to K.G. and P.M. is gratefully acknowledged.
Figure 1
Double-reciprocal plot of initial rate measurements in steady-state experiments as a function of NADPH at 2 (σ), 5 (λ), 10 (ν), and 30 (τ) μM of 2-hydroxy-p-naphthoquinone (from top to bottom).

Figure 2
UV/Vis absorbance spectra of wild type YhdA and the four protein variants.

Figure 3
Panel A and B: 6.5 μL protein solution with 15 μM and 60 μM, respectively, were applied onto each lane.

Figure 4
Crystal structure of wild type YhdA from Bacillus subtilis (PDB entry: 1NNI). The oligomeric state can be described as a dimer of dimers. One dimer is formed by the green and magenta chains, the second by the pink and blue chains. The FMN cofactors are shown as spheres. The figure was prepared using the program PyMOL (http://www.pymol.org/).

Figure 5
Schematic representation of the observed oligomeric states and dimer-dimer interactions in different YhdA variants. In each case one two-fold symmetric dimer subunit is shown in its surface representation, whereas the other is shown in a cartoon representation. Two views are presented, which are rotated by 90° around the x-axis. Wild type YhdA as well as the double replacement variants form stable tetramers with the two dimers rotated by about 60° relative to each other (left). In the single replacement variants (right) the two dimers are oriented parallel to each other. The latter interaction is only present in the crystal. The figure was prepared using the program PyMOL (http://www.pymol.org/).

Figure 6
Stereo representation of the superposition of all YhdA protomers found in the crystal structures of the wild type (PDB entry 1nni) and the different variants. In total, 21 structures are shown in different colours. The two sites of amino acid exchanges are indicated. The protomer structures are very similar to each other (see text for details) and differ only in some loop regions especially around residue 109 and 137. The figure was prepared using the program PyMOL (http://www.pymol.org/).
Figure 7
Close-up view of a portion of the dimer-dimer interface. One subunit is shown in light-blue, the other in magenta. The FMN cofactor is shown in yellow, hydrogen bonding interactions are indicated using dashed green lines. In wild type YhdA a salt bridge between Lys-109 and Asp-137 is formed across this interface. In the K109D/D137K variant, the number of interactions is greatly reduced and the salt bridge cannot be formed anymore. The figure was prepared using the program PyMOL (http://www.pymol.org/).

Figure 8
“Bfactor-putty” representation of structures of different YhdA variants (A: wild type, B: D137L, C: K109L and D: K109D/D137K) focusing in the glycine-rich loop around residue 109. Orange to red colours and a wider tube indicate regions with higher B-factors, whereas shades of blue and a narrower tube indicate regions with lower B-factors. The FMN-cofactor is shown is a stick-representation. The figure was prepared using the program PyMOL (http://www.pymol.org/).
**Tables**

**Table 1**

Extinction coefficients of wt-YhdA and the four protein variants at 450 nm

<table>
<thead>
<tr>
<th>Protein</th>
<th>$\varepsilon_{450}$ (M$^{-1}$ cm$^{-1}$)</th>
<th>$\lambda_{\text{max}}$ (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wt</td>
<td>11,690</td>
<td>451</td>
</tr>
<tr>
<td>D137L</td>
<td>10,660</td>
<td>452</td>
</tr>
<tr>
<td>K109L</td>
<td>11,070</td>
<td>450</td>
</tr>
<tr>
<td>K109L/D137L</td>
<td>10,760</td>
<td>452</td>
</tr>
<tr>
<td>K109D/D137K</td>
<td>10,720</td>
<td>453</td>
</tr>
</tbody>
</table>

**Table 2**

Native molecular mass estimation by molecular sieve chromatography and dynamic light scattering (DSL) and apparent unfolding temperatures $T_m$ in °C as determined by CD spectroscopy and differential scanning calorimetry (DSC)

<table>
<thead>
<tr>
<th>Protein</th>
<th>$V_E$ (mL)</th>
<th>molecular mass (kDa)</th>
<th>DSL$^\dagger$ (kDa)</th>
<th>$T_m$ (CD)</th>
<th>$T_m$ (DSC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wt</td>
<td>56.69</td>
<td>72.7</td>
<td>85</td>
<td>87*</td>
<td>93</td>
</tr>
<tr>
<td>D137L</td>
<td>66.29</td>
<td>33.5</td>
<td>53</td>
<td>84</td>
<td>95</td>
</tr>
<tr>
<td>K109L</td>
<td>64.61</td>
<td>44.0</td>
<td>68</td>
<td>88</td>
<td>95</td>
</tr>
<tr>
<td>K109L/D137L</td>
<td>64.21</td>
<td>45.1</td>
<td>72</td>
<td>89</td>
<td>95</td>
</tr>
<tr>
<td>K109D/D137K</td>
<td>59.45</td>
<td>61.0</td>
<td>88</td>
<td>89</td>
<td>&gt;95</td>
</tr>
</tbody>
</table>

The void volume of the column was determined to $V_v = 44.04$ mL;$^\dagger$ values given are the average of two independent measurements; * value taken from [8]
Table 3
Steady-state and rapid reaction parameters for wt-YhdA and protein variants.

Turnover measurements were carried out with NADPH and oxygen as substrates. The rate of reduction and oxidation was measured with NADPH and 2-hydroxy-p-napthoquinone (2OHpNQ), respectively.

<table>
<thead>
<tr>
<th>Protein</th>
<th>turnover $k_{\text{red}}$ (s$^{-1}$)</th>
<th>$K_M$ (NADPH) (mM)</th>
<th>reduction $k_{\text{red}}$ (s$^{-1}$)</th>
<th>$K_D$ (NADPH) (mM)</th>
<th>oxidation $k_{\text{ox}}$ (s$^{-1}$)</th>
<th>$K_D$ (2OHpNQ) (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wt</td>
<td>57.9 ± 6.7</td>
<td>0.52 ± 0.09</td>
<td>100 ± 3</td>
<td>0.54 ± 0.05</td>
<td>90 ± 9.7</td>
<td>0.27 ± 0.05</td>
</tr>
<tr>
<td>D137L</td>
<td>0.28 ± 0.02</td>
<td>0.65 ± 0.07*</td>
<td>0.45$^\nu$</td>
<td>-</td>
<td>115 ± 7.1</td>
<td>0.21 ± 0.02</td>
</tr>
<tr>
<td>K109L</td>
<td>0.02 ± 0.004</td>
<td>0.42 ± 0.14*</td>
<td>n.d.</td>
<td>-</td>
<td>96 ± 28.8</td>
<td>0.31 ± 0.018</td>
</tr>
<tr>
<td>K109L/D137L</td>
<td>0.55 ± 0.12</td>
<td>1.37 ± 0.35*</td>
<td>3.07 ± 0.3</td>
<td>2.29 ± 0.42</td>
<td>103 ± 10.9</td>
<td>0.24 ± 0.044</td>
</tr>
<tr>
<td>K109D/D137K</td>
<td>0.16 ± 0.04</td>
<td>0.69 ± 0.18*</td>
<td>n.d.</td>
<td>-</td>
<td>124 ± 6.6</td>
<td>0.12 ± 0.014</td>
</tr>
</tbody>
</table>

n.d., not determined (rates were very small compared to wt-YhdA); $^*$, accurate determination of $K_M$ values was hampered by low activity of the variants; $^\nu$, rate of reduction increased linearly to $k_{\text{red}}$ = 0.45 sec$^{-1}$ at 4 mM NADPH.
Table 4
Data collection and refinement statistics

<table>
<thead>
<tr>
<th></th>
<th>K109L</th>
<th>D137L</th>
<th>K109D/D137K</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Data collection</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Space group</td>
<td>$P2_1$</td>
<td>$P1$</td>
<td>$P2_1$</td>
</tr>
<tr>
<td><strong>Cell dimensions</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$a$, $b$, $c$ (Å)</td>
<td>47.54, 66.24, 220.75</td>
<td>51.49, 56.08, 64.18</td>
<td>68.63, 170.13, 93.29</td>
</tr>
<tr>
<td>$\alpha$, $\beta$, $\gamma$ (°)</td>
<td>90.0, 90.0, 90.0</td>
<td>84.1, 77.0, 74.5</td>
<td>90.0, 92.3, 90.0</td>
</tr>
<tr>
<td>Resolution (Å)</td>
<td>29.0-3.20</td>
<td>20.0-2.40</td>
<td>30.0-2.11</td>
</tr>
<tr>
<td>$R_{sym}$</td>
<td>0.161 (0.597)</td>
<td>0.057 (0.274)</td>
<td>0.096 (0.460)</td>
</tr>
<tr>
<td>$I$ / $\sigma I$</td>
<td>10.4 (2.9)</td>
<td>12.8 (3.2)</td>
<td>15.8 (2.0)</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>99.5 (99.5)</td>
<td>95.0 (88.8)</td>
<td>96.7 (52.5)</td>
</tr>
<tr>
<td>Redundancy</td>
<td>4.9 (4.5)</td>
<td>2.0 (1.9)</td>
<td>3.6 (2.0)</td>
</tr>
<tr>
<td><strong>Refinement</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resolution (Å)</td>
<td>29.0-3.20</td>
<td>19.9-2.50</td>
<td>29.3-2.11</td>
</tr>
<tr>
<td>No. reflections</td>
<td>12155</td>
<td>24878</td>
<td>119393</td>
</tr>
<tr>
<td>$R_{work}$ / $R_{free}$</td>
<td>0.212/0.262</td>
<td>0.199/0.251</td>
<td>0.185/0.221</td>
</tr>
<tr>
<td>No. atoms</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td>5124</td>
<td>5175</td>
<td>15379</td>
</tr>
<tr>
<td>Cofactor</td>
<td>124</td>
<td>124</td>
<td>372</td>
</tr>
<tr>
<td>Water</td>
<td>-</td>
<td>319</td>
<td>977</td>
</tr>
<tr>
<td><strong>B-factors</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td>58.0</td>
<td>36.5</td>
<td>36.1</td>
</tr>
<tr>
<td>Cofactor</td>
<td>53.6</td>
<td>28.3</td>
<td>29.4</td>
</tr>
<tr>
<td>Water</td>
<td>-</td>
<td>39.3</td>
<td>40.7</td>
</tr>
<tr>
<td><strong>R.m.s. deviations</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bond lengths (Å)</td>
<td>0.003</td>
<td>0.002</td>
<td>0.002</td>
</tr>
<tr>
<td>Bond angles (°)</td>
<td>0.6</td>
<td>0.6</td>
<td>0.6</td>
</tr>
</tbody>
</table>

*Values in parentheses are for highest-resolution shell.
Scheme 1
Schematic representation of the four salt-bridges in the YhdA tetramer (colour coding as in Figure 5)
Figures

Figure 1
Figure 2
Figure 3

A

B
Figure 7
Figure 8