Mutation network-based understanding of pleiotropic and epistatic mutational behavior of *Enterococcus faecalis* FMN-dependent azoreductase

Jinyan Sun\textsuperscript{a,b,1}, Ohgew Kweon\textsuperscript{a,1}, Jinshan Jin\textsuperscript{a}, Gui-Xin He\textsuperscript{c}, Xiyu Li\textsuperscript{c}, Carl E. Cerniglia\textsuperscript{a}, Huizhong Chen\textsuperscript{a,*}

\textsuperscript{a} Division of Microbiology, National Center for Toxicological Research, US Food and Drug Administration, Jefferson, AR 72079, USA

\textsuperscript{b} State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan 430071, China

\textsuperscript{c} Department of Biomedical and Nutritional Sciences, University of Massachusetts Lowell, Lowell, MA 01854, USA

**ABSTRACT**

We previously identified a highly active homodimeric FMN-dependent NADH-preferred azoreductase (AzoA) from *Enterococcus faecalis*, which cleaves the azo bonds (R-N=N-R) of diverse azo dyes, and determined its crystal structure. The preliminary network-based mutational analysis suggested that the two residues, Arg-21 and Asn-121, have an apparent mutational potential for fine-tuning of AzoA, based on their beneficial pleiotropic feedbacks. However, epistasis between the two promising mutational spots in AzoA has not been obtained in terms of substrate binding and azoreductase activity. In this study, we further quantified, visualized, and described the pleiotropic and/or epistatic behavior of six single or double mutations at the positions, Arg-21 and Asn-121, as a further research endeavor for beneficial fine-tuning of AzoA. Based on this network-based mutational analysis, we showed that pleiotropy and epistasis are common, sensitive, and complex mutational behaviors, depending mainly on the structural and functional responsibility and the physicochemical properties of the residue(s) in AzoA.

1. Introduction

Azo dyes, characterized by the presence of one or more azo groups, are the largest and most versatile dye class [1–4]. Since an azo group in a natural product is rare [1] and industrially produced azo dyes usually resist biodegradation in conventional aerobic sewage treatment plants, they are considered persistent pollutants [2,5]. Azoreductase, widely distributed in the bacterial world, is a key enzyme responsible for biodegradation of azo dyes by breaking of the nitrogen double bonds (-N=N-) to generate aromatic amines via hydrazo intermediates [6–15]. Studying the molecular mechanism of azoreductase might lead to better understand toxicity, mutagenicity and carcinogenicity of the azo dyes and the development of effective bioremediation method to degrade azo dyes in environment.

AzoA from *Enterococcus faecalis* is a typical group I azoreductase, the polymeric flavin-dependent NADH-preferred azoreductases [16]. In our previous synergetic studies based on three-dimensional structure, computational analysis, and reverse genetics coupled with site-directed mutagenesis deciphered the binding site and binding mode of the substrates, FMN, NADH and a model azo dye, methyl red, and the catalytic mechanism of AzoA [6,7,17]. As shown in Fig. 1, AzoA is homodimeric with two separate active sites at the interface between the two monomers, and the FMN lies inside each active site [17]. FMN, stabilized by 22 amino acid residues, transfers four electrons from NADH to the azo dye substrate, resulting in the degradation of the azo dye substrate [6]. In AzoA, the substrates NADH and methyl red are stabilized by about 12 and 19 amino acid residues, respectively, and lie against the flavin isoalloxazine ring of FMN at angles of 45° and 35°, respectively. Conclusively, the sequential functional interaction of three substrates, FMN, NADH, and methyl red is essential for successful biodegradation of azo dyes via azo reduction (Fig. 1).

Pleiotropy (mutations at loci that affect more than one trait) and epistasis (interaction between mutations) have been recognized to play a prominent role in enzyme engineering in the laboratory (i.e., directed evolution, semi-rational or rational design) and in natural enzyme evolution [18–20]. In our previous study, pleiotropy and epistasis were used to analyze the interaction between AzoA and its substrates. The phenotypic feedbacks of 13 single mutations (e.g., substrate binding and azoreductase activity) were mapped to a mutation network [7]. The network-based mutational analysis suggested that the two residues,
Arg-21 and Asn-121, have an apparent mutational potential for fine-tuning of AzoA, based on their beneficial pleiotropic feedbacks, i.e., enhancing ligand binding affinity and azoreductase activity. However, epistasis between the two promising mutational spots in AzoA has not been obtained in terms of substrate binding and azoreductase activity. In this study, we further quantified, visualized, and described the pleiotropic and/or epistatic behavior of six single or double mutations at the positions, Arg-21 and Asn-121, as a further research endeavor for beneficial fine-tuning of AzoA.

2. Materials and methods

2.1. Materials

Methyl red, NADH, FMN, dimethyl sulfoxide (DMSO) and isopropyl β-D-thiogalactopyranoside (IPTG) were purchased from Sigma-Aldrich. *Escherichia coli* BL21-Gold (DE3) pLysS and pET11a (Stratagene) were used as the host and expression vectors, respectively. Luria-Bertani liquid medium with chloramphenicol (50 μg/ml) and ampicillin (50 μg/ml) was used for cultivation of recombinant *E. coli*.

2.2. Site-directed mutagenesis of azoreductase

The original construct, wild type pAzoA [16], was used as the template for site-directed mutagenesis using the QuikChange II XL site-directed mutagenesis kit (Stratagene). The primers with the corresponding mutations were used in the PCR reactions (Table 1). PCR reactions were performed in a Mastercycler gradient (Eppendorf). The amplification conditions were one cycle of 95 °C for 2 min, 20 cycles with 50 s of melting at 95 °C, 50 s of annealing at 60 °C, and 7 min of extension at 68 °C for each cycle, and one final extension cycle at 68 °C for 10 min. The parental plasmids were digested using *Dpn*I at 37 °C for 1 h. Then the DNA was transformed to XL-10Gold competent cells. The plasmids with the desired mutations were extracted using QIAprep Spin Miniprep Kit (Qiagen) and confirmed by sequencing. All the mutant plasmids were used to transform to competent *E. coli* BL21-Gold (DE3) pLysS cells.

Table 1

| Mutant | Primer Sequences (5′ → 3′) |
|--------|----------------------------|
| R21G   | TCAGCTCAGTTGGCTGTTAGAAACAT  |
|        | ATGTTTCTAAACCAACTGAGCTGTTA  |
| N121A  | GTAGATACGATCGATTGGTATTGTATG  |
| N121Q  | GTAGATACGATCGATTGGTATTGTATG  |
| R21K   | TCAGCTCAGTTGGCTGTTAGAAACAT  |
|        | ATGTTTCTAAACCAACTGAGCTGTTA  |

Underlined nucleotides indicate mutations incorporated into primers.

2.3. Recombinant protein expression and purification

The mutant azoreductases were expressed in *E. coli* as described previously [6]. Cells were harvested by centrifugation at 4000 × g for 15 min and resuspended in 20 ml potassium phosphate buffer (25 mM, pH 7.1). The cells were disrupted by sonication for 5 min at 0 °C with a Vibra-Cell VCX 400 sonifier (Sonics and Materials). The cell debris was removed by centrifugation at 12,000 × g for 20 min and ammonium sulfate was added to the collected supernatants to a final concentration of 0.5 M. Then the mixture was centrifuged and filtered for purification. The azoreductase samples were purified as described previously [7].

2.4. Azoreductase activity assays

Azoreductase activity assays were performed as described previously [6]. The activities of AzoA wild type and mutants were analyzed by measuring the reduction of methyl red at 430 nm at room temperature. The reaction mixture was 1956 μl potassium phosphate buffer (pH 7.1), 2 μl methyl red (25 μM), 20 μl NADH (0.1 mM), 2 μl FMN (0.5 μM), and 20 μl azoreductase. The amount of azoreductase needed for the reduction of 1 μmol methyl red per minute was defined as one unit of activity. Specific azoreductase activity is defined as units of activity per mg protein. The protein was quantified using the Bicinchoninic acid assay (Pierce) with bovine serum albumin (BSA) as the standard. SDS-PAGE was carried out using precast gels (12%, Bio-Rad).
2.5. Kinetic parameters

Initial velocities of the enzymatic reaction of azoreductase were measured by varying the concentrations of one substrate, methyl red (5–60 µM) or NADH (0.05–1.0 mM), while the concentration of the other substrate remained fixed (1 mM NADH, 30 µM methyl red). The parameters \( K_m \) and \( V_{max} \) were obtained from Lineweaver-Burk plots [6].

3. Results

3.1. Expression and purification of the mutant proteins

To test the pleiotropic and epistatic effects of single and dual mutations, we constructed single and double mutants by site-directed mutagenesis at positions Arg-21 and Asn-121 in AzoA from *E. faecalis*. For a comparable mutation set, in this study, the amino acid residues Arg-21 and Asn-121 were substituted by the amino acids with common physico-chemical properties, lysine and glutamine, respectively. AzoA wild-type and 6 AzoA mutants (N121Q, R21K, R21G/N121Q, R21G/N121Q, R21K/N121A, and R21K/N121Q) were cloned into pET11a and expressed by consecutive chromatography using anion exchangers HiPrep Q XL and Mono Q followed by a gel filtration step on HiLoad Superdex 75. Purified mutants were 23 kDa on SDS-PAGE same as wild type AzoA (Fig. 2). The supernatants of cell lysis from AzoA mutants overexpressed cells displayed a bright yellow color, indicating cosynthesis of flavin during the protein expression. On the basis of spectroscopy and protein molecular weight, the molar ratio between FMN and purified proteins of the wild-type and mutants were similar (~ 2), suggesting that two FMN molecules bind to an AzoA (wild-type and mutants) (Table 2).

3.2. Kinetic characterization of the AzoA mutants

The enzymatic activities of 6 AzoA mutants were characterized in comparison with wild type AzoA and two AzoA mutants (R21G, N121A) constructed in our previous study. Table 2 shows the kinetic parameters of the wild-type AzoA and AzoA mutants. The \( K_m \) values for methyl red of single mutants N121Q and R21K were 29 µM and 16.3 µM, for NADH 79.5 µM and 190.4 µM, respectively, and their specific azoreductase activities were 136% and 171%, which were higher than that of wild-type. As for double mutants of R21K/N121A and R21K/N121Q, the \( K_m \) values for methyl red were 31 µM and 33.8 µM, for NADH 266.6 µM and 115.4 µM, respectively. However, their specific azoreductase activities were 41.4% and 40% of that of wild-type AzoA.

3.3. Mutation network of AzoA

To visualize pleiotropic or epistatic mutational behavior, we reconstructed a mutation network of the nine mutants, showing dynamic functional perturbations at the four functional categories—NADH binding, methyl red binding, FMN binding, and azoreductase activity (Fig. 3). As shown in the mutation network, all nine mutants showed dynamic pleiotropic behavior in the functional categories, with the exception of FMN binding, with no drastic mutational effect. In the enzyme activity category, five mutants (three single mutants [R21G, N121A, and N121Q] and two double mutants [R21G/N121A and R21G/N121Q]) are located in the inner region than the wild-type AzoA (i.e., enhanced azoreductase activity by substitution[s]). Among the five mutants with enhanced azoreductase activity, only two mutants, R21G and R21G/N121A, were positioned in the inner region in all the functional categories (Fig. 3).

In the single substitution mutants, with exception of R21K, the mutants R21G, N121A, and N121G with improved azoreductase activity are located in the inner region of the enzyme activity category but are located in the outer region of the substrate binding categories (i.e., decreased substrate binding affinity). On the other hand, the R21K is located in the outer region in the enzyme activity category, but is positioned in the inner region of the NADH and methyl red binding categories (i.e., increased the binding affinity of NADH and methyl red by the substitution).

The overall connection patterns between the four functional categories of the double mutants showed apparent non-additive effects, indicating epistatic effects in combination of the two single mutations. Different types of epistasis, such as reciprocal epistasis, sign epistasis, positive epistasis, and negative epistasis, were observed. No case of positive epistasis, but three cases of negative epistasis between beneficial mutations, were observed in the methyl red binding and azoreductase activity of the mutant R21G/N121A and in the azoreductase activity of the mutant R21G/N121A (Table 2 and Fig. 3). Two cases of reciprocal sign epistasis were observed, in the NADH binding of the mutant R21G/N121A and in the methyl red binding of the mutant R21K/N121A, respectively. Several cases of sign epistasis between a beneficial mutation and a deleterious mutation were observed in all functional categories. The mutants R21G/N121A and R21G/N121Q are located in the inner region of the enzyme activity category (i.e., improved azoreductase activity), whereas the other double mutants R21K/N121A and R21K/N121Q are positioned in the outer region of the enzyme activity category (i.e., decreased azoreductase activity). No conserved pleiotropic and epistatic feedbacks related to their changed azoreductase activity from the four double mutants were observed.

4. Discussion

A comprehensive understanding of pleiotropy of mutations and epistasis among mutations on evolutionary trajectories of enzymes is necessary to decipher mechanisms of protein adaptation and natural evolution. On the other hand, using available structural and functional data, a systematic prediction of the possible pleiotropic and epistatic behavior of enzymes of interest is an initial key step for successful
The two residues, Arg-21 and Asn-121, have no direct contact and are located distantly on the AzoA molecule (~23.8 Å). In addition, the structural and functional responsibility of Arg-21 and Asn-121 is interesting, due to no apparent overlaps in functional responsibility of the two residues. As we determined, the substitutions with amino acids with the opposite properties of the side chain in size and hydrophobicity at Arg-21 and Asn-121, showed dynamic mutational effects depending on the four functional categories, FMN-binding, NADH-binding, methyl red-binding, and azoreductase activity. Interestingly, the two mutants R21G and N121A shared the same pleiotropic feedback pattern: a slightly decreased FMN binding, methyl red binding affinity, but increased methyl red binding affinity with no significant change in FMN binding. The sum of the pleiotropic behavior seems counterintuitive, i.e., generating the mutant azoreductases with improved activity. Therefore, the comparatively drastic phenotypic fluctuations of R21G and N121A to the subtle impacts of R21K and N121Q provide not only direct pleiotropic evidence but also an insight into the productive pleiotropic trade-off for beneficial fine-tuning of AzoA. It is evident that pleiotropy is a pervasive, sensitive, and complex mutational behavior in AzoA. No completely conserved pleiotropic feedback pattern for enhancing azoreductase activity was observed, although the functional category “methyl red-binding affinity” showed a strong connection with the azoreductase activity. More mutational case studies with reasonable analytical resolution should give a better understanding of the general propensity of mutational pleiotropic behavior in AzoA.

The two residues, Arg-21 and Asn-121, have no direct contact and are located distantly on the AzoA molecule (~23.8 Å). In addition, there is no apparent overlapping functional contribution between the two residues, i.e., functional contribution of Asn-121 for binding of FMN and methyl red but no direct contribution of Arg-21 to binding of any ligands. Considering the structural and functional responsibility of the residues, pervasive epistasis caused by the mutational combinations between Arg-21 and Asn-121 is interesting in terms of its causation. Firstly, considering their structurally remote locations, the detected epistasis suggests non-additive combination effects between indirect structural impacts of Arg-21 and direct functional impacts of Asn-121. In AzoA, such a long-range indirect mutational interaction should be

---

**Table 2**

Kinetic parameters of wild-type E. faecalis AzoA and the mutants.

| Enzyme           | FMN | \(K_m\) (µM) | \(K_m\) \(^{NADH}\) (µM) | \(V_{max}\) (U/mg) | \(k_{cat}\) (s⁻¹) | \(k_{cat}/K_m\) \(^{FMN}\) (s⁻¹ µM⁻¹) | \(k_{cat}/K_m\) \(^{NADH}\) (s⁻¹ µM⁻¹) | Relative specific activity (%) |
|------------------|-----|--------------|---------------------------|-------------------|-----------------|--------------------------------|--------------------------------|-----------------------------|
| Wild-type        | +   | 24           | 143                       | 84                | 59.1            | 2.46                         | 0.41                          | 100                         |
| R21G             | +   | 12.6         | 380                       | 200               | 140             | 11.11                        | 0.37                          | 382                         |
| N121A            | +   | 8.2          | 292                       | 158               | 111             | 13.54                        | 0.38                          | 170                         |
| N121Q            | +   | 29           | 370.9                     | 103.2             | 72.6            | 2.5                          | 0.2                           | 115                         |
| R21K             | +   | 19.5         | 121.8                     | 65.2              | 45.9            | 2.35                         | 0.28                          | 80.2                        |
| 21G/N121A        | +   | 10.8         | 79.5                      | 106.2             | 74.7            | 5.23                         | 0.71                          | 136                         |
| 21G/N121Q        | +   | 16.3         | 190.4                     | 135.4             | 95.3            | 5.85                         | 0.5                           | 171                         |
| 21K/N121A        | +   | 31           | 266.6                     | 61.1              | 43              | 1.39                         | 0.16                          | 41.2                        |
| 21K/N121Q        | +   | 33.8         | 115.4                     | 63                | 44.3            | 1.31                         | 0.38                          | 40                          |

* Kinetic data of wild-type. R21G and N121A are from our previous results and for the purpose of comparison [7].

---

The likelihood of pleiotropy of residue mutations usually depends on various factors, such as functional and/or structural responsibility of residues in the protein. In addition, the physicochemical properties of the residue in the context (i.e., nature of the mutation) largely govern the degree of pleiotropic impact—giving a detectable phenotype(s). In this perspective, the pleiotropic behavior of mutations at Arg-21 and Asn-121 is intriguing, due to no apparent overlaps in functional responsibility of the two residues. As we determined, the substitutions with amino acids with the opposite properties of the side chain in size and hydrophobicity at Arg-21 and Asn-121, showed dynamic mutational effects depending on the four functional categories, FMN-binding, NADH-binding, methyl red-binding, and azoreductase activity. Interestingly, the two mutants R21G and N121A shared the same pleiotropic feedback pattern: a slightly decreased NADH binding affinity, but increased methyl red binding affinity with no significant change in FMN binding. The sum of the pleiotropic behavior seems counterintuitive, i.e., generating the mutant azoreductases with improved activity. Therefore, the comparatively drastic phenotypic fluctuations of R21G and N121A to the subtle impacts of R21K and N121Q provide not only direct pleiotropic evidence but also an insight into the productive pleiotropic trade-off for beneficial fine-tuning of AzoA. It is evident that pleiotropy is a pervasive, sensitive, and complex mutational behavior in AzoA. No completely conserved pleiotropic feedback pattern for enhancing azoreductase activity was observed, although the functional category “methyl red-binding affinity” showed a strong connection with the azoreductase activity. More mutational case studies with reasonable analytical resolution should give a better understanding of the general propensity of mutational pleiotropic behavior in AzoA.
mediated by ligand-ligand interaction (i.e., FMN-NADH and/or FMN-methyl red). The FMN-mediated epistatic interactions can be further supported by the pleiotropic evidence of Arg-21 with no direct functional contribution for binding of any ligands, showing apparent fluctuations of binding affinity of NADH and methyl red. The several types of epistasis observed, including sign epistasis and reciprocal sign epistasis, additionally suggest that the FMN-mediated epistatic interactions between the residues are sensitive and sophisticated in the FMN-dependent AzoA. In AzoA, FMN generates an interaction network with about 22 amino acid residues, eight of which, including Asn-121, function in binding of the FMN isoalloxazine ring [6,7]. Although AzoA seems to have a certain degree of tolerance to mutational perturbations in FMN binding [6,7], it is evident that even subtle changes in the binding mode of FMN (especially the isoalloxazine ring) in the active sites can cause detectable pleiotropic and epistatic impacts.

There are several approaches to create the tailored biocatalysts (e.g., with improved enzyme activity or new catalytic capabilities): random approaches (directed evolution), semi-rational and rational (data-driven) design [18–20,22–27]. Among the engineering approaches, rational design has several attractive advantages, including an increased chance of favorable mutations and a considerable reduction of the library size and so less effort and time for the screening of the library [27]. Despite these apparent advantages, however, purely rational design is still limited by i) our limited structural and functional understanding and ii) a relatively low degree of predictability of mutational impacts [27]. Conclusively, the initial key step for the successful rational design of enzymes of interest is to propose the most promising mutations, and such endeavor needs a successful systematic integration between the structure/function information and possible pleiotropic and epistatic impacts. As revealed in this study, a rational, design-based fine-tuning for enhancing azoreductase activity of AzoA seems to be challenging, due mainly to the high degree of pleiotropic and epistatic complexity (i.e., a low degree of predictability). In the previous study and this one, we observed several single or double mutational cases to enhance the azoreductase activity of AzoA. Arg-21 and Asn-121 were unexpected mutational spots with beneficial mutational effects on azoreductase activity of AzoA [6,7]. Moreover, in the mutational combinations of the two residues, if Arg-21 was substituted by glycine, the azoreductase activity was enhanced, regardless of the substitution of Asn-121. Such unexpected effects and mutational feedbacks inform us of our limited understanding of structure-function-mutation relationships and suggest more systematic studies of pleiotropy and epistasis for successful fine-tuning of AzoA.

In conclusion, this study was an initial research endeavor for network-based understanding of the pleiotropic and epistatic mutational behavior, from systematic linking between mutation(s) and the corresponding phenotypic feedback(s) to pleiotropic and epistatic understanding of the relationships via network-based visualization. In AzoA, pleiotropy and epistasis are common, sensitive, and complex mutational behaviors, governed mainly by i) the structural and functional responsibility and ii) the physicochemical properties of the residues(s). More systematic combinatorial research activities, such as generating more wet-lab mutational data and converting it to an in silico prediction system, are required to better control pleiotropic and epistatic mutational behaviors, essential for successful rational design of proteins.

Acknowledgments

We thank Drs. John Sutherland and Kidon Sung for their critical review of this manuscript. This study was funded by the National Center for Toxicological Research (E0762101), United States Food and Drug Administration, and supported in part by appointments (JS and JJ) to the Postgraduate Research Fellowship Program by the Oak Ridge Institute for Science and Education through an interagency agreement between the U.S. Department of Energy and the U.S. Food and Drug Administration (FDA). The findings and conclusions in this publication are those of the authors and do not represent FDA positions or policies.

Appendix A. Transparency document

Transparency document associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2017.10.008.

References

[1] A. Stolz, Basic and applied aspects in the microbial degradation of azo dyes, Appl. Microbiol. Biotechnol. 56 (2001) 69–80.
[2] H. Chen, Recent advances in azo dye degrading enzyme research, Curr. Protein Pept. Sci. 7 (2006) 101–111.
[3] K.T. Chung, S.E. Stevens Jr., C.E. Cerniglia, The reduction of azo dyes by the intestinal microflora, Crit. Rev. Microbiol. 18 (1992) 175–190.
[4] R.L. Steingley, W. Zou, T.M. Heinitz, H. Chen, C.E. Cerniglia, Metabolism of azo dyes by human skin microbiota, J. Med. Microbiol. 59 (2010) 108–114.
[5] J. Feng, C.E. Cerniglia, H. Chen, Toxicological significance of azo dye metabolism by human intestinal microflora, Front. Microbiol. (Elite Ed.) 4 (2012) 568–586.
[6] H. Chen, H. Xu, O. Kweon, S. Chen, C.E. Cerniglia, Functional role of Trp-105 of Enterococcus faecalis azoreductase (AzoA) as resolved by structural and mutational analysis, Microbiology 154 (2008) 2659–2667.
[7] J. Feng, O. Kweon, H. Xu, C.E. Cerniglia, H. Chen, Probing the NADH- and methyl red-binding site of a FMN-dependent azoreductase (Azo) from Enterococcus faecalis, Arch. Biochem. Biophys. 520 (2012) 99–107.
[8] C.E. Cerniglia, J.P. Freeman, W. Franklin, L.D. Pack, Metabolism of benzidine and benzidine-congener based dyes by human, monkey and rat intestinal bacteria, Biochem. Biophys. Res. Commun. 107 (1982) 1224–1229.
[9] C.E. Cerniglia, Z. Zhuo, B.W. Manning, T.W. Federle, R.H. Helrich, Mutagenic activation of the benzidine-based dye direct black 38 by human intestinal microflora, Mutat. Res. 175 (1986) 11–16.
[10] K.T. Chung, Azo dyes and human health: a review, J. Environ. Sci. Health C Environ. Carcinog. Ecotoxicol. Rev. 34 (2016) 233–261.
[11] S. Bhmull, H.J. Knackmuss, A. Stolz, Molecular cloning and characterization of the gene coding for the aerobic azoreductase from Xenophilus azovarans KF46F, Appl. Environ. Microbiol. 68 (2002) 3948–3955.
[12] M. Nakashita, C. Yamane, N. Ishida, Y. Kitade, Putative ACP phosphodiesterase gene (acpD) encodes an azoreductase, J. Biol. Chem. 276 (2001) 46394–46399.
[13] Y. Suzuki, T. Yoda, A. Ruhul, W. Sugiyura, Molecular cloning and characterization of the gene coding for azoreductase from Bacillus sp. OY1-2 isolated from soil, J. Biol. Chem. 276 (2001) 9059–9065.
[14] J.M. Morrison, C.M. Wright, G.H. John, Identification, isolation and characterization of a novel azoreductase from Clostridium perfringens, Anaerobe. 18 (2012) 229–234.
[15] C.J. Wang, C. Hagemeier, N. Rahman, E. Lowe, M. Noble, M. Coughtrie, et al., Molecular cloning, characterization and ligand-bound structure of an azoreductase from Pseudomonas aeruginosa, J. Mol. Biol. 375 (2007) 1213–1228.
[16] H. Chen, R.F. Wang, C.E. Cerniglia, Molecular cloning, overexpression, purification, and characterization of an aerobic FMN-dependent azoreductase from Enterococcus faecalis, Protein Expr. Purif. 34 (2004) 302–310.
[17] Z.J. Liu, H. Chen, N. Shaw, S.L. Hopper, L. Chen, S. Chen, et al., Crystal structure of an aerobic FMN-dependent azoreductase (AzoA) from Enterococcus faecalis, Arch. Biochem. Biophys. 463 (2007) 68–77.
[18] J. Kaur, R. Sharma, Directed evolution: an approach to engineer enzymes, Crit. Rev. Biotechnol. 26 (2006) 165–199.
[19] B. Ontman, A. Hintze, C. Adami, Impact of epistasis and pleiotropy on evolutionary adaptation, Proc. Biol. Sci. 279 (2012) 247–256.
[20] C.M. Milton, N. Tokuriki, How mutational epistasis impacts predictability in protein evolution and design, Protein Sci. 25 (2016) 1260–1272.
[21] P.C. Phillips, Epistasis – the essential role of gene interactions in the structure and evolution of genetic systems, Nat. Rev. Genet. 9 (2008) 855–867.
[22] J.A. de Visser, T.F. Cooper, S.F. Elena, The causes of epistasis, Proc. Biol. Sci. 278 (2011) 3617–3624.
[23] R. Kobayashi, N. Hirano, S. Kanaya, M. Haruki, Enhancement of the enzymatic activity of Escherichia coli acetyl esterase by a double mutation obtained by random mutagenesis, Biosci. Biotechnol. Biochem. 76 (2012) 2082–2088.
[24] D. Dell’Orco, P.G. De Benedetti, F. Fanelli, In silico screening of mutational effects on enzyme-protein inhibitor affinity: a docking-based approach, BMC Struct. Biol. 7 (2007) 37.
[25] P.C. Ng, S. Henikoff, Predicting the effects of amino acid substitutions on protein function, Annu. Rev. Genomics Hum. Genet. 7 (2006) 61–80.
[26] K.S. Goo, C.S. Chua, T.S. Sim, Relevant double mutations in bioengineered Streptomyces clavuligerus deacetoxycephalosporin C synthase result in higher binding specificities which improve penicillin bioconversion, Appl. Environ. Microbiol. 74 (2008) 1167–1175.
[27] K. Steiner, H. Schwab, Recent advances in rational approaches for enzyme engineering, Comput. Struct. Biotechnol. J. 2 (2012) e201209010.