Functional roles of Tryptophan residues in diketoreductase from Acinetobacter baylyi

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Diketoreductase (DKR) from Acinetobacter baylyi contains two tryptophan residues at positions 149 and 222. Trp-149 and Trp-222 are located along the entry path of substrate into active site and at the dimer interface of DKR, respectively. Single and double substitutions of these positions were generated to probe the roles of tryptophan residues. After replacing Trp with Ala and Phe, biochemical and biophysical characteristics of the mutants were thoroughly investigated. Enzyme activity and substrate binding affinity of W149A and W149F were remarkably decreased, suggesting that Trp-149 regulates the position of substrate at the binding site. Meanwhile, enzyme activity of W222F was increased by 1.7-fold while W222A was completely inactive. In addition to lower thermostability of Trp-222 mutants, molecular modeling of the mutants revealed that Trp-222 is vital to protein folding and dimerization of the enzyme. [BMB Reports 2012; 45(8): 452-457]

INTRODUCTION

Diketoreductase (DKR) is a NAD(P)H-dependent oxidoreductase that catalyzes the reduction of ethyl-3,5-dioxo-6-benzyloxyhexanoate to ethyl-(3R,5S)-dihydroxy-6-benzyloxyhexanoate with excellent stereoselectivity (Fig. S1), and the dihydroxy product containing two asymmetric centers is a useful chiral intermediate for the synthesis of statin drugs (1-7). DKR is a homodimeric protein with a molecular mass of 30 kDa for each subunit composed of 283 amino acids. The enzyme shares 35.7% amino acid identity with human heart L-3-hydroxyacyl-CoA dehydrogenase (HAD) (8). Therefore, using crystal structures of HAD (PDB ID 1F0Y) as a template, a homology model of DKR was built, and the quality of the model structure was evaluated by PROCHECK and ERRAT to be reliable. Based on the model structure, the active site consisting of Ser-122, His-143 and Glu-155 was confirmed by mutagenesis study in our previous report (9). After investigating the interaction between DKR and diketo substrate by fluorescence spectroscopy, we found that the fluorescence intensity of DKR was decreased upon the binding between the substrate and the enzyme, suggesting that at least one tryptophan (Trp) residue is closely located in the substrate-binding site. Because DKR only contains two Trp residues at positions 149 and 222, it is possible to identify the individual roles of the Trp residues. Additionally, sequence alignment of DKR and HAD indicates that these two Trp residues are unique to DKR. In the model structure of DKR, Trp-149 is positioned in the loop close to the substrate binding site of N-terminal domain, whereas Trp-222 is located at the hydrophobic dimer interface of the C-terminal domain (Fig. S2) (9). Because Trp is an important residue in proteins for activity, folding, stability and ligand binding (10-14), exploration of the roles of Trp-149 and Trp-222 in the enzyme would be helpful to better understand binding and catalytic mechanism of DKR.

In the present study, both Trp residues in DKR were replaced by alanine (Ala) or phenylalanine (Phe) by site-directed mutagenesis. Because Ala has the smallest side chain, mutation with Ala would produce little conformational alteration but completely lose aromatic-aromatic interaction and hydrogen bonding. On the other hand, substitution with Phe retains comparable aromatic-aromatic interaction with weaker hydrogen bonding capacity compared to Trp. Subsequently, the effects of these replacements indicated that Trp-149 is located along the entry path of the substrate into active site and stabilizes the substrate at the active site, and Trp-222 is a major contributor to the fluorescence and is critical to the stability of dimer formation and overall conformation for enzyme function.

RESULTS AND DISCUSSION

Expression and purification of mutant enzymes The expression yields of soluble mutant proteins were 140-160 mg/L culture, comparable to the yield of wild-type
DKR (Fig. S3A). An identical procedure was successfully used to purify the DKR mutants including W149A, W149F, W222F and W149F/W222F. However, W222A could not be purified under the same condition (Fig. S3B), suggesting that the replacement of Trp-222 by Ala possibly disrupts the quaternary structure and proper folding.

**Kinetic characterization of mutant enzymes**

Kinetic constants for wild-type DKR and the mutated enzymes are listed in Table 1. The kinetic parameters of wild-type enzyme and Trp-149 mutants showed a notable increase of apparent $K_m$ from 271.1 ± 36.7 μM to 648.6 ± 12.5 and 617.7 ± 54.8 μM. With regard to the turnover number ($k_{cat}$), Trp-149 mutants only remained 13% and 19% compared to wild-type. These kinetic data strongly suggested that substitutions at this position could alter substrate binding and hinder catalytic efficiency. Without a strong interaction between residue 149 and the substrate, W149A was strongly suggested that substitutions at this position could alter substrate-DKR intermediate formation and subsequent dihydroxy production, and the indole ring of Trp-222 may exert hydrophobic interaction in the protein structure and contribute to a conformational change in DKR during the catalysis.

**Fluorescence properties of Trp-149 and Trp-222**

The fluorescence emission spectra of wild-type DKR and mutants are presented in Supplementary Fig. 4. Table 2 lists the positions of the respective emission spectra maximum ($\lambda_{max}$) and relative fluorescence intensity. Assuming that the emission spectrum of a multi-Trp-containing protein is the sum of the fluorescence spectra of individual Trp residues, we would expect a decrease in the normalized fluorescence after removal of one of the emitting species. However, this was only seen in the replacement of Trp-222. Compared to wild-type DKR, Trp-222 exhibited unchanged fluorescence intensity in Trp-149 mutants, while the fluorescence in mutants W222F and W149F/W222F was hardly observed. On the other hand, the overall secondary structures of the mutants were not altered by mutations because of their similar Far-UV CD spectra compared to wild-type (Fig. S5). Therefore, Trp-222 was the main fluorescence source in DKR molecule. In addition, $\lambda_{max}$ values of wild-type DKR and Trp-222 mutants were at 315 nm and 312 nm respectively, whereas W222F displayed a maximum at 308 nm. This blue shift of Trp-149 mutants suggests that Trp-149 is potentially exposed to solvent (15).

To verify the solvent exposure of Trp residues in DKR, quenching experiments with acrylamide (Acr) and KI were performed. Supplementary Fig. 6 shows the Stern-Volmer relationship for Acr and KI quenching, and Table 2 lists the quenching constants. The Stern-Volmer plot for Acr was linear, to postulate that Trp-149 is involved in promoting substrate-DKR intermediate formation and subsequent dihydroxy production. Functional roles of Tryptophan residues in diketoreductase from *Acinetobacter baylyi* Yan Huang, et al.

### Table 1. Kinetic analysis of wild-type DKR and mutants

<table>
<thead>
<tr>
<th></th>
<th>Wild-type</th>
<th>W149A</th>
<th>W149F</th>
<th>W222F</th>
<th>W149F &amp; W222F</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_{cat}$ (s$^{-1}$)</td>
<td>8.4 ± 0.5</td>
<td>1.1 ± 0.2</td>
<td>1.6 ± 0.1</td>
<td>14.6 ± 1.0</td>
<td>1.8 ± 0.1</td>
</tr>
<tr>
<td>$K_m$ (μM)</td>
<td>271.1 ± 36.7</td>
<td>648.6 ± 12.5</td>
<td>617.7 ± 54.8</td>
<td>421.6 ± 63.9</td>
<td>330.4 ± 30.7</td>
</tr>
<tr>
<td>$k_{cat}/K_m$ (10$^3$ s$^{-1}$ · M$^{-1}$)</td>
<td>31.0 ± 1.3</td>
<td>1.7 ± 0.1</td>
<td>2.6 ± 0.2</td>
<td>35.0 ± 1.3</td>
<td>5.3 ± 0.3</td>
</tr>
</tbody>
</table>

### Table 2. Fluorescent properties of wild-type DKR and mutants

<table>
<thead>
<tr>
<th></th>
<th>$\lambda_{max}$ (nm)</th>
<th>Relative fluorescence intensity</th>
<th>Acr</th>
<th>KI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\lambda_{max}$ (nm)</td>
<td>$K_m$ (M$^{-1}$)</td>
<td>$I_0$</td>
<td>$K_q$ (M$^{-1}$)</td>
</tr>
<tr>
<td>Wild-type</td>
<td>315</td>
<td>1.0</td>
<td>8.3 ± 0.4</td>
<td>1</td>
</tr>
<tr>
<td>W149A</td>
<td>312</td>
<td>1.1</td>
<td>8.3 ± 0.3</td>
<td>1</td>
</tr>
<tr>
<td>W149F</td>
<td>312</td>
<td>1.1</td>
<td>7.5 ± 0.2</td>
<td>1</td>
</tr>
<tr>
<td>W222F</td>
<td>308</td>
<td>0.3</td>
<td>8.6 ± 0.3</td>
<td>1</td>
</tr>
</tbody>
</table>

$^a$ ND: not determined corresponding to the non-linear Stern-Volmer relationship.
indicating that all Trp residues in either wild-type or mutants could be quenched completely by Acr, and the Lehrer’s plot further supported it. In the case of quencher KI, all the proteins except W222F displayed upward curving upon high concentration of the quencher. In Table 2, 79%, 26%, 41% and 100% of total fluorescence of native and mutants were accessible to 1−, respectively, confirming that Trp-149 is exposed to solvent and Trp-222 is in a relatively hydrophobic environment.

Thermal stability after Trp-222 replacement
Wild-type DKR displayed the consistent activities in the temperature range of 25-45°C (Fig. 1), and the activity steeply declined above 45°C. The W149A and W149F showed a comparable decline of activity compared to wild-type DKR during 25-55°C, while W222F and W149F/W222F were much less stable and their activities were dramatically deteriorated at 35°C. It was obvious to see that the Trp-Phe substitution affected the 3-D structural stability of DKR, decreasing its thermal stability without notable changes in secondary structure. Because hydrogen bonding and hydrophobic interaction are the major forces contributing to the thermodynamic stability of a protein (16-19), the decrease of thermostability of W222F and W149F/W222F could be caused by reduced hydrophobicity. In addition, the packing interactions involving Trp-222 at the domain-domain interface might significantly contribute towards the thermal stability.

Interactions between Trp-149 and His-147 on substrate binding
In the docking models, the substrate is located at the cleft between N- and C-domain, and Trp-149 is located along the entry path of the substrate into active site. Trp-149 may play a role in the orientation and stabilization of substrate at active site through Π-Π interactions with the phenyl group of the substrate. As shown in Fig. 2A, Trp-149 and His-147 adopt a parallel-displaced geometry with imidazole placed in the stacked fashion over the phenyl ring, pyrrole ring and a fused C=C bond of indole ring, respectively. When the substrate was docked into the enzyme, the aromatic stack pairing of indole-imidazole-phenyl trimer stabilized the substrate at the binding site. However, when Trp-149 was replaced, the absence or weaker aromatic-aromatic interactions were produced by W149A and W149F, resulting in the shift of His-147 and movement of substrate away from the catalytic site. This observation is also in accordance with the previous report (20) and could explain why the maximum activity of DKR was towards aromatic ketones or aromatic keto-esters with medium length of the side chains.

Influence of Trp-222 on conformational stability
Contacts between the two subunits of DKR are mainly hydrophobic (Fig. 2B). The hydrophobic pocket is formed by the side chains of Leu-196, Leu-200, Ala-204, Ala-205 and Leu-207, and Trp-222 forms hydrogen bonds with Ile-218 and Asp-219, inserting its side chain into the hydrophobic pocket. A protein could be destabilized when a large hydrophobic residue in the core of the protein is truncated to a smaller side chain, which is caused by the creation of one or more cavities of varying sizes (21-24). The replacement of Trp-222 with Ala caused a major difference in α-helix of C-domain from wild-type enzyme as shown in Fig. 2C, in which interrupted hydrophobic interactions with the β-strands of the opposing subunit. Meanwhile, this mutation created a large cavity between the hydrophobic pockets of α-helix, leading to the disruption of the formation of dimer interface. Both of them contributed to the dissociation of the two subunits, which could be the reason for W222A that could not be purified by the same purification procedure as for wild-type enzyme. Because interactions that influence the energetics of subunit association also impact the catalytic function, the supernatants of W222A were not surprising to be inactive.

While the replacement with Phe did not impact the gross structure and positions of the hydrophobic residues in mutant W222F (Fig. 2C, D), a cavity within the enzyme core from the smaller side chain of Phe was produced, and the edge to face type of aromatic-aromatic interaction was decreased, resulting in a looser hydrophobic core than wild-type enzyme. Moreover, the changes in thermal stability of the mutants could be correlated with the size of cavity created by the mutation. The significant effect of W222F was on the enzyme activity, showing almost two-fold increase of activity. According to the model structure of DKR, the diketo ester substrate makes essential contacts with both subunits. It was proposed that enzyme activity can be affected not only by residues in or near the substrate binding site but also residues with distances of 15-20 Å from the site (25). Although Trp-222 was not close to active site, the activity increase of W222F might be attributed to local conformational changes that affect the

**Fig. 1.** Thermal stability of wild-type DKR and mutants. Enzyme activity was determined by an initial rate measurement with preheated enzyme solution using 0.25 mM of diketo ester as substrate in a 0.1 M sodium phosphate buffer, pH 6.0.
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Electrostatic or hydrophobic interactions between the subunits. In addition, a wider substrate-binding pocket exhibits more flexibility, resulting in a positive modulation on catalytic efficiency of the enzyme. Therefore, DKR dimer interface appeared to be optimal for the stability and catalytic function.

In conclusion, this study has provided an insight into the roles of Trp-149 and Trp-222 in DKR architectures and functions. The mutations resulted in significant changes in catalytic activity and protein stability. Trp-149 may play an important role in substrate binding and Trp-222 may take part in maintaining the correct dimer formation for enzyme catalysis. Because of the enhancement of enzyme activity by W222F, further studies are undertaken to screen more variants using W222F as a template for potential industry applications.

MATERIALS AND METHODS

Materials

The oligonucleotides used in site-directed mutagenesis were synthesized by Invitrogen Bio Inc., (Shanghai, China). Axy prep plasmid miniprep kit came from Axygene Biotech Ltd., (USA). IPTG (isopropyl-β-D-thiogalactopyranoside), NAD(P)H, and acetoacetyl-CoA were purchased from Sigma Chemical Co.. E. coli strains DH5α and BL21 (DE3) were obtained from Tiangen Biotech Co. Ltd. (Beijing, China).

Site-directed mutagenesis

Two overlapping complementary primers containing the desired nucleotide changes were designated for each mutation (Supplemental Table 1). Mutations were introduced into the expression vector pET22b (+)-DKR using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, USA) following the manufacturer’s protocol.

Protein expression and purification

E. coli BL21 (DE3) host cells transformed with the desired plasmid were grown at 37°C in LB broth by selection with ampicillin. Protein expression and purification were the same as previously described (9). Briefly, the enzymes were induced by 0.1 mM IPTG at 15°C for 14 h and purified by a HiTrap DEAE FF and a Sephadex G-100 column on an AKTA purifier 900 (Amersham Biosciences). Purity of the protein samples was examined by 12% SDS-PAGE gels with Commassie blue staining. Protein concentration was determined by a BCA kit (CoWin Biotech Co. Ltd., Beijing).
Enzyme assay and kinetic analysis
Enzyme activity of DKR was determined using a UV-1700 array spectrophotometer (Shimadzu, Kyoto, Japan) as described by Wu et al. (1). A standard assay mixture contains 0.1 mM potassium phosphate buffer (pH 6.0), 0.15 mM NADH, 0.25 mM ethyl 3, 5-diketo-6-benzyloxy hexanoate and 2 μg purified enzyme. For kinetic analysis, nine concentrations (0.10, 0.15, 0.20, 0.30, 0.40, 0.50, 0.60, 0.75 and 1.0 mM) of substrate or different quantities of NADH (0.03, 0.06, 0.09, 0.12, 0.15, 0.18, 0.24 and 0.30 mM) were prepared in the standard assay conditions. The values of \( K_{\text{m}} \) and \( k_{\text{cat}} \) and means ± S.D. were calculated from three independent experiments by a nonlinear regression and were plotted using the Michaelis-Menten equation.

Circular dichroism
Circular dichroism (CD) spectra in the Far-UV region (190-250 nm) were obtained using a Jasco-810 spectropolarimeter (8). CD experiments were carried out using 0.2 mg ml \(^{-1}\) of wild type and mutant enzyme in 0.1 M potassium phosphate buffer (pH 6.0) at room temperature. Three scans were collected for each sample.

Fluorescence spectroscopy
Steady state fluorescence measurements were performed at room temperature using a RF-5301PC (Shimadzu, Japan) spectrofluorimeter using a 1 cm quartz cell (9). The excitation and emission monochromators were set at 5 nm slit widths. Excitation was at 295 nm to selectively excite tryptophan residues. Emission was monitored between 290 and 400 nm. The average of three separate scans was analyzed, and contribution of the buffer was subtracted.

Quenching experiments with KI (0-1.8 M) and Acr (0-0.2 M) were accomplished in 3 ml of protein solution. The fluorescence intensity changes were recorded at the maximum emission wavelength for the mutant DKR and corrected for dilution by equation 1 (26): \( F_{\text{corr}} = F_{\text{obs}} / (V_0 + dV) / V_0 \). \( F_{\text{obs}} \) is the fluorescence observed prior to correction for dilution effects, \( V_0 \) is the total volume of protein solution, and \( dV \) is the total volume of quencher solution added during titration. The quenching data were plotted according to the Stern-Volmer equation (27): \( F_0 / F = 1 + K_{\text{sv}} [Q] \) (2), where \( F_0 \) and \( F \) are the fluorescence intensities in the absence and presence of the quencher, respectively; \([Q]\) is the molar concentration of acrylamide; and \( K_{\text{sv}} \) is the Stern-Volmer constant for collisional quenching.

Eq.(2) was modified by Lehrer (28), in the case of proteins where two types of tryptophan fractions are present, one accessible and the other inaccessible to the quencher: \( F_0 / \Delta F = \frac{1}{F_0} + \frac{1}{F_0} [Q] \) (3), where \( F_0 \), \([Q]\) and \( F \) were the same as in Eq. (2), \( F_0 \) was the fraction of accessible Trp residues, and \( K_{\text{sv}} \) was the quenching constant. The data were fitted by linear regression using Origin 8.0.

Thermostability determination
The reaction solution comprised of 0.25 mM of diketo ester substrate diluted in 0.1 mM potassium phosphate buffer, pH 6.0, containing 0.15 mM NADH. Ten microliter aliquots of the enzyme solution were preheated at various temperatures (25, 30, 35, 40, 45, 50, 55 and 60°C) for 30 min. The enzyme activity was determined by the initial rate measurement.

Molecular modeling
Structures of the mutants were generated based on the modeled structure of DKR with alanine and phenylalanine substitutions at residues 149 and 222 by Discovery Studio/Protein Modeling/Build Mutants program (Accelrys Inc., San Diego, CA, USA) as previously described (9). Energy minimization was employed to reduce structure conflicts. The models with the lowest energy value and least number of outliers in the Ramachandran plot were chosen as the final structures. Structural quality of the model was checked with PROCHECK and ERRAT (http://nihserver.mbi.ucla.edu/SAVS/).

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