Structural double-mutant cycle analysis of a hydrogen bond network in ketosteroid isomerase from Pseudomonas putida biotype B

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INTRODUCTION

Site-directed mutagenesis can be employed to understand the role of a specific residue at the molecular level for the structural or functional analysis of a protein [1,2]. To evaluate the quantitative effect of the double mutation on the structural or functional property of a protein, double-mutant cycle analysis has been used extensively to evaluate the effect of the mutation [3]. This method has been applied to the studies on protein folding and/or catalysis of various proteins. If the change of free energy in the double mutation of two residues is different from the sum of those of individual mutations, then the two residues are supposed to be coupled by an intermolecular or intramolecular interaction.

Coupling energy generally reflects the extent to which two residues interact with each other for the catalysis and stability of an enzyme [4]. Double-mutant cycle analysis may be theoretically ideal if no structural rearrangement occurs within the cycle or if the energy associated with the structural change is cancelled in the double-mutant cycle [5,6]. However, these assumptions are not applicable to most cases, and the crystal structure of each mutant within the double-mutant cycle needs to be compared. Quantitative effects of the second mutation on a mutant enzyme were classified previously into several categories with respect to the first mutation [7]. Even though the coupling energy between two residues can be measured by the double-mutant cycle, whether or not the measured interaction represents true coupling energy needs to be evaluated, based on the structural changes of the mutants. The crystal structure of each mutant within the double-mutant cycle was found to be very useful in interpreting the measured coupling energy [8]. Structures of single- and double-mutant barnases were essential in determining whether the interaction which had been measured previously represented the true coupling energy.

KSI (Δ3-3-ketosteroid isomerase) is one of the most proficient enzymes, catalysing the allylic isomerization reaction at a rate comparable with the diffusion limit [9,10]. KSIs from two different bacterial species, Pseudomonas putida and Comamonas testosterone, have been most intensively investigated in terms of structure–function relationship [11–20]. Although the two KSIs share only 34% sequence identity, three-dimensional structures of the two KSIs determined at high resolution by X-ray crystallography [14,15] and that of the C. testosterone KSI by NMR [16,17] indicated that their overall structures are remarkably similar to each other. In addition, the crystal active-site residues

Abbreviations used: B factor, average temperature factor; KSI, ketosteroid (= oxoesteroid) isomerase; PLA2, phospholipase A2; RMSD, root-mean-square deviation; WT, wild-type.

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The atomic co-ordinates of D99L (Asp99 → Leu) ketosteroid isomerase (1W00), Y14F/D99L ketosteroid isomerase (1W02), Y30F/D99L ketosteroid isomerase (1VZZ) and Y55F/D99L ketosteroid isomerase (1W01) were deposited in the Protein Data Bank.

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Expression and purification of mutant KSIs

Mutant KSIs were overexpressed in Escherichia coli BL21 (DE3) (Novagen) harbouring an expression vector plasmid containing a mutant KSI gene, and were purified as described previously [19]. The purity of KSI was confirmed by the identification of a single band in SDS/polyacrylamide gels stained with Coomassie Blue.

Equilibrium unfolding

The equilibrium constant ($K_U$) and the free energy change ($\Delta G_{ul}$) for denaturation were determined according to a two-state model of denaturation by utilizing the following equations as described previously [20]:

$$K_U = 2P_f \cdot \left[ F_{i,j}^2 / (1 - F_{i,j}) \right]$$

$$\Delta G_{ul} = -RT \cdot \ln(K_U) = \Delta G_{ul}^{H_2O} - m \cdot \text{[urea]}$$

where $P_f$ is the total protein concentration, $F_{i,j}$ is the apparent fraction of the unfolded form, $\Delta G_{ul}^{H_2O}$ is the free energy change in the absence of urea and $m$ is a measure of dependency of $\Delta G_{ul}$ on urea concentration.

Double-mutant cycle analysis

The difference of free energies of activation ($\Delta \Delta G_{i,j}$) was determined by substituting the catalytic rate constants in eqn (3), and the difference of free energies of unfolding ($\Delta \Delta G_{i,j}^{H_2O}$) was obtained by eqn (4) as described previously [3–8]:

$$\Delta \Delta G_{i,j} = -RT \cdot \ln(k_{cat,mt} / k_{cat,wt})$$

$$\Delta \Delta G_{i,j}^{H_2O} = \Delta G_{i,j}^{H_2O}_{mt} - \Delta G_{i,j}^{H_2O}_{wt}$$

where $\Delta G_{i,j}^{H_2O}_{wt}$ and $\Delta G_{i,j}^{H_2O}_{mt}$ are Gibbs free energy change for the unfolding in the absence of urea at 25 °C for WT and a mutant KSI respectively. The coupling energy, $\Delta G_i$, was determined by eqn (5):

$$\Delta G_i = \Delta G_{i+1} + \Delta G_{i-1}$$

Double-mutant cycle analysis

The difference of free energies of activation ($\Delta \Delta G_{i,j}$) was determined by substituting the catalytic rate constants in eqn (3), and the difference of free energies of unfolding ($\Delta \Delta G_{i,j}^{H_2O}$) was obtained by eqns (3) and (4) [7]. $\Delta G_i$ is the difference between the effect of the double mutation ($\Delta G_{i+1}$) and the sum of those of two single mutations ($\Delta G_i + \Delta G_j$). The effect of the second mutation on the first was classified according to the categories as described previously [7]: the synergistic effect for $\Delta G_{i+2} > \Delta G_i + \Delta G_j$ ($\Delta G_i > 0$) and the partially additive effect for $\Delta G_i < \Delta G_{i+2} < \Delta G_i + \Delta G_j$ ($\Delta G_i < 0$).

Crystallization and structure determination

Crystals of D99L, Y14F/D99L, Y32F/D99L and Y55F/D99L mutant KSIs were grown by the hanging-drop diffusion method at room temperature (22 °C) as described previously [20]. Diffraction data of Y55F/D99L KSI were obtained by a DIP2020 area detector with graphite-monochromated CuKα X-rays which were generated by a MacScience M18XHF rotating anode generator operated at 90 mA and 50 kV at room temperature. The diffraction data for D99L, Y14F/D99L and Y30F/D99L mutant KSIs were collected at 100 K by the Beamline 6B at the Pohang Accelerator Laboratory. The diffraction data of each mutant KSI were processed using HKL software, and data reduction, merging and scaling were carried out with the programs DENZ0 and SCALPELL as described previously [21]. The structure was determined by molecular replacement utilizing the highly refined monomeric model of the uninhibited KSI and further refinement was carried out with X-PLOR as described previously [22].

Solvent-accessible area

The solvent-accessible area was calculated from the atomic coordinates of the protein by use of a software program, Molecular Surface, which was supplied with a Quanta software...
Table 1 Changes in free energy of catalysis and stability of KSI by mutagenesis

<table>
<thead>
<tr>
<th>KSI</th>
<th>$k_{cat}$ (sec$^{-1}$)</th>
<th>$\Delta G_o$,$^\circ$ (kJ/mol)</th>
<th>$\Delta G_{a}$,$^\circ$ (kJ/mol)</th>
<th>$\Delta G_{H_2O}$,$^\circ$ (kJ/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>21.230 ± 610*</td>
<td>101.7</td>
<td>101.7</td>
<td>-</td>
</tr>
<tr>
<td>Y14F</td>
<td>13.3 ± 0.6</td>
<td>18.4</td>
<td>83.3</td>
<td>18.4</td>
</tr>
<tr>
<td>Y30F</td>
<td>17.800 ± 700</td>
<td>0.4</td>
<td>101.7</td>
<td>0</td>
</tr>
<tr>
<td>Y55F</td>
<td>3510 ± 60°</td>
<td>4.6</td>
<td>87.0</td>
<td>14.7</td>
</tr>
<tr>
<td>Y14F/D99L</td>
<td>2200 ± 9°</td>
<td>11.3</td>
<td>85.8</td>
<td>15.9</td>
</tr>
<tr>
<td>Y14F/D99L</td>
<td>0.67 ± 0.01†</td>
<td>25.5</td>
<td>72.8</td>
<td>28.9</td>
</tr>
<tr>
<td>Y30F/D99L</td>
<td>40.7 ± 1.4</td>
<td>15.5</td>
<td>74.5</td>
<td>27.2</td>
</tr>
<tr>
<td>Y55F/D99L</td>
<td>1.2 ± 0.4</td>
<td>24.3</td>
<td>68.6</td>
<td>33.1</td>
</tr>
</tbody>
</table>

* Values from Kim et al. [20].
† Values from Choi et al. [30].

Table 2 Coupling energy of two residues in the active site of KSI

<table>
<thead>
<tr>
<th>Mutant KSI</th>
<th>Catalysis (kJ/mol)</th>
<th>Stability (kJ/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y14F/D99L</td>
<td>-4.2 ± 0.2</td>
<td>-5.4 ± 0.8</td>
</tr>
<tr>
<td>Y30F/D99L</td>
<td>3.8 ± 0.2</td>
<td>113 ± 1.3</td>
</tr>
<tr>
<td>Y55F/D99L</td>
<td>8.4 ± 0.8</td>
<td>25 ± 0.4</td>
</tr>
</tbody>
</table>

Figure 2 Double-mutant cycles for Y14F/D99L, Y30F/D99L and Y55F/D99L KSIs

Thermodynamic double-mutant cycles show free energy changes in kJ/mol caused by the respective mutation. The value in each mutational step represents the free energy change for the catalysis (A) or stability (B) as determined by eqns (3) or (4).

(Atualys) [23]. The probe radius for the calculation was 1.4 Å (1 Å = 0.1 nm).

RESULTS

Preparation of mutant enzymes

All mutant KSIs were produced in soluble form. Each mutant KSI could be bound specifically to the deoxycholate-affinity resin during the purification of the mutant KSI by affinity chromatography as described previously [19]. Purified proteins were homogeneous as judged by SDS/PAGE analysis (results not shown). Each mutant KSI showed a single band of identical size corresponding to the molecular mass of 14 kDa on SDS/PAGE.

Double-mutant cycle analysis

In order to assess the degree of coupling between amino acids constituting the hydrogen bond network in the active site of KSI, the $\Delta G_o$,$^\circ$ and $\Delta G_{H_2O}$,$^\circ$ values were obtained by applying eqns (3) and (4) for single- or double-mutant KSI of three mutant sets, Y14F/D99L, Y30F/D99L and Y55F/D99L (Table 1). The $\Delta G_o$,$^\circ$ value of Y14F/D99L KSI was 25.5 kJ/mol, which was smaller than the sum (29.7 kJ/mol) of the $\Delta G_o$,$^\circ$ values of single mutants, Y14F and D99L KSIs (Figure 2). The $\Delta G_{H_2O}$,$^\circ$ value of Y14F/D99L KSI was 28.9 kJ/mol, which was smaller than the sum (34.3 kJ/mol) of the $\Delta G_{H_2O}$,$^\circ$ values of single mutants, Y14F and D99L KSIs. The effect of either the Y30F/D99L or the Y55F/D99L mutation was distinctly different from that of the Y14F/D99L mutation. The $\Delta G_o$,$^\circ$ value of Y30F/D99L KSI was 15.5 kJ/mol, which was much larger than the sum (11.7 kJ/mol) of the $\Delta G_{H_2O}$,$^\circ$ values of single mutants, Y30F and D99L KSIs. However, the $\Delta G_{H_2O}$,$^\circ$ value of Y30F/D99L KSI was 27.2 kJ/mol, which was much larger than the sum (15.9 kJ/mol) of those of respective single mutants. In the case of Y55F/D99L KSI, the $\Delta G_o$,$^\circ$ and the $\Delta G_{H_2O}$,$^\circ$ values, 24.3 kJ/mol and 33.1 kJ/mol respectively, were larger than the sum (15.9 kJ/mol for catalysis and 30.6 kJ/mol for stability) of those of single mutants. The coupling energies ($\Delta G$) were determined for three double mutants, Y14F/D99L, Y30F/D99L and Y55F/D99L, utilizing eqn (5) (Table 2). The $\Delta G$ values of catalysis were $-4.2$ kJ/mol for Y14F/D99L KSI, 3.8 kJ/mol for Y30F/D99L KSI and 8.4 kJ/mol for Y55F/D99L KSI. The $\Delta G$ values of stability were $-5.4$ kJ/mol, 11.3 kJ/mol and 2.5 kJ/mol for Y14F/D99L, Y30F/D99L and Y55F/D99L KSIs respectively.

Structural analysis of mutant KSIs

In order to interpret the results of double-mutant cycle analysis for the catalysis and stability, we determined the crystal structures of D99L, Y14F/D99L, Y30F/D99L and Y55F/D99L KSIs (Table 3). In the active site of WT KSI, the carboxy group of Asp$^99$ and the hydroxy group of Tyr$^{14}$ could form hydrogen bonds with Water$^{504}$ [14]. The hydroxy group of Tyr$^{14}$ can form a hydrogen-bond with that of Tyr$^{35}$, which can also be hydrogen-bonded to that of Tyr$^{30}$ (Figure 1). When the crystal structures of WT and mutant KSIs were superimposed on each other to investigate structural differences (Figure 3), RMSD (root-mean-square deviation) values were determined to be 0.24 Å for Y14F/D99L KSI, 0.35 Å for Y30F/D99L KSI and 0.38 Å for Y55F/D99L KSI, indicating that the overall structures of mutant KSIs were similar to each other. Distances between residues involved in the hydrogen bond network were determined (Table 4). In Y14F/D99L KSI, the hydrogen bond between the hydroxy group of Tyr$^{35}$ and that of Tyr$^{30}$ was retained (Figure 3A). Closer examination of Y14F/D99L KSI around the site of the mutation revealed that the phenol ring of Phe$^{14}$ rotated approx. 17° towards Tyr$^{35}$ (Figure 3A). This movement resulted in the positional shift of Tyr$^{55}$ and Tyr$^{30}$, shortening the distance of the hydrogen bond between the hydroxy group of Tyr$^{35}$ and that of Tyr$^{30}$ (Table 4).

The Y30F/D99L mutation resulted in the loss of two hydrogen bonds, Asp$^{99}$...Water$^{504}$ and Tyr$^{55}$...Tyr$^{30}$. The crystal structure of Y30F/D99L KSI revealed that the phenolic ring of Tyr$^{14}$ turned towards Tyr$^{35}$ slightly, and the phenolic ring of Tyr$^{35}$ also turns towards Tyr$^{30}$.
Table 3 Crystallographic statistics for mutant KSIs

<table>
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<tr>
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<tbody>
<tr>
<td>Space group</td>
<td>P2_12_12</td>
<td>C2_2_2</td>
<td>P2_12_12</td>
<td>P2_12_12</td>
</tr>
<tr>
<td>Unit cell [a,b,c (Å)]</td>
<td>35.651, 73.166, 95.615</td>
<td>35.556, 94.859, 73.524</td>
<td>35.279, 72.958, 95.855</td>
<td>36.496, 75.942, 92.312</td>
</tr>
<tr>
<td>α, β, γ (°)</td>
<td>90.0, 90.0, 90.0</td>
<td>90.0, 90.0, 90.0</td>
<td>90.0, 90.0, 90.0</td>
<td>90.0, 90.0, 90.0</td>
</tr>
<tr>
<td>Resolution (Å)</td>
<td>23.1/27.9</td>
<td>23.1/28.5</td>
<td>23.1/23.9</td>
<td>21.2/26.8</td>
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<tr>
<td>R factor/R free (%)</td>
<td>23.1/28.5</td>
<td>21.2/26.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RMSD from ideal</td>
<td>0.006</td>
<td>0.006</td>
<td>0.006</td>
<td>0.006</td>
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<tr>
<td>Bond lengths (Å)</td>
<td>1.124</td>
<td>1.481</td>
<td>1.188</td>
<td>1.187</td>
</tr>
<tr>
<td>Bond angle (°)</td>
<td>135</td>
<td>95</td>
<td>87</td>
<td>38</td>
</tr>
<tr>
<td>Average B factor</td>
<td>23.739</td>
<td>43.567</td>
<td>20.636</td>
<td>31.227</td>
</tr>
</tbody>
</table>

Figure 3 Stereoview of the active site residues in WT and mutant KSIs

(A) WT KSI (light grey) and Y14F/D99L KSI (dark grey). (B) WT KSI (light grey) and Y30F/D99L KSI (dark grey). (C) WT KSI (light grey) and Y55F/D99L KSI (dark grey). Quanta software (Accelrys) was employed for the superimposition of the structures. MolScript was used to draw the structures. The residues in parentheses are the ones which replaced the original amino acid of WT KSI.

rotated slightly with respect to Phe30 (Figure 3B). However, Y55F/D99L KSI showed the removal of three hydrogen bonds, Water504···Asp99, Tyr14···Tyr55 and Tyr55···Tyr30, by mutation. Closer examination of the crystal structures revealed that the phenolic ring of Tyr14 in Y55F/D99L KSI was found to make a rotation of nearly 16° with respect to Tyr55 and the phenyl ring of Phe55 in Y55F/D99L KSI was also found to rotate approx. 8° with respect to Tyr30. These conformational changes in Tyr14 and...
Tyr\textsuperscript{30} shortened the distance between the two residues, so that a new hydrogen bond between Tyr\textsuperscript{14} and Tyr\textsuperscript{30} could be formed, since the distance between the hydroxy group of Tyr\textsuperscript{14} and that of Tyr\textsuperscript{30} was observed to be less than 3.0 Å (Table 4).

**DISCUSSION**

Double-mutant cycle analyses were carried out to investigate the interaction between amino acids which constitute a hydrogen bond network in the active site of KSI. The hydrogen bond network Asp\textsuperscript{99}···Water\textsuperscript{504}···Tyr\textsuperscript{14}···Tyr\textsuperscript{30} connects two critical catalytic residues, Tyr\textsuperscript{14} and Asp\textsuperscript{99}, with Tyr\textsuperscript{14}, Tyr\textsuperscript{30} and a water molecule in the highly apolar active site of KSI. Previous studies revealed that the hydrogen bond network could be crucial for KSI to maintain the proper active-site geometry for both function and stability [20,24].

Phenylalanine and leucine were chosen instead of alanine for the mutagenesis in the replacement of tyrosine and aspartic acid respectively, in order to prevent the formation of a destabilizing cavity, since those residues were located in the buried hydrophobic active site [25–27].

The $\Delta G_o$ values of Y14F/D99L KSI were smaller than the sum of the $\Delta G_o$ values of single mutants, Y14F and D99L KSIs (Figure 2), with the coupling energy ($\Delta G_c$) being negative, suggesting that the effects of the second mutation on the first one should be partially additive for both catalysis and stability. The negative coupling energy indicates that the degree of destabilization by the Y14F/D99L mutation is smaller than the sum of those by two single mutations. The effect of the Y14F/D99L mutation on $k_{cat}$ suggested that Tyr\textsuperscript{14} and Asp\textsuperscript{99} might interact positively to facilitate the same step [7]. Because two catalytic residues were found to be involved in the stabilization of a transition state during the catalysis of KSI [9,10], the effect of the double mutation could be caused by the co-operative binding of the transition state by Tyr\textsuperscript{14} and Asp\textsuperscript{99} with the coupling energy of $-4.2$ kJ/mol, which reflected the amount of co-operativity of the two amino acids (Table 2).

The crystal structure of Y14F/D99L KSI showed that the hydrogen bond network involving Asp\textsuperscript{99}···Water\textsuperscript{504}···Tyr\textsuperscript{14}···Tyr\textsuperscript{30} was disrupted by the loss of three hydrogen bonds, Tyr\textsuperscript{14}···Water\textsuperscript{504}, Asp\textsuperscript{99}···Water\textsuperscript{504} and Tyr\textsuperscript{14}···Tyr\textsuperscript{30}. The small RMSD value between the crystal structure of WT KSI and that of Y14F/D99L KSI indicated that the overall structure was not changed significantly. The crystal structure of Y14F/D99L KSI showed that the disruption of hydrogen bonds in PLA\textsubscript{2} (phospholipase A\textsubscript{2}) [28,29]. The $\Delta G_o$ ($22.6$ kJ/mol) of the Y52F/Y73F PLA\textsubscript{2} was smaller than the sum ($25.1$ kJ/mol) of the $\Delta G_o$ values of single mutants [28]. The crystal structure of Y52F/Y73F PLA\textsubscript{2} clearly demonstrated that disruption of hydrogen bonds between tyrosine residues in PLA\textsubscript{2} caused the increase in hydrophobic interactions of the phenyl groups [29]. In the cases of both KSI and PLA\textsubscript{2}, a partially additive effect of the double mutation could be observed similarly when the double mutation could generate an extra interaction, such as a hydrophobic interaction.

The $\Delta G_o$ values of Y30F/D99L KSI were larger than the sum of the $\Delta G_o$ values of single mutants (Figure 2), Y30F and D99L KSIs respectively. The positive $\Delta G_o$ value indicated that the effect of the Y30F/D99L mutation was synergistic for both catalysis and stability. Hence the effect of the Y30F/D99L mutation suggested that Tyr\textsuperscript{14} and Asp\textsuperscript{99} should interact negatively for the catalysis and stability. The small RMSD value between the crystal structure of WT KSI and that of Y30F/D99L KSI indicated that the overall structure of Y30F/D99L KSI was not changed significantly by the mutation. Double replacements of Tyr\textsuperscript{30} and Asp\textsuperscript{99} with phenylalanine and leucine respectively disrupted the hydrogen bond network. However, the synergistic effect ($3.8$ kJ/mol) of the Y30F/D99L mutation for catalysis was small, since the positioning of Tyr\textsuperscript{30} was not significantly changed (Figure 3B). Since Tyr\textsuperscript{14} and Asp\textsuperscript{99} are located at either end of the hydrogen bond network, no direct co-operative interaction between these two residues could be expected for catalysis or stability. However, the two residues could affect the catalysis and stability indirectly, since they are integral components for the hydrogen bond network in the active site. The synergistic effect of the Y30F/D99L mutation could be explained by the negative interaction between Tyr\textsuperscript{30} and Asp\textsuperscript{99} for both stability and catalysis, even if the interaction is indirect. Tyr\textsuperscript{30} of the *P. putida KSI* was replaced homologously with phenylalanine in the *C. testosteroni* KSI, suggesting that Tyr\textsuperscript{30} might not be critical for the integrity of the hydrogen bond network [20]. Negligible values of $\Delta G_c$ between WT KSI and Y30F KSI, i.e. 0.4 kJ/mol for catalysis and 0.0 kJ/mol for stability, demonstrated that Tyr\textsuperscript{30} could be replaced with phenylalanine with little damage to catalysis and stability. Therefore the D99L mutation could dominantly affect the catalysis in the case of the Y30F/D99L mutation.

The $\Delta G_o$ values of Y55F/D99L KSI were larger than the sum of those of single mutants, Y55F and D99L KSIs. The positive $\Delta G_o$ value indicated that the effect of the Y55F/D99L mutation was synergistic for both catalysis and stability, similar to that of the Y30F/D99L mutation (Figure 2). RMSD between the crystal structure of WT KSI and that of Y55F/D99L KSI indicated that the overall structure was not changed significantly. The crystal

| Table 4 Distances (Å) between the residues or between the residue and water molecule of WT and mutant KSIs |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Water\textsuperscript{504}···Tyr\textsuperscript{14}(O\textsubscript{γ}) | 2.8             | –               | 3.4             | 3.3             | 2.7             | –               | 2.6             | 2.8             |
| Water\textsuperscript{504}···Asp\textsuperscript{99}(O\textsubscript{δ2}) | 2.8             | 3.3             | 3.1             | 3.1             | –               | –               | –               | –               |
| Tyr\textsuperscript{14}(O\textsubscript{η})···Tyr\textsuperscript{30}(O\textsubscript{η}) | 2.6             | 2.7             | –               | 2.6             | –               | 2.6             | –               | –               |
| Tyr\textsuperscript{30}(O\textsubscript{η})···Tyr\textsuperscript{55}(O\textsubscript{η}) | 2.7             | 2.6             | –               | 2.6             | 2.5             | –               | –               | –               |
| Tyr\textsuperscript{30}(O\textsubscript{η})···Asp\textsuperscript{99}(O\textsubscript{δ2}) | 4.1             | –               | 3.0             | 4.3             | –               | 4.3             | –               | 2.9             |

* Distances were obtained from the structures deposited in the Protein Data Bank; WT (1OPY), Y14F (1EA2), Y30F (1DMQ) and Y55F (1DMM).
structure of Y55F/D99L KSI showed that the double mutation not only disrupted the hydrogen bond network, but also significantly altered the positioning of Tyr14, as judged by the change of distance between the hydroxy group of Tyr14 and that of Tyr30 from 4.1 Å in WT KSI to 2.9 Å in Y55F/D99L KSI (Table 4). The synergistic effect of the Y55F/D99L mutation suggests that Tyr55 and Asp99 interact negatively for the catalysis and stability, as shown similarly in the Y30F/D99L mutation. Since Tyr55 is linked to Asp99 through Tyr14 and Water56 in the hydrogen bond network, the synergistic effect of the double mutation could be caused by the disruption of the hydrogen bond network and more importantly by the significant change in the positioning of Tyr14 (Figure 3C). The proper positioning of Tyr14 might be very crucial to the maintenance of the hydrogen bond network, which is critical to both catalysis and stability of KSI.

The importance of Tyr14, a critical catalytic residue, in the catalysis of KSI was reported previously [24,30–33]. Tyr14 is supposed to be able to polarize the 3-carbonyl group in the steroid substrate in order to facilitate the enolization of the substrate during the catalysis of KSI [9,10]. The crystal structure of WT KSI indicated that the average temperature factor (B factor) of the carbon atoms in the phenyl ring of Tyr14 was 6.3 Å², indicating that Tyr14 is very rigid [24]. Relaxation studies of the backbone dynamics in KSI also revealed that the motion of Tyr14 was restricted significantly [31–33]. Although the kcat of Y55F KSI was decreased 6-fold compared with that of WT KSI, the additional replacement of Tyr55 with phenylalanine restored the catalytic activity up to half of the level of WT KSI [20]. The pseudoreversion of the catalytic activity could be explained by the crystal structure of Y30F/Y55F KSI, since the Y30F mutation in addition to the Y55F mutation reoriented Tyr14 into the position occupied originally in WT KSI. Moreover, the B factor (10.1 Å²) of the carbon atoms in the phenyl ring of Tyr14 in Y30F/Y55F KSI was decreased compared with that of Tyr14 (13.0 Å²) in Y55F KSI. The crystal structures of Y30F/D99L and Y55F/D99L KISIs indicated that the B factor values of carbon atoms in the phenyl ring of Tyr14 in the two mutant KSIs, 12.2 Å² and 12.8 Å² respectively, were increased as compared with that in WT KSI. The decreased rigidity of the phenolic group in Tyr14 might weaken the proper interaction between the steroid substrate and Tyr14. Tyr14 could stabilize the enolate form of the steroid intermediate during the catalytic reaction [9,10]. The significant change of the positioning of Tyr14, as well as the increase of the B factor of carbon atoms in Tyr14 due to the loss of the hydrogen bond between Tyr14 and Tyr55, is consistent with the reason why the coupling energy from Y55F/D99L KSI was larger than that of Y30F/D99L KSI in the catalysis of KSI.

Interestingly, the crystal structure of Y55F/D99L KSI showed that a new hydrogen bond could be formed between Tyr14 and Tyr30 (Table 4). The newly formed hydrogen bond seemed to contribute to the further decrease of ΔGc for stability compared with the ΔG value, which might be expected without the hydrogen bond. A similar effect of the hydrogen bond strength on stability was reported previously [34,35]. In barnase, the solvent-inaccessible phenolic hydroxy group of Tyr53, which forms hydrogen bonds with two uncharged amide groups of the protein, contributed 5.9 kJ/mol to the stability of the protein [34]. In bovine pancreatic PLA2, the conformational stability affected by several mutations of residues involved in the hydrogen bond network was investigated [35]. The hydrogen bond between Tyr53 and Asp99 contributes 8.4–12.6 kJ/mol to the conformational stability in bovine pancreatic PLA2. These results suggest that the loss of the hydrogen bond could destabilize the protein by approx. 8.4–12.6 kJ/mol. Hence the formation of a new hydrogen bond in Y55F/D99L KSI might explain the reason why the coupling energy calculated in Y55F/D99L KSI (2.5 kJ/mol) was smaller than that in Y30F/D99L KSI (11.3 kJ/mol) (Table 2).

In conclusion, double-mutant cycle analyses of the residues constituting a hydrogen bond network in the active site of KSI demonstrated that the hydrogen bond network is important for both catalysis and stability. The negative coupling energy of Y14F/D99L KSI suggested that Tyr14 and Asp99 should interact positively for the catalysis and stability. The positive coupling energy of both Y30F/D99L and Y55F/D99L KISIs suggested that either Tyr14 and Asp99 or Tyr55 and Asp99 mutation should interact negatively for the catalysis and stability. The Y55F/D99L mutation of KSI was found to significantly impair the proper positioning of Tyr14.

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Double-mutant cycle analysis of ketosteroid isomerase


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