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Role of hydrogen bond network in the function, stability, and structure of ketosteroid isomerase from *Pseudomonas putida*

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H-bond networks that are formed by polar or charged residues play a critical role in the structure, catalytic activity and stability of proteins. Ketosteroid isomerase (KSI) catalyzes the allylic isomerization of a variety of Δ^5-3-ketosteroids by the intramolecular transfer of a proton from the 4β-position to the 6β-position via a dienolate intermediate. An H-bond network in KSI consists of two catalytic residues (Tyr14 and Asp99), Tyr30, Tyr55, and a water molecule in the highly hydrophobic active site. In this review, we summarize our current understanding of the role of the H-bond network in the activity, stability, and structure of KSI. Both mutational and structural studies suggest that the H-bond newtork could contribute to the catalysis and stability of KSI through the maintenance of the active-site geometry and the provision of structural support.

INTRODUCTION

Hydrogen bonds are ubiquitous in nature and are very important in chemical and biological systems (Desiraju and Steiner, 2001; Jeffrey and Saenger, 1991). Hydrogen bonds are formed and broken between hydrogen bond donors and acceptors and play important roles in protein folding, protein and nucleic acid structures, and enzymatic catalysis (Bordo and Argos, 1994; Rose and Wolfenden, 1993; Schowen et al., 2000; Shan and Herschlag, 1999). Occasionally, polar or charged residues form one or more hydrogen bonds with adjacent residues or water molecules to make an H-bond network. Such a network plays a critical role in the fine-tuned protein structure. H-bond networks are frequently found within active sites, where they contribute to catalysis by stabilizing the transition state (Kicska et al., 2002; Mortensen et al., 1994; Sigala et al., 2013). In addition, the dynamics of H-bond networks can drive the allosteric transitions of proteins and proton-coupled electron transfer (Chakrabarti et al., 2014; Sigala et al., 2009). Moreover, H-bond networks are important for maintaining protein stability (Bak and Elliott, 2013; Federici et al., 2009; Yuan et al., 2013).

Ketosteroid isomerase (KSI) is a small dimeric protein that catalyzes the allylic isomerization of a variety of Δ^5-3-ketosteroids by the intramolecular transfer of a proton from the 4β-position to the 6β-position via a dienolate intermediate (Figure 1) (Ha et al., 2001; Pollack et al., 1999). Two homologous enzymes from two different bacterial sources, *Comamonas testosteroni* and *Pseudomonas putida*, have been studied extensively to investigate the mechanistic enzymology of steroid biochemistry (Kraut et al., 2006; Pollack, 2004; Talalay and Wang, 1955; Zhao et al., 1996b). Recently, human glutathione transferase was observed to catalyze the isomerization of Δ^5-3-ketosteroids, which suggests that it could play a role in the biosynthetic pathway of steroid hormones (Johansson and Mannervik, 2001). Moreover, plant KSI from *Digitalis lanata* was found to show similar properties to bacterial KSIs (Meitinger et al., 2015).

KSI has several advantages as a model system for studying protein folding, activity, and stability. Compared with other proteins, KSI is easily expressed and purified by the affinity chromatographic method together with size exclusion chromatography (Jang et al., 2004; Lee et al., 2008b). Therefore, proteins single- or double-labeled with ^{13}C or ^{15}N are easily overexpressed and are obtained in higher yields than other proteins. Backbone NMR resonance assignments were performed for both KSIs and used to study their folding and catalysis (Joe et al., 2012; Lee et al., 2008a; Zhao et al., 1996a). Moreover, various crystal structures of mutant KSIs were determined to enable the better understanding of structure-function relationships (Choi et al., 2000; Kim et al., 2000; Kraut et al., 2010; Schwans et al., 2013).

The crystal and solution structures of *C. testosteroni* KSI and *P. putida* KSI revealed that the overall structures of both KSIs were almost identical, though the sequence identity between the

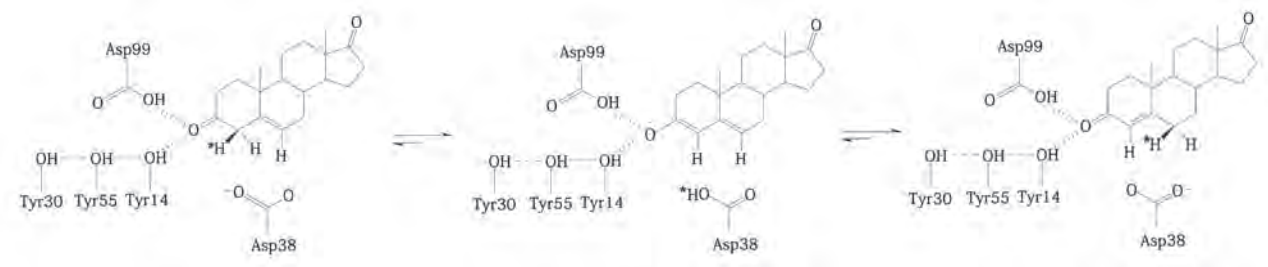

FIGURE 1 | The isomerization reaction catalyzed by KSI. The proton at C4 of the substrate is transferred to Asp38 to generate a dienolate intermediate, and then transferred to the C6 of the substrate. Both Tyr14 and Asp99 stabilize the dienolate intermediate by forming hydrogen bonds with the steroid.

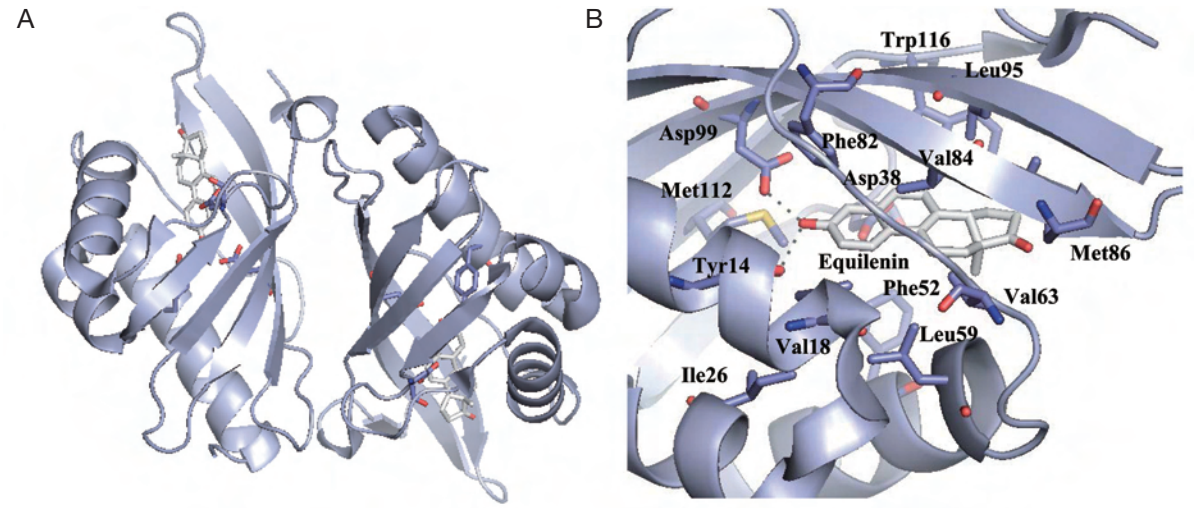

FIGURE 2 | Three-dimensional structure of KSI. (A) Ribbon diagram of the dimeric structure of KSI in complex with equilenin (PDB code 1OH0). Three catalytic residues (Tyr14, Asp38, and Asp99) and equilenin are shown as stick models. (B) Closed-up view of the active site. The active-site cavity is lined mainly with hydrophobic residues such as Val18, Phe52, Phe82, Val84, Met112, and Trp116. The equilenin and active-site residues are shown as stick models. The hydrogen bonds are shown as dotted lines. The figure was displayed using the PyMOL program.

KSIs is only 34 % (Kim et al., 1997; Wu et al., 1997). Scytalone dehydratase, nuclear transport factor-2, and naphthalene 1,2-dioxygenase beta subunit had similar protein folds according to the structural classification of proteins (Murzin, 1998). The crystal structures of the KSIs revealed that three α-helix and six β-sheets constituted each cone-shaped monomer (Kim et al., 1997). Long, narrow patches of β-sheets of each monomer interact with each other, and the interface between monomers is formed between the convex surfaces of the β-sheets (Figure 2A). According to the PISA server (Krissinel and Henrick, 2007), the buried surface area between monomers is approximately 1040 $Å^2$, which could provide favorable interactions between monomers. Mutational studies on the residues involved in the dimeric interface revealed that the dimeric interface residues contribute to the stability and structural integrity of KSI (Nam et al., 2003).

The active site of KSI is very hydrophobic for effective substrate binding. Most of the active-site cleft is composed of hydrophobic residues such as Val18, Ile26, Phe52, Leu59, Val63, Phe82, Val84, Met86, Leu95, Met112, and Trp116 (the residues of *P. putida* KSI are numbered according to the sequence of *C. testosteroni* KSI throughout the text). These hydrophobic active-site residues are suitable for hydrophobic interaction with the ring moieties of steroid as shown in the crystal structure of *P. putida* KSI in complex with equilenin, an intermediate analogue (Figure 2B) (Kim et al., 1997).

In apo-form KSI, two critical catalytic residues (Tyr14 and Asp99), Tyr30, Tyr55, and a water molecule (Wat504) form an H-bond network (Asp99···Water504···Tyr14···Tyr55···Tyr30) in the highly apolar active site (Figure 3A) (Kim et al., 2000). In the presence of equilenin, the steroid displaces the Wat504 molecule and interacts directly with Tyr14 and Asp99 without inducing any notable conformational change (Figure 3B). This H-bond network is also conserved in *C. testosteroni* KSI except for position 30, which is not tyrosine but phenylalanine. Interestingly, the three tyrosine residues are hydrogen bonded to each other to make a tyrosine triad. In this review, we focus on the role of the H-bond network in KSI in catalysis, stability, and structure. In addition, we

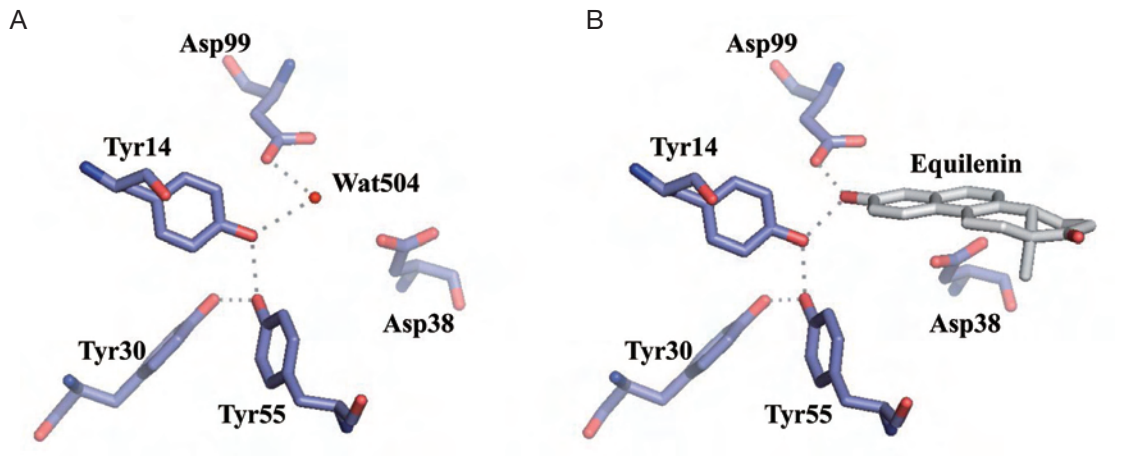

FIGURE 3 | An H-bond network in the active site of KSI. (A) Tyr14, Tyr30, Tyr55, Asp99, and Wat504 form the H-bond network in apo-form KSI. (B) The Wat504 molecule is replaced by the C3-O of equilenin in the steroid-bound form. The residues involved in the H-bond network and the steroid are shown as stick models. The water molecule is shown as a red sphere, and the hydrogen bonds are represented by dotted lines.

discuss the double-mutant cycle analysis of the H-bond network, the compensatory effects among three tyrosine-to-phenylalanine mutations, and the role of the phenyl rings of the tyrosine triad in maintaining the H-bond network.

CONTRIBUTION OF THE H-BOND NETWORK TO CATALYSIS, STABILITY, AND STRUCTURE IN KSI

A Tyr14 residue was first identified as the residue involved in stabilizing the oxyanion (C3-O) of the dienolate intermediate during steroid isomerization (Kuliopulos et al., 1989). A kinetic study of Y14F KSI from *P. putida* revealed that the Y14F mutation significantly affected catalysis by lowering the k_{cat} by approximately 2000-fold because the Tyr14 residue participates in the allylic isomerization by providing a hydrogen bond to the dienolate intermediate (Table 1) (Kim et al., 1997). Structural comparison between the wild-type (WT) and Y14F KSIs revealed that the Y14F mutation caused a structural perturbation of the active site (Figure 4A) (Choi et al., 2001). The Y14F mutation led to loss of two hydrogen bonds between the hydroxyl group of Tyr14 and the Wat504 molecule and between the hydroxyl group of Tyr14 and the Tyr55 residue. The mutation also caused to the phenyl ring of residue 14 to move into a more hydrophobic

TABLE 1 | Activity and stability parameters of KSIs

KSI	k_{cat} (sec^{-1})	K_M (μM)	k_{cat} / K_M (M^{-1}s^{-1})	$\Delta G_U^{H_2O}$ (kcal/mol)
WT	21230 ± 810 [a]	49.9 ± 1.3	4.3×10^8	24.3 [a]
Y14F	13.3 ± 0.6 [a]	17.1 ± 3.1	7.8×10^5	19.9 [a]
Y30F	17800 ± 70 [a]	55.2 ± 2.0	3.3×10^8	24.3 [a]
T55F	3510 ± 60 [a]	23.0 ± 1.0	1.5×10^8	20.8 [a]
D99L	220 ± 9 [a]	25.8 ± 0.8	8.5×10^6	20.5 [a]
Y14F/D99L	0.67 ± 0.01 [b]	98.2 ± 6.9	6.8×10^3	17.4 [c]
Y30F/D99L	40.7 ± 1.4 [a]	73.5 ± 11.8	5.5×10^5	17.8 [a]
Y55F/D99L	1.2 ± 0.4 [a]	61.9 ± 6.2	1.9×10^4	16.4 [a]
Y14F/Y30F	302 ± 18 [b]	78.7 ± 6.5	3.8×10^6	23.3 [d]
Y30F/Y55F	10680 ± 350 [a]	50.2 ± 5.5	2.1×10^8	24.3 [a]
Y14F/Y55F	360 ± 14 [b]	28.7 ± 4.1	1.3×10^7	21.8 [d]

The effects of the mutations on k_{cat} and k_{cat}/K_M were similar because the K_M values of mutant KSIs were within about 2-fold of the K_M value of WT KSI.
[a] Data from Kim et al., (Kim et al., 2000). [b] Data from Choi et al., (Choi et al., 2001). [c] Data from Jang et al., (Jang et al., 2004). [d] Data from Cha et al., (Cha et al., 2013).

environment and to move the Wat504 molecule away from its original location. Moreover, the Y14F mutation decreased the stability of KSI. The unfolding free-energy change of Y14F KSI was lowered by 4.4 kcal/mol compared with WT KSI (Table 1) (Kim et al., 2000). Those results suggested that the removal of the hydroxyl group from the Tyr14 residue could significantly affect both catalysis and stability due to the loss of a hydrogen bond between Tyr14 and the steroid substrate or between Tyr14 and Wat504.

A mutation of the Tyr55 residue, which constitutes the H-bond network with Tyr14 and Tyr30, into phenylalanine decreased k_{cat} by 6-fold (Kim et al., 2000), indicating that the effect of the Y55F mutation on catalysis was not significantly large compared with the Y14F mutation. The unfolding free-energy change of Y55F KSI was lowered by 3.5 kcal/mol compared with the WT KSI (Kim et al., 2000). The decrease in stability for Y55F KSI was smaller than for Y14F KSI. Although two hydrogen bonds (Tyr14···Tyr55 and Tyr55···Tyr30) were abolished by the mutation, the hydrogen bonds (Tyr14···Wat504···Asp99) were still maintained in Y55F KSI, as shown by the crystallographic data. Those data indicated that the effects of the Y55F mutation on catalysis and stability were smaller than the effects of Y14F KSI, as the H-bond network in Y55F KSI was not greatly distorted by the mutation.

Although two tyrosine residues (Tyr14 and Tyr55) are conserved in *C. testosteroni* KSI and *P. putida* KSI, the residue at position 30 is not conserved. The residue at position 30 in *C. testosteroni* KSI is phenylalanine, as mentioned in the introduction. The mutation of Tyr30 into phenylalanine did not affect the catalysis or stability (Kim et al., 2000). In addition, structural comparison between the WT and Y30F KSIs did not show any difference in the configuration of the H-bond network (Kim et al., 2000). Therefore, the removal of the hydroxyl group from the Tyr30 residue might not disrupt the H-bond network.

The Asp99 residue also plays a critical role in catalysis by stabilizing the dienolate intermediate, together with the Tyr14 residue (Thornburg et al., 1998; Wu et al., 1997). The catalytic activity of D99L KSI was decreased by 97-fold compared with WT KSI (Choi et al., 2000). Moreover, the stability of D99L KSI was significantly affected by the mutation. The unfolding free-energy change of D99L KSI was decreased by 3.8 kcal/mol compared to the WT KSI (Kim et al., 2000). However, the mutation of Asp99 into leucine did not induce any structural changes without the loss of a hydrogen bond between Asp99 and Wat504. The crystal structure of D99L KSI revealed that the hydrogen bonds (Wat504···Tyr14···Tyr55···Tyr30) were not disrupted by the mutation (Jang et al., 2004). Therefore, the hydrogen bond between Asp99 and Wat504 might contribute substantially to the catalysis and stability of KSI.

DOUBLE-MUTANT CYCLE ANALYSIS OF THE H-BOND NETWORK IN KSI

The simultaneous mutation of the Tyr14 and Asp99 residues significantly affected the catalysis of KSI (Kim et al., 2000). From the double-mutant cycle analysis, the effect of the Y14F/D99L mutation on catalysis should be partially additive, which suggests that Tyr14 and Asp99 might interact positively (Mildvan et al., 1992). Because the Tyr14 and Asp99 residues were found to stabilize the dienolate intermediate by forming hydrogen bonds, the effect of the Y14F/D99L mutation could be caused by the cooperative binding of Tyr14 and Asp99 to the intermediate (Jang et al., 2004). The effect of the Y14F/D99L mutation on stability also corresponded to the partially additive effect. The solvent-accessible area of residue 14 in Y14F/D99L KSI was smaller than that observed in Y14F KSI or WT KSI. This decreased solvent-accessible area indicated an increase in hydrophobic interactions with hydrophobic residues around the active site, which could explain that the degree of destabilization by the double mutation is smaller than the sum of that by each single mutation (Jang et al., 2004). From these analyses, the partially additive effects of the Y14F/D99L mutation on catalysis and stability could originate from the simultaneous participation of the Tyr14 and Asp99 residues in the allylic isomerization step and the extra hydrophobic interaction in the active-site microenvironment.

The Y30F/D99L mutation also affected the catalytic power and stability of KSI (Kim et al., 2000). Double-mutant cycle analysis revealed that the effects of the Y30F/D99L mutation on catalysis and stability were synergistic (Jang et al., 2004). However, the synergistic effect was small, as the conformation of Tyr14 was not significantly affected by the double mutation. Although there was no direct interaction between the Tyr30 and Asp99 residues, the two residues could indirectly affect the catalysis and stability. Moreover, the mutational effect on catalysis in Y30F/D99L KSI could be dominantly affected by the D99L mutation because the Y30F mutation caused little damage to catalysis and stability (Kim et al., 2000).

Remarkably, the Y55F/D99L mutation resulted in a significant decrease in k_{cat}, which was lower than for Y14F KSI (Kim et al., 2000). The double mutation also significantly decreased the stability (Kim et al., 2000). Similar to the Y30F/D99L mutation, the effect of the Y55F/D99L mutation was synergistic for catalysis and stability (Jang et al., 2004). Structural comparison between the WT and Y55F/D99L KSIs revealed that the Y55F/D99L mutation not only disrupted the H-bond network but also significantly changed the conformation of Tyr14 (Jang et al., 2004). The side chain of Tyr14 was found to rotate into the Phe55 residue. Because the Tyr55 residue is linked to the Asp99 residue through Tyr14 and Wat504 in the H-bond network, the synergistic effect of the Y55F/D99L mutation could be due to the distorted H-bond network and the altered conformation of the Tyr14 residue. Moreover, the rigidity of the phenol ring in the Tyr14 residue was decreased, which could prevent the proper interaction between the Tyr14 residue and the substrate. The changes in the conformation and rigidity of the Tyr14 residue could explain why the catalytic activity of Y55F/D99L KSI was

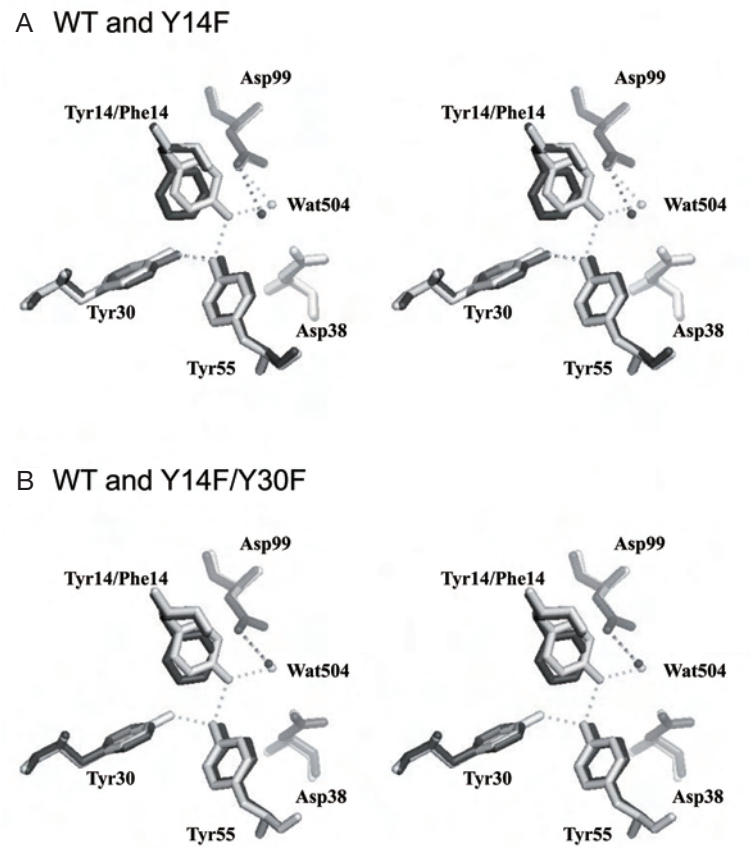

FIGURE 4 | Stereoview of the superposition of the active-site residues in WT and mutant KSIs. (A) WT KSI (light gray; PDB code 1OPY) and Y14F (dark gray; PDB code 1EA2); (B) WT KSI (light gray) and Y14F/Y30F (dark gray; PDB code 4K1U). The active-site residues are represented as stick models, and the water molecules is shown as spheres. The hydrogen bonds are represented by dotted lines.

lower than the catalytic activity of Y14F KSI.

COMPENSATORY EFFECTS AMONG TYROSINE-TO-PHENYLALANINE MUTATIONS

Adding the Y30F mutation to Y14F resulted in catalytic reversion. The Y30F mutation increased the catalytic activity of Y14F KSI by 23-fold (Choi et al., 2001). Structural comparison between the Y14F and Y14F/Y30F KSIs showed that the additional Y30F mutation partially recovered the perturbation of the active site by the Y14F mutation (Figure 4B) (Cha et al., 2013). The phenyl ring of residue 14 and the Wat504 molecule were partially returned to their original positions, leading to the restoration of the hydrogen bond between Asp99 and Wat504. The Y30F mutation also rescued the stability of Y14F KSI by increasing the hydrophobic interactions within the active site (Cha et al., 2013). Those date suggested that the Y30F mutation could rescue the catalytic activity and stability of Y14F KSI via the structural restoration of the active site and the improvement of hydrophobic interactions with neighboring residues.

The additional Y30F mutation also restored the catalytic activity and stability of Y55F KSI. The activity of Y30F/Y55F KSI was increased to half the level of WT KSI (Kim et al., 2000). In addition, the stability of Y30F/Y55F KSI was restored to the level of WT KSI (Kim et al., 2000). Closer examination of the crystal structure of Y30F/Y55F KSI revealed that the additional Y30F mutation resulted in the reorientation of the Tyr14 residue into its original location (Kim et al., 2000). Moreover, the side chains of residue 30 and residue 55 were restored to their original positions. Therefore, the Y30F mutation might recover the decreased catalytic activity in Y55F KSI by reconstructing the configurations of the active-site residues. The recovery of stability in Y30F/Y55F KSI might result from additional hydrophobic interactions in the hydrophobic active site environment. Although the Y30F/Y55F mutation results in the loss of two hydrogen bonds (Tyr14···Tyr55 and Tyr30···Tyr55), the additional hydrophobic interactions might compensate for the mutational effect on stability in Y55F KSI.

Interestingly, the Y55F mutation also rescued the defective effects of Y14F KSI. The catalytic activity of Y14F/Y55F KSI was increased by 27-fold compared with Y14F KSI (Choi et al., 2001). Similarly, the recovery of catalytic activity by the Y55F mutation was also observed in *C. testosteroni* KSI (Li et al., 1993). Crystallographic data revealed that the recovery of catalytic activity by the Y55F mutation could be due to active-site geometry restoration (Cha et al., 2013). Reorientation of the phenyl ring in residue 14 and restoration of the hydrogen bond between Asp99 and Wat504 were observed in Y14F/Y55F KSI.

CONTRIBUTION OF BENZYL RINGS OF TYROSINE TRIAD TO THE MAINTENANCE OF THE H-BOND NETWORK

Mutations of the three tyrosine residues into serine significantly affected the catalytic activity. The k_{cat} values of Y14S, Y30S, and Y55S KSIs were decreased by 33-, 4-, and 51-fold, respectively, compared with WT KSI (Nam et al., 2001). The three tyrosine-to-serine mutations also significantly affected the stability of KSI. The stability measurement of each serine mutant revealed that the stabilities of the Y14S, Y30S, and Y55S KSIs were decreased by 11.9, 13.7, and 9.5 kcal/mol, respectively, relative to WT KSI (Nam et al., 2001). Remarkably, the mutational effects of the three tyrosine-toserine mutations on stability were larger than the effects of the tyrosine-tophenylalanine mutations, which suggest that the phenyl rings of tyrosine residues contribute more significantly to the conformational stability of KSI than the hydroxyl groups. Those results suggested that the removals of phenyl rings in the tyrosine triad could significantly affect both

the catalytic activity and the stability of KSI.

Circular dichroism spectrum showed that the three tyrosine-to-serine mutations also affected the molar ellipticity at 222 nm (θ_{222nm}). The decreases in θ_{222nm} for the Y14S, Y30S, and Y55S KSIs were 3800, 5200, and 3400 deg cm^2 $dmol^{-1}$, respectively, compared with WT KSI (Nam et al., 2001). However, the far UV spectra of the Y14F, Y30F, and Y55F KSIs were similar to the far UV spectrum of WT KSI. Therefore, removing the phenyl rings from the three tyrosine residues could significantly induce structural changes by perturbing the α-helices. The crystal structure of Y55S KSI revealed that α-helix A3 displayed poor electron densities for Y55S KSI due to the perturbation of the α-helix (Nam et al., 2001). In addition, structural comparison between the WT and Y55S KSIs showed that the Y55S mutation resulted in a positional shift of the Tyr14 residue toward the Tyr30 residue and shortened the distance between the Tyr14 and Tyr30 residues. Therefore, this positional shift disrupted the H-bond network, which might be correlated with the decreased activity of the Y55S KSI.

Interestingly, the Y55S mutation also affected the volume of the active-site cavity. When the volume of the active-site cavity was calculated using the CASTp server (Dundas et al., 2006), the cavity volumes of the WT and Y55S KSIs were 584 and 746 $Å^3$, respectively (Cha et al., 2014). Synchrotron X-ray scattering experiments also showed that the active-site cavity in Y55S KSI was larger than in WT KSI in solution (Cha et al., 2014). The enlarged cavity of Y55S KSI might originate from the conformational changes of flexible regions around the active site, including the flexible α-helix A3 region. The K_M value of Y55S KSI was 1.3-fold higher than that of WT KSI (Nam et al., 2001), suggesting that the Y55S mutation could affect the substrate binding. Therefore, the abnormally enlarged active site in Y55S KSI might prevent the proper binding of substrate in the active site, leading to the reduced catalytic activity. Those data suggested that the phenyl ring of Tyr55 residue might play a critical role in maintaining the local structure near the active site. Taken together, the phenyl rings of the three tyrosine residues could contribute to the activity and stability of KSI by maintaining the H-bond network and the structural integrity near the active site.

CONCLUSION

The H-bond network plays an important role in the catalytic activity and protein stability of KSI by providing hydrogen bonds to ligands and/or adjacent residues. Structural analysis showed that the H-bond network contributed to both function and stability by maintaining the fine-tuned active site geometry and providing structural support. Double-mutant cycle analyses based on crystal structures revealed that the H-bond network was important for the catalysis and stability of KSI. Interestingly, three tyrosine-to-phenylalanine mutations at the residues that constitute the H-bond network compensated for each other, thereby escaping the defective effects of a disrupted network. Combined with the solution X-ray scattering and crystallographic data, the mutational studies on the role of the aromatic moieties of the active-site tyrosine residues demonstrated that the phenyl groups contribute to the catalysis and stability by providing the structural integrity around the active site.

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AUTHOR INFORMATION

The authors declare no potential conflicts of interest

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