Protein engineering to improve the thermostability of glucoamylase from Aspergillus awamori based on molecular dynamics simulations

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Twelve mutations were constructed to improve the thermostability of glucoamylase from Aspergillus awamori based on the results of molecular dynamics simulations. The thermal unfolding of the catalytic domain followed a putative hierarchical behavior. In addition, the unfolding of the 13 α-helices obeyed the random ordered mechanism, in which the α-helices 8, 1 and 11 unfolded more rapidly than the others. The catalytic center was well protected by the (α/β)6-barrel at simulation temperatures up to 600 K, whereas the catalytic base, E400, migrated from its original interior pocket to the surface of the catalytic domain by surmounting the hydrophobic barrier provided by α-helices 12 and 13 at 800 K. The disulfide bonds engineered to ‘lock’ the α-helix 11 on the surface of the catalytic domain dramatically increased the thermostability. Substituting G396 and G407 with Ala residues slightly increased the thermostability, whereas their specific activity and catalytic efficiency were reduced. This indicates that the introduced residues with higher hydrophobicity were favorable in the loop between α-helices 12 and 13, whereas they partially destroyed the hydrogen bond and salt linkage network in the catalytic center. α-Helices 12 and 13 can be stabilized by introducing residues with higher hydrophobicity, except for the H391M mutation.

Keywords: catalytic efficiency/hydrophobic barrier/hydrophobicity/specific activity/unfolding

Introduction

Glucoamylase [α-(1,4)-β-glucan glucohydrolase, EC 3.2.1.3] (GA), isolated from different organisms such as fungi, yeasts and bacteria, is one of the most widely used enzymes in industry (Liu, 2001). It catalyzes the hydrolysis of α-1,4-glucosidic linkages from the non-reducing ends of starch and related oligo- and polysaccharide chains (Meagher and Reilly, 1989). The three-dimensional structure of the catalytic domain (CD) of Aspergillus awamori var. X100 GA has been solved by x-ray crystallography in its native state at different pH values and in complexes with different inhibitors (Aleshin et al., 1992, 1994a,b, 1996; Harris et al., 1993; Stoffer et al., 1995). In addition, the solution structure of the starch-binding domain (SBD) has been determined by NMR spectroscopy (Jacks et al., 1995; Sorimachi et al., 1996, 1997). Furthermore, the GA gene from A. awamori has been cloned and expressed in Saccharomyces cerevisiae (Innis et al., 1985), which has made genetic manipulation of GA a convenient task.

The structural and functional relationship of proteins is of great interest in modern biochemistry. Previously, necessary structural and functional information for design of engineered GA variants with desired properties was mostly provided by amino acid sequence alignments from different organisms (Coutinho and Reilly, 1994). A better understanding of protein folding/unfolding processes allows us to predict more precisely a specific site or region critical to GA’s thermostability. In the food industry, a highly thermostable form of GA that could be used at higher temperatures is desired not only because the reaction rate can be increased, but also because the viscosity of the reaction syrups and the chance of microbial contamination can be reduced.

Previous mutagenesis strategies to obtain a GA with higher thermostability include (1) introducing extra disulfide bonds (Li et al., 1998), (2) reducing the number of possible conformations in the unfolded state of protein by replacing Gly with other residues (Chen et al., 1996), (3) reducing the backbone flexibility by introducing a Pro residue (Li et al., 1997) and (4) decreasing flexibility and increasing O-glycosylation in the 440–470 belt region (Liu et al., 2000). In addition, GA has also been engineered to increase its selectivity towards α-1,4-linked oligosaccharides (Fang et al., 1998a,b; Liu et al., 1998, 1999).

 Usually, proteins begin to unfold owing to the increased intramolecular motions caused by increased temperature. GA undergoes irreversible thermal inactivation at moderately acidic pH and 75°C (Liu et al., 2000). Neither deamidation (Chen et al., 1994a,b) nor Asp-X peptide bond hydrolysis (Chen et al., 1995) were found during thermostabilization of GA. In addition, mismatched disulfide bonds were not observed at 75°C and pH 3.5 and 4.5 (Liu et al., 2000). These results indicate that some other mechanism must be responsible for the thermodenaturation of the CD. This mechanism appears to be a change in the secondary or tertiary structure of the CD, such as the unfolding that destroys the integrity of the catalytic center (Williamson et al., 1992; Liu et al., 2000). In order to gain insight into the unfolding mechanism prior to the rational design of the mutations to increase the thermostability of GA, molecular dynamics (MD) simulations of the CD from A. awamori var. X100 GA were performed in the present study.

Based on the results from the MD simulations, 12 mutations were constructed to improve the thermostability of GA. α-Helix 11 is the third helix to undergo the thermal unfolding process in the MD simulations. It is located on the surface of the CD and is not included in the well-defined (α/β)6-barrel (Figure 1). The MD simulations show that this helix is extremely unstable even at low simulation temperatures. The Cα distances between residues 276 and 347 and 298 and 354 are 5.01 and 5.08 Å, respectively, in the crystallographic structure, which are within the interval for an ideal disulfide bond (i.e. 4.83–5.45 Å) (Richardson, 1981). In the present study, we tried to engineer extra disulfide bonds between residues A276 and S347 and residues S298 and L354 to ‘lock’...
We replaced these two Gly residues by Ala to reduce the flexibility of the polypeptide chain in this region, resulting in G396A, G407A and G396A/G407A GAs. In addition, the stability of the α-helix plays an important role in determining the thermostability of the CD. The result from the MD simulations shows that the unfolding of the 13 α-helices follows a random mechanism with the unfolding order as α8→α1→α11→α7→α10→α3→α12→α13→α4→α5→α9→α6→α2. E400 is located between α-helices 12 and 13 (Figure 2), which are the seventh and eighth to unfold, respectively. In the present study, we tried to increase the helix stability by substituting the amino acid residues in the C- and N-terminal regions of α-helices 12 and 13, respectively, by residues with higher hydrophobicity, resulting in the following mutant GAs: S386L, E389M, T390L, H391M, T416L and S418L.

Materials and methods

Materials

YEpPM18, a yeast expression vector carrying the wild-type (wt) GA cDNA and the S.cerevisiae C468 (α leu2–3 leu2–112 his3–11 his3–15 mal–) (Innis et al., 1985) was a generous gift from Cetus. All restriction enzymes and buffers were purchased from Promega (Madison, WI). Maltose and other chemicals were obtained from Sigma (St. Louis, MO). Acaorbos was a gift from Miles Laboratories.

Molecular dynamics simulations

The initial X-ray crystallographic structure of the CD from A.awamori var. X100 GA was taken from the Protein Data Bank (PDB entry 1GLM) (Aleshin et al., 1992). The energy minimizations and MD simulations of the CD were conducted with the InsightII program (Accelrys, San Diego, CA) with the force field Discover CVFF (consistent valence force field) (Peng et al., 1997; Hwang et al., 1998; Maple et al., 1998) in an SGI O200 workstation with 64-bit HIPS RISC R12000 270 MHz CPU and PMC-Sierra RM7000A 350 MHz processor (Silicon Graphics, Mountain View, CA). The X-ray crystallographic structure of the CD was subjected to energy minimization calculations in vacuum by the steepest descent method with 3000 iterations followed by the Newton–Raphson method with 5000 iterations to be used as the starting lowest energy structure for further structural comparison. The CD was then submitted to 200 ps MD simulations in vacuum after equilibrating for about 10 ps at pH 4.5 and 300, 400, 600 and 800 K, respectively, using the Discover module of the InsightII program. The temperature and pressure were maintained constant for each MD simulation by weak coupling with 5000 iterations to be used as the template in the Altered Sites II vector to be used as the template in the Altered Sites II in vitro mutagenesis system (Fang et al., 1998b). The mutagenesis oligonucleotide primers synthesized by GenAsia (Si-Chi, Taipei, Taiwan) are listed in Table I. All mutations were verified by DNA sequencing.

Protein engineering of GA

An Xbal–HindIII fragment of pGEM-GA containing the wt GA cDNA (Fang and Ford, 1998) was inserted into the Promega pALTER-I vector, resulting in a GA cDNA-containing vector to be used as the template in the Altered Sites II in vitro mutagenesis system (Fang et al., 1998b). The mutagenesis oligonucleotide primers synthesized by GenAsia (Si-Chi, Taipei, Taiwan) are listed in Table I. All mutations were verified by DNA sequencing.

Fig. 1. View of A.awamori var. X100 GA, showing the locations of the corresponding mutations made in A.awamori GA (as listed in Table I). The catalytic acid, E179, and the catalytic base, E400, are shown in ball and stick form. The locations of N- and C-termini are indicated. α-Helices 12 and 13 are represented in pink and yellow, respectively. This figure was generated using the InsightII program.

Fig. 2. View of the local environment surrounding α-helix 11 (represented in brown) of A.awamori var. X100 GA. The residues S347 and L357, located in the N- and C-terminal regions of α-helix 11, are shown in ball and stick form. The Cα distances between residues 347 and 276 and 354 and 298 are 5.01 and 5.08 Å, respectively. This figure was generated using the InsightII program.
Expression and purification of GA

The wt and mutated GA genes were subcloned into the yeast expression vector YEpPM18 and subsequently transformed into S. cerevisiae C468 by electroporation. The mutant and wt GAs were produced in shaker flasks at 30°C, 170 r.p.m. for 5 days. The cultures were centrifuged to remove the yeast cells and the supernatants were concentrated, dialyzed against 0.5 M NaCl–0.1 M NaOAc buffer (pH 4.5) with a 10 kDa MWCO Amicon S1 spiral ultrafiltration cartridge. The concentrates were applied to acarbose (a generous gift from Dr C. Ford of Iowa State University) affinity chromatography for purification of the enzymes (Chen et al., 1994a). Bound enzyme was eluted with 1.7 M Tris buffer at pH 7.6, then dialyzed against water and concentrated to 10 ml by ultrafiltration. The molecular weights of GAs were confirmed with SDS–PAGE, which was carried out using 0.75 mm thick 10% polyacrylamide gels following the method of Garfin (Garfin, 1990).

Thiol titration

Wt, A276C/S347C, S298C/L354C and A276C/S347C/S298C/L354C GAs at 2 mg/ml were denatured by boiling in denaturing solution containing 2% SDS, 0.08 M sodium phosphate (pH 8.0) and 0.5 mg/ml EDTA (Habeeb, 1972) with or without 50 mM dithiothreitol (DTT) (Pollitt and Zalkin, 1983) for 10 min. The denatured GAs (reduced or non-reduced) were carried out using 0.75 mm thick 10% polyacrylamide gels following the method of Garfin (Garfin, 1990).

Specific activity and kinetic parameters

Protein concentration was determined with the Pierce bicinchoninic acid protein assay with Pierce bovine serum albumin as standard (Smith et al., 1985). GA specific activity was determined at 50°C, pH 4.5, with 117 mM maltose as substrate in 0.05 M NaOAc buffer. Aliquots of the assay mixture were quenched at several time points by addition of 4 M Tris–HCl, pH 7.0, to a final concentration of 1.1 M Tris–HCl. Glucose concentration was determined in the quenched reaction mixtures by the glucose oxidase–peroxidase method. One unit (IU) of activity was defined as the amount of enzyme that produces 1 mmol of glucose per minute under the assay conditions. Kinetic parameters, $k_{cat}$ and $K_M$, for maltose were obtained at 50°C, pH 4.5, in 0.05 M NaOAc buffer by the ENZFITTER program (Fang et al., 1998a).

Irreversible thermostability

Purified wt and mutant GAs in 0.05 M NaOAc buffer at pH 4.5 were incubated at six temperatures at 2.5°C intervals from 65 to 77.5°C. Seven samples were taken periodically from each experiment and promptly chilled on ice, followed by GA activity assay after 24 h of cooling (Liu et al., 1998). The inactivation rate constants ($k_d$) were obtained from a semilogarithmic plot of residual activity versus inactivation time. The activation free energies ($\Delta G^\ddagger$) for thermostiuctivation were obtained from a semilogarithmic plot of $k_d$ versus 1/$T$.

Results

Molecular dynamics simulations

Figure 3 shows the linear correlation between the predicted melting temperature ($T_m$) and the unfolding order of the 13 $\alpha$-helices in the CD. $T_m$ was defined as the simulation
temperature at which the average residual helicity (Figure 4) of a specific $\alpha$-helix is 50% relative to the crystallographic structure. The helicity was predicted according to the Kabsch–Sander algorithm (Kabsch and Sander, 1983) and the average helicity was calculated by averaging the helicity over the 200 ps simulation time. The unfolding of these 13 $\alpha$-helices followed the random ordered mechanism, where $\alpha$-helices 11, 12 and 13 were found to be the third, seventh and eighth to unfold, respectively. In addition, the catalytic base E400 was found to migrate from the interior pocket to the surface of the CD, resulting in the destruction of the catalytic center at the simulation temperature 800 K by the root-mean-square deviation (r.m.s.d.) analysis (data not shown) and snapshot illustrations (Figure 5). The entire CD kept expanding while the 13 $\alpha$-helices kept losing their helicities. This suggested that the supplied kinetic energy was high enough for the

Fig. 5. Snapshots of the CD at (a) 0, (b) 100 and (c) 200 ps during the MD simulation at 800 K. $\alpha$-Helices 12 and 13 are shown in brown and blue, respectively. The location of the catalytic base, E400 (stick and ball representation), is also indicated. These figures were generated using the InsightII program.
catalytic center to overcome the hydrophobic barrier provided by α-helices 12 and 13. Thus, any strategy to stabilize these two α-helices may result in an increase in GA thermostability.

Examination of the formation of engineered disulfide bonds

The newly engineered disulfide bonds were demonstrated by thiol titration. By comparing the numbers of free thiol groups before and after DTT treatment, the total number of disulfide bonds in wt, A276C/S347C, S298C/L354C and A276C/S347C/S298C/L354C GAs were deduced to be four, five, and six, respectively (Table II). There are three and one disulfide bonds in the CD and the SBD of the wt GA, respectively (Aleshin et al., 1992; Sorimachi et al., 1996). Hence the introduced Cys residues formed disulfide bonds as expected in the desired positions.

Specific activities and kinetic parameters for wt and mutant GAs

Table III shows the specific activities and kinetic parameters for wt and mutant GAs. The specific activities and catalytic efficiencies (kcat/KM) for all mutant GAs are slightly higher than those of wt GAs, except for G396A, G407A, G396A/G407A and H391M GAs. The A276C/S347C/S298C/L354C GA showed the highest catalytic efficiency, whereas the H391M GA had the lowest specific activity. Generally, there is a positive correlation between specific activity and catalytic efficiency amongst the wt and mutant GAs.

Thermostability for wt and mutant GAs

The irreversible thermoinactivation of wt and mutant GAs followed first-order kinetics (Liu et al., 2000). A semilogarithmic plot of the inactivation rate constants (kd) of wt, A276C/S347C/S298C/L354C and H391M mutant GAs versus inactivation temperature is shown in Figure 6 (data for other mutant GAs not shown). Mutant GAs usually had lower inactivation rates than wt GA at all inactivation temperatures except for H391M GA. The changes of the activation free energies for thermostoinactivation (Δ∆G‡) for mutant GAs relative to wt GA at 65, 70 and 75°C are listed in Table III. All mutant GAs except for H391M GA showed the highest Δ∆G‡ values, indicating that they were more thermostable than wt GA. Amongst these mutations, A276C/S347C/S298C/L354C GA was the most thermostable one, having the highest Δ∆G‡ values. The thermostability for most of the mutant GAs was increased less than that for wt GA at higher temperatures except for A276C/S347C, S298C/L354C and A276C/S347C/S298C/L354C GAs.

Discussion

In order to gain insight into the structure–function relationship, MD simulations with the temperature jump technique (Williams et al., 1996; Thompson et al., 1997) were performed to investigate the unfolding mechanism of the CD from A. awamori var. X100 GA. Instead of a simultaneous event, the unfolding of the CD followed a putative hierarchical manner, in which the heavily O-glycosylated belt region from residues T440 to A471 acted as the initiation site, followed by the destruction of the α-helix secondary structure and then the collapse of the catalytic center pocket. In addition, the unfolding of the 13 α-helices obeyed the random order mechanism with the unfolding order as α8→α1→α11→α7→α10→α3→α12→α13→α4→α5→α9→α6→α2 predicted by Tm (Figure 3) (i.e. the higher the Tm was, the slower the unfolding occurred). The catalytic center pocket was well protected by the highly hydrophobic (α/α)6-barrel at simulation temperatures up to 600 K. E400, the catalytic base, migrated from its original interior pocket to the surface of the CD by overcoming the hydrophobic barrier provided by its adjacent α-helices 12 and 13. The results from MD simulations allowed the rational design of mutations to increase the thermostability of GA.

 Twelve mutations to increase the thermostability of GA were constructed in this work. A276C/S347C, S298C/L354C and A276C/S347C/S298C/L354C GAs were designed to ‘lock’ the unstable α-helix 11 on the surface of the CD. G396A, G407A and G396A/G407A GAs were constructed to reduce the flexibility of the polypeptide chain between α-helices 12 and 13. S386L, E389M, T390L, H391M, T416L and S418L GAs were made to enhance the stability of α-helices 12 and 13. Previous studies have shown that thermostability can be dramatically improved by engineering extra disulfide bonds into small (Betz, 1993) and large proteins (Li et al., 1998). In this study, we found that α-helix 11, which is located on the surface of the CD and does not belong to the (α/α)6-barrel secondary structure motif, is extremely unstable. It fluctuated very quickly and lost its helicity in a relatively short period of simulation time. By examining the X-ray crystallographic structure, we found that S347 and L354, both located in the α-helix 11, are very close to A276 and S298, respectively and the distance between them (i.e. 5.01 and 5.08 Å, respectively) are very suitable for engineering extra disulfide bonds. A276C/S347C, S298C/L354C and A276C/S347C/S298C/L354C GAs, with one, one and two extra disulfide bonds, respectively, all showed higher specific activity, catalytic efficiency and thermostability relative to wt GA (Table III). Classical theory suggests that the mechanism by which disulfide bonds stabilize proteins is by decreasing the configurational entropy of the
unfolded state of the protein (Betz, 1993). More recent work suggests that the native entropic and enthalpic effects should also be taken into consideration besides unfolded entropic effects (Li et al., 1998). The cross-linkages between $\alpha$-helix 11 and its adjacent region resulted in favorable entropic contributions to the free energy for thermonactivation by 4.0–5.6 kcal/mol. This indicates that the engineered disulfide bonds successfully decrease the native state entropy of GA. Previous studies have shown that the engineered disulfide bonds in a flexible region contribute to a protein’s thermostability more than those in a rigid region (Tidor and Karplus, 1993; Li et al., 1998). In the present study, we also show that the thermostability of GA can be greatly increased by rigidifying the flexible region of $\alpha$-helix 11.

The process of GA thermonactivation is dominated by the formation of incorrect protein conformations (Munch and Tritsch, 1990), such as ‘scrambled’ structures at higher temperatures (Li et al., 1998). Previous study has shown that the thermostability of GA can be increased up to 75°C by replacing Gly residues with Ala to reduce helix flexibility (Chen et al., 1996). In the present study, G396 and G407, both located in the loop region between $\alpha$-helices 12 and 13, were replaced with Ala and the resulting mutant GAs were denoted G396A, G407A and G396A/G407A. The specific activity and catalytic efficiency of these mutations decreased, whereas the thermostability of these mutations increased slightly compared with wt GA. Previous study has replaced G396 by Ser and resulted in changes in the backbone conformation that destabilized the protein (Flory et al., 1994). Although a Ser residue is more rigid than Gly, its polar characteristics may not suitable for the highly hydrophobic environment in the loop region, resulting in a negative effect on the thermostability of GA. In the present study, we replaced G396 with Ala, the hydrophobicity of which is 1.8 compared with −0.8 for Ser according to Kyte and Doolittle (Kyte and Doolittle, 1982). The Ala residue seems to be more favorable to be located in this highly hydrophobic region. In addition, the backbone flexibility for an Ala residue is less than that for a Gly residue, leading to stabilization of the CD. The catalytic center pocket of the CD is defined by the residues R54, D55, L177, E179, R305 and E400 through hydrogen bonds and a salt linkage network (Aleshin et al., 1994b). The replacement of the two Gly residues near the catalytic center by Ala residues may partially destroy the non-covalent bonding network, leading to decreased specific activity and catalytic efficiency.

Experimental studies aimed at deciphering the factors that stabilize helices have been intensively conducted. For example, aromatic (Shoemaker et al., 1987), hydrogen-bonded (Richardson and Richardson, 1988) and hydrophobic interactions (DeGrado and Lear, 1985) appear to stabilize $\alpha$-helices. In addition, electrostatic interactions between charged side chains and either helical dipole or another charged residue located one turn of helix away are also important in determining helix stability (Marqusee and Baldwin, 1987). Site-directed mutagenesis of proteins is a powerful tool for determining helix-stabilizing interactions. In principle, this method might also be applied to obtain a thermodynamic scale for the relative helical preferences of the individual amino acid residues by substituting all possible amino acids at a given position in an $\alpha$-helix of a natural protein. In practice, the data would be difficult to interpret because helices in proteins are typically involved in multiple hydrogen-bonded, van der Waals and electrostatic interactions. Thus, mutation of a given residue would change not only the conformational preference but also other interactions. In the present study, we aimed at studying the hydrophobic effects on stabilizing $\alpha$-helices by substituting the residues with about the same van der Waals volume but higher hydrophobicity in the C- and N-terminal half of $\alpha$-helices 12 and 13, respectively. Except for H391M GA, S386L, E389M, H3, T390L, H4, H4, H391M, H5, T416L; H6, S418L.

### Table III

Specific activities and kinetic parameters for wt and mutant GAs measured at 50°C, pH 4.5, using 117 mM maltose in 0.05 M NaOAc as substrate, and changes in the activation free energy for thermonactivation ($\Delta G^\ddagger$) at 65, 70 and 75°C for mutant GAs relative to wt GA.

<table>
<thead>
<tr>
<th>GA form</th>
<th>Specific activity (IU/mg GA)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$K_M$ (mM)</th>
<th>$k_{cat}/K_M$ (s$^{-1}$ mM$^{-1}$)</th>
<th>$\Delta G^\ddagger$ (kJ/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>21.1 ± 0.3$^b$</td>
<td>8.88 ± 0.21</td>
<td>0.78 ± 0.05</td>
<td>14.4</td>
<td>4.0 4.3 4.5</td>
</tr>
<tr>
<td>S1</td>
<td>22.8 ± 0.6</td>
<td>9.01 ± 0.11</td>
<td>0.67 ± 0.02</td>
<td>13.4</td>
<td>4.2 4.4 4.7</td>
</tr>
<tr>
<td>S2</td>
<td>33.5 ± 0.7</td>
<td>9.26 ± 0.15</td>
<td>0.69 ± 0.04</td>
<td>14.7</td>
<td>4.8 5.3 5.6</td>
</tr>
<tr>
<td>S3</td>
<td>19.8 ± 0.4</td>
<td>7.99 ± 0.32</td>
<td>0.83 ± 0.02</td>
<td>9.6</td>
<td>1.7 1.3 0.9</td>
</tr>
<tr>
<td>G1</td>
<td>20.2 ± 0.3</td>
<td>8.52 ± 0.16</td>
<td>0.85 ± 0.03</td>
<td>10.0</td>
<td>1.1 1.0 0.7</td>
</tr>
<tr>
<td>G2</td>
<td>20.5 ± 0.5</td>
<td>8.13 ± 0.20</td>
<td>0.91 ± 0.06</td>
<td>8.9</td>
<td>1.5 1.2 1.1</td>
</tr>
<tr>
<td>H1</td>
<td>21.6 ± 0.9</td>
<td>9.13 ± 0.15</td>
<td>0.75 ± 0.04</td>
<td>12.2</td>
<td>2.4 2.0 1.7</td>
</tr>
<tr>
<td>H2</td>
<td>21.4 ± 0.6</td>
<td>9.22 ± 0.17</td>
<td>0.77 ± 0.03</td>
<td>12.0</td>
<td>2.8 2.3 2.0</td>
</tr>
<tr>
<td>H3</td>
<td>21.9 ± 0.6</td>
<td>8.98 ± 0.22</td>
<td>0.72 ± 0.02</td>
<td>12.5</td>
<td>1.9 1.7 1.4</td>
</tr>
<tr>
<td>H4</td>
<td>18.4 ± 0.3</td>
<td>7.78 ± 0.30</td>
<td>0.88 ± 0.06</td>
<td>8.8</td>
<td>−0.5 −0.8 −1.1</td>
</tr>
<tr>
<td>H5</td>
<td>22.2 ± 0.8</td>
<td>9.01 ± 0.21</td>
<td>0.73 ± 0.02</td>
<td>12.3</td>
<td>3.5 3.3 2.2</td>
</tr>
<tr>
<td>H6</td>
<td>21.8 ± 0.5</td>
<td>9.17 ± 0.29</td>
<td>0.70 ± 0.05</td>
<td>13.1</td>
<td>3.1 2.8 2.4</td>
</tr>
</tbody>
</table>

$^a$GA forms: wt, wild type; S1, A276C/S347C; S2, S298C/L354C; SS, A276C/S347C/S298C/L354C; G1, G396A; G2, G407A; GG, G396A/G407A; H1, S386L; H2, E389M; H3, T390L; H4, H391M; H5, T416L; H6, S418L.

$^b$Standard error.
Removing the positive charge of His residue in the H391M GA dramatically reduces the activity and thermostability of the CD, meaning that this charged residue is necessary to maintain the charge distribution in the packing void of the catalytic center pocket.

In summary, rational design of mutations aimed at improving the thermostability of GA was successfully achieved based on the results of the MD simulations. Eleven out of 12 mutations constructed in this study increased the thermostability of GA more or less. Introducing disulfide bonds in a highly flexible region in the polypeptide chain of GA dramatically decreased the thermonactivation rate. Residues with higher hydrophobicity stabilized α-helices, leading to increased thermostability of GA. The specific activity and catalytic efficiency may be reduced by interrupting the hydrogen bond and salt linkage network in the catalytic center.

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