Involvement of Single Residue Tryptophan 548 in the Quaternary Structural Stability of Pigeon Cytosolic Malic Enzyme

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Pigeon cytosolic malic enzyme has a double dimer quaternary structure with three tryptophanyl residues in each monomer distributed in different structural domains. The enzyme showed a three-state unfolding phenomenon upon increasing the urea concentration (Chang, H. C., Chou, W. Y., and Chang, G. G. (2002) J. Biol. Chem. 277, 4663–4671). At urea concentration of 4–4.5 M, where the intermediate form was detected, the enzyme existed as partially unfolded dimers, which were easily polymerized. Mn²⁺ provided full protection against the polymerization. To further characterize this phenomenon, three mutants of the enzyme (W129, W321, and W548), each with only one tryptophanyl residue left, were constructed. All these mutants were successfully overexpressed in Escherichia coli cells and purified to homogeneity. The results indicate that a single residue of all mutants revealed a three-state urea unfolding process in the absence of Mn²⁺. In the presence of 4 mM Mn²⁺, W548 and wild type (WT) enzymes shifted to monomeric form, while W129 and W321 were still bimorphic. Similar results were obtained from the fluorescence spectral changes, except for W321, which showed monomeric denaturation curve with or without Mn²⁺. Analytical ultracentrifugation analysis indicated that the mutant enzymes were polymerized at 4.5 M urea, and Mn²⁺ provided protective effect on W548 and WT enzymes only. Other mutants with mutated Trp-548 polymerized at 4.5 M urea in the absence or presence of 4 mM Mn²⁺. The above results indicate that a single residue, Trp-548, in the subunit interface region, is responsible for the integrity of the quaternary structure of the pigeon cytosolic malic enzyme.

Pigeon cytosolic malic enzyme is a tetramer with a double dimer quaternary structure, a structure common to all malic enzymes (1–4) (Fig. 1). For pigeon enzyme, the dimer and the tetramer quaternary structure, a structure common to all malic enzyme, was verified in all recombinant malic enzymes.

Expression and Purification of the Recombinant Pigeon Liver Malic Enzyme—The recombinant DNA plasmid was transformed into BL21 bacterial strain. Enzyme synthesis was induced by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside into the LB medium. After 20-h incubation at 25 °C, the cells were harvested by centrifugation at 6000 × g for 10 min. Purification of the recombinant pigeon liver malic enzyme was carried out according to the protocol described previously.
All purified enzymes were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to examine the purity. Malic enzyme activity was assayed according to the published procedure (15). Apparent Michaelis constants for the substrate and cofactors were determined by varying the concentration of one substrate (or cofactors) around its $K_m$ value while maintaining other components constant and at the saturation levels. The kinetic parameters were obtained by fitting the experimental data to appropriate kinetic models. The calculation was carried out with Sigma Plot 5.0 program (Jandel, San Rafael, CA).

Enzyme Denaturation in Urea Solution—WT or mutant malic enzymes preincubated with or without 4 mM Mn$^{2+}$/H$_{2}$O$_{2}$ were denatured with various concentrations of freshly prepared urea in Tris acetate buffer (0.1 M, pH 7.4) at 25 °C for 1 h. The unfolding of the enzyme was monitored by fluorescence and circular dichroism spectroscopic techniques. Fluorescence spectra of the recombinant malic enzymes were analyzed with a PerkinElmer Life Sciences LS 50B luminescence spectrometer at 25 °C. All spectra were corrected for the buffer absorption. Both the red shift and the fluorescence intensity changes were analyzed.


The average emission wavelength (\( \lambda_{av} \)) was calculated according to the following equation, 

\[
\lambda_{av} = \frac{\sum_{i=1}^{N} (F_i \cdot \lambda_i)}{\sum_{i=1}^{N} F_i}
\]  

(Eq. 1)

in which \( F \) is the fluorescence intensity and \( \lambda \) the emission fluorescence wavelength.

The CD measurements were made with a Jasco J-810 spectropolarimeter using a 0.1-cm path length cell under constant \( N_2 \) flush. Three repetitive scans between 250 and 200 nm were averaged. Parallel spectra of urea solution without protein were also recorded and subtracted from the sample spectra. Mean residue ellipticity (\( \Phi \)) was calculated by the following equation,

\[
\Phi = |\varphi_{222}M_{MW}/10dc|
\]  

(Eq. 2)

### Table I

<table>
<thead>
<tr>
<th>Kinetic parameters for the WT and tryptophan mutant pigeon liver malic enzymes</th>
<th>( K_{m,Mg} )</th>
<th>( K_{m,Mal} )</th>
<th>( K_{m,NADP} )</th>
<th>( k_{cat} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>0.26 ± 0.03</td>
<td>0.29 ± 0.03</td>
<td>2.49 ± 0.16</td>
<td>112 ± 11</td>
</tr>
<tr>
<td>W129</td>
<td>0.22 ± 0.01</td>
<td>0.37 ± 0.02</td>
<td>16.1 ± 2.63</td>
<td>116 ± 10</td>
</tr>
<tr>
<td>W321</td>
<td>0.18 ± 0.01</td>
<td>0.39 ± 0.02</td>
<td>1.91 ± 0.15</td>
<td>136 ± 9</td>
</tr>
<tr>
<td>W548</td>
<td>0.12 ± 0.02</td>
<td>0.39 ± 0.06</td>
<td>0.61 ± 0.13</td>
<td>125 ± 22</td>
</tr>
</tbody>
</table>
Characterization of Polymerization of Malic Enzyme during Urea Denaturation by Analytical Ultracentrifugation and Dynamic Light Scattering Measurements—The sedimentation coefficients of the enzyme under various conditions were estimated by a Beckman-Coulter XL-A analytical ultracentrifuge with an An60Ti rotor. Sedimentation velocity was performed at 20°C and 40,000 rpm with standard double sectors aluminum centerpieces. The UV absorption of the cells was scanned every 5 min for 2 h. The data were analyzed with the SedFit version 8.5 program (19–21) (web site: www.analyticalultracentrifugation.com/). The solvent density and viscosity in the presence of urea were corrected with the UltraScan version 5.0 (22) (web site: www.ultrascan.uthscsa.edu/). A partial specific volume of 0.7403 was used for malic enzyme (23). All samples were visually checked for clarity after ultracentrifugation.

RESULTS

Characterization of the Tryptophan Mutants of Pigeon Liver Malic Enzyme—The fluorescence of the indole moiety is highly sensitive to its environment. This makes tryptophan an ideal residue to monitor the conformational changes of protein molecule (25). Each subunit of the pigeon malic enzyme contains three tryptophanyl residues, located at positions 129, 321, and 548, respectively. Changing any two Trp to Phe results in a mutant with only one Trp left. When excited at 295 nm, only
the structural change around that Trp will be reflected by the fluorescence measurement.

All these three tryptophan mutants and the WT malic enzyme gave similar far-UV CD spectrum (Fig. 2A), indicating that the substitutions do not alter the secondary structure of the enzyme.

The kinetic parameters of these mutants are shown in Table I. Since none of the mutated Trp residues are located at the active site, the $K_m$ values of the mutants do not differ significantly from those of WT. The $K_{m,NADP}$ value of W129 increased by a factor of 6.5 and that for W548 decreases by a factor of 4 than that of the WT (Table I). These results are conceivable, since W321 was closest to the NADP$^+$ binding site (Fig. 1) but was changed to phenylalanine in the W129 and W548 mutants. The $k_{cat}$ values of all mutants, however, were similar to that of the WT value. The above results, therefore, indicated that the tryptophan to phenylalanine substitution does not affect the structural and functional integrity of the enzyme.

**Fluorescence Spectral Properties of the Tryptophan Mutants of Pigeon Liver Malic Enzyme**—The fluorescence emission maximum ($\lambda_{ex} = 295$ nm) for the WT, W129, W321, and W548 were located at 333, 324, 338, and 329 nm, respectively (Fig. 2B). All these spectra were shifted to 358 nm when denatured with 8.3 M urea. The fluorescence intensities of the mutants, however, were drastically decreased due to the fact that only one tryptophanyl residue remained in each mutant (Fig. 2B). At equal protein concentration, the sum of the mutant fluorescence emission spectra does not match to the WT value. It suggests small structural perturbations of the mutations. Alternatively, it may imply that in WT there is some degree of energy transfer of the tryptophanyl residues, probably between Trp-129 of subunit “a” and Trp-548 of subunit “d” (see “Discussion”) (25).

**Denaturation of WT and Tryptophan-mutated Malic Enzymes Monitored by the Intrinsic Fluorescence Changes**—Urea-induced denaturation of the WT and tryptophan mutated malic enzymes were monitored by the change in fluorescence emission spectrum at an excitation wavelength of 295 nm. The results were plotted by taking the average emission wavelength (Fig. 3, A–D) and integrated fluorescence area (Fig. 3, E–H) versus urea concentration. These results are similar to those previously reported for WT enzyme where excitation at 280 nm was performed (11). Biphasic and monophasic unfolding processes were observed for the WT enzyme in the absence and presence of Mn$^{2+}$, respectively. The corresponding $[\text{urea}]_{1/2}$ values of WT were 2.5, 5.2, and 4.6 M, respectively, for the biphasic and monophasic denaturation unfolding process. The W129 yielded biphasic, but W321 gave a monophasic unfolding curve. Only W548 gave identical results with those of WT. Metal ion provides protection on WT and W548 (Fig. 3, A, D, E, and H). No protective effect was found for W129 or W321 (Fig. 3, B, C, F, and G).

The increase in the characteristic integrated fluorescence area between 3 and 6 M urea in the WT enzyme in the absence of Mn$^{2+}$ is even more prominent in W129 (Fig. 3F). It indicates that Trp-129 has a different microenvironment from the other

![Fig. 7. Distribution of tryptophanyl residues in pigeon liver malic enzyme. A, surface potential model of the enzyme. Only one subunit is shown. The negatively charged regions are shown in red, and the positively charged regions are shown in blue. The visible inside region represents the interfacial area of the subunit. Trp-129 is buried, but Trp-548 sticks out into a pocket from the other subunit. Trp-321 is buried near the NADP$^+$ binding site region and is invisible in this figure. B, enlargement of the local area showing the possible interaction of Trp-129 and Trp-548 from different subunits. C, stereo view of Trp-129(a) and Trp-548(d) showing the nearly coplanar orientation of the two aromatic rings. The color codes are the same as in B. This figure was generated with RasMol version 2.7 (33).](http://www.jbc.org/)

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two tryptophanyl residues in the intermediate state. Lacking this Trp, W321 and W548 do not have this characteristic peak (Fig. 3, G and H).

**Unfolding of WT and Tryptophan-mutated Malic Enzymes Monitored by Far-UV CD Changes**—The unfolding process of WT and tryptophan-mutated malic enzymes were further monitored by CD spectral change (Fig. 4). The changes in far-UV CD monitored at 222 nm were converted to folded fractions and then plotted against urea concentration. All four enzymes were unfolded via a three state pathway, indicating the presence of at least one intermediate. In the presence of 4 mM Mn$^{2+}$, W129 and W321 still showed a biphasic phenomenon but W548 and WT changed to monophasic.

**Quaternary Structural Changes of WT and Tryptophan-mutated Malic Enzymes during Urea Denaturation**—According to our previous results, the intermediate state involves polymerization of the partially unfolded dimers and Mn$^{2+}$ provides full protection up to 4 mM urea for WT (11). Interestingly, the intermediate form was not observed in W321 during the urea denaturation process, which was monitored by fluorescence (Fig. 3, C and G). These data are consistent with the involvement of interfacial region in the intermediate state.

We then checked the substituted mutation effect on the quaternary structure with analytical ultracentrifugation. All tryptophan mutants and WT showed a major peak with similar quaternary structure with analytical ultracentrifugation. All interfacial region in the intermediate state.

**Dynamic Light Scattering Experiment**—The above results clearly indicate polymerization of the enzyme molecule at intermediate concentration of urea. The enzyme solution, even incubated with 4.5 mM urea for 1 h, however, was visually transparent. Dynamic light scattering technique was employed to further characterize the polymerization phenomenon.

The dimensions of the tetrameric malic enzyme are 11 × 11 × 5.5 nm determined by x-ray crystallography (2) or 10.2 × 10.8 × 7 nm observed in electron microscopic analysis (23). The ellipsoidal monomer of the enzyme has dimensions of 4.8 × 5.4 × 7 nm (23). When examining by the dynamic light scattering technique, WT malic enzyme revealed monodispersed particle with size diameter of 9 nm, compatible with tetramer in solution. Incubating the enzyme with 4.5 mM urea for 30 min produced particles of 6.3 nm in diameter, consistent with dissociation of the enzyme into dimers or monomers. Prolong incubation, however, will result in polymerization as manifested by the increasing of particle size upon incubation time. A similar phenomenon was observed for mutant malic enzyme. The particle size of W321, for example, was 4.6 nm when incubating with 4.5 mM urea for 30 min. The particle size increased to 25.8 nm after 1 h. Prolonged incubation resulted in aggregation.

The unfolding process of malic enzyme in urea solution, thus, is dissociation of tetramer to partially unfolded dimers and then monomers. The partially unfolded dimers or monomers are easily polymerized and finally aggregated. The signals detected in CD or fluorescence spectroscopy during urea denaturation thus could be partly due to the light scattering effect of the polymers. For this reason the unfolding data shown in Figs. 3 and 4 is only qualitatively discussed. No quantitative analysis of the unfolding thermodynamics was attempted.

**DISCUSSION**

We have constructed three tryptophan mutants of pigeon liver malic enzyme to assess the structural role of different domains of the enzyme. All mutants have similar CD spectra, kinetic parameters, and sedimentation coefficients with the WT (Fig. 2A, Table I, and Fig. 5, respectively). The overall tertiary and quaternary structures of the enzyme seem undisturbed by the mutation.

With these mutants, we explored the contribution of individual tryptophanyl residue by monitoring the CD and fluorescence spectral changes during denaturation process. Only the W548 mutant gave identical results to the WT enzyme (Figs. 3 and 4). This suggests that the local environment of Trp-548 but not Trp-129 or Trp-321 is highly correlated to the observed quaternary structural change. The interface region shown in Figs. 1B and 7A clearly indicates that Trp-129 is buried and Trp-321 is far away from the interfacial region. On the other hand, the Trp-548 residue sticks out from the subunit and is located in a deep, hydrophobic pocket from the subunit of another dimer and thus involves in the tetramer interaction (Fig. 7A (1)). The tetrameric pigeon malic enzyme has been demonstrated to dissociate into dimers and monomers at a lower pH (5, 6). Examination of the crystal structure of the enzyme shows the presence of a pair of histidyl residues, His-117 and His-202, in which His-117 is located in the binding pocket for Trp-548 (1). It is possible that protonation of these histidyl residues at a lower pH can affect the binding of the Trp-548 residue in the tetramer interface, which in turn, destabilized the tetramer interface. For this reason, substitution of Trp-548 to phenylalanine in W129 and W321 mutants may interfere the interaction with histidyl residues and thus induced some dissociation of malic enzyme as noted in Fig. 5. Furthermore, Trp-548 is adjacent to Trp-129 from domain A of another subunit. The distance between Trp-129(a) and Trp-548(d) is 15 Å (Fig. 7B). It suggests that in WT there is some degree of energy transfer between Trp-129 of subunit a and Trp-548 of subunit d (26, 27). The nearly coplanar orientation between Trp-129 and Trp-548 would optimize their mutual interactions (Fig. 7C). This could explain the sum of individual mutant’s fluorescence is larger than WT, in which some quenching might have occurred between Trp-129 and Trp-548 (Fig. 2B). The same reason can be extended to explain the characteristic peak for the intermediate unfolding state detected by integrated fluorescence area, which is enhanced in W129 due to the lack of Trp-548 quenching (Fig. 3F).

The Trp-321 is located at domain C, near, but not directly to the NADP$^+$ binding motif, which is far from the interfacial region (Fig. 1). Mutation of this tryptophanyl residue in the W129 and W548 mutants do show altered $K_m$,NADP. However, only those enzyme mutants with a chromophore at the dimer or tetramer interfacial region (Trp-129 or Trp-548) will the unfolding intermediate state be detected. For this purpose, CD still can detect the unfolding intermediate in W321 as shown in Fig. 4. These results demonstrate the usefulness of CD spectra
in the studies of protein conformational changes (28). The pattern of secondary structural change (Fig. 4) during denaturation of the three tryptophan mutants was parallel to the quaternary structural change. All intermediate signals in the secondary structural change of the tryptophan mutants were correlated to polymerization in the urea denaturation. We demonstrate here that a single tryptophanyl residue, Trp-548, near the C terminus is responsible for the quaternary structure stability of the pigeon malic enzyme.

The partially unfolded intermediate state is unstable and is easily polymerized (Fig. 6). Metal ion provides full protection against the polymerization up to 4 M urea in WT (11). This is even pronounced in W548, in which Mn\(^{2+}\) exhibited full protection even at 4.5 M urea (Fig. 6H). W129 and W321 mutants were polymerized even in the presence of Mn\(^{2+}\). The involvement of metal ion in the quaternary structure is common (29).

In human mitochondrial malic enzyme, binding of metal ion, La\(^{3+}\), to the enzyme has been demonstrated to induce rigid-body movements of residues in domains A and D, which are located at the dimer and tetramer interface of the enzyme, respectively, and thus change the subunit association affinity (30) (Fig. 1). The protection of the partially unfolded state from polymerization (aggregation) by a metal ion has important biological significance. In this simple way, the partially unfolded intermediate form will have time to fold properly without diversion to unwanted sidetrack under in vivo conditions.

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