

Equilibrium Unfolding Mechanism of Chicken Muscle Triose Phosphate Isomerase

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Abstract: Triose phosphate isomerase (TIM) was prepared and purified from chicken breast muscle. The equilibrium unfolding of TIM by urea was investigated by following the changes of intrinsic fluorescence and circular dichroism spectroscopy, and the equilibrium thermal unfolding by differential scanning calorimetry (DSC). Results show that the unfolding of TIM in urea is highly cooperative and no folding intermediate was detected in the experimental conditions used. The thermodynamic parameters of TIM during its urea induced unfolding were calculated as $\Delta G^\circ = 3.54 \text{ kcal}\cdot\text{mol}^{-1}$, and $m_G = 0.67 \text{ kcal}\cdot\text{mol}^{-1}\cdot\text{M}^{-1}$, which just reflect the unfolding of dissociated folded monomer to fully unfolded monomer transition, while the dissociation energy of folded dimer to folded monomer is probe silence. DSC results indicate that TIM unfolding follows an irreversible two-state step with a slow aggregation process. The cooperative unfolding ratio, $\Delta H_{\text{cal}}/\Delta H_{\text{vH}}$, was measured close to 2, indicating that the two subunits of chicken muscle TIM unfold independently. The *van't Hoff* enthalpy, ΔH_{vH} , was estimated as about $200 \text{ kcal}\cdot\text{mol}^{-1}$. These results support the unfolding mechanism with a folded monomer formation before its tertiary structure and secondary structure unfolding.

Keywords: Chicken muscle triose phosphate isomerase, urea, fluorescence, CD, DSC, equilibrium unfolding mechanism.

INTRODUCTION

Triose phosphate isomerase (TIM, EC 5.3.1.1.) is a glycolytic enzyme that interconverts D-glyceraldehyde 3-phosphate and dihydroxyacetone phosphate [1]. Recently it has been found that this enzyme could associate with red cell membrane and microtubules. Deficiency of this enzyme, including certain mutations is associated haemolytic anaemia which is coupled with progressive, severe neurological misfolding disease and death in early childhood [2, 3]. Crystal structures for TIM from 14 various organisms have been determined (for example, chicken [4], yeast [5], *Trypanosoma cruzi* [6], *Escherichia coli* [7], *Entamoeba histolytica* [8], human [9]). Both primary and three-dimensional structures of TIM show a high degree of conservation. All the wild-type TIMs studied so far are homodimers, with the exception of those from *Pyrococcus woesei* and *Methanothermobacter fervidus*, which are homotetramers [10]. Each subunit folds into a single eight-stranded β/α -barrel domain. Residues involved in substrate binding and catalysis are all located in the same subunit. Although dimerization is required for its catalytic activity, this enzyme is not allosterically regulated and exhibits classical Michaelis-Menten kinetics [11]. The fine structure difference of triose phosphate isomerase from different species made this enzyme an excellent target for drug design [12]. Thus the structure and function relationship of this enzyme has been studied widely and deeply by both experiments and theoretical simulations.

Studies on denaturant-induced unfolding mechanism of TIM from different sources have indicated that its equilibrium unfolding mechanism is TIM species dependent ([13] and reference therein). Rabbit muscle TIM, the best studied one for its unfolding/refolding pathway, showed a two-state equilibrium unfolding process during GdnHCl denaturation with its unfolding transition curve moving to high denaturant direction when TIM concentrations were increased [14]. However, the protein concentration dependent unfolding of rabbit muscle TIM was not observed under hydrostatic pressure denaturation conditions [15]. Recently it has been shown that the TIM from *Saccharomyces cerevisiae* dissociates into partially folded monomers in the presence of moderate concentration of GdnHCl [16], and TIM from *Trypanosoma cruzi* unfolds in GdnHCl through both stable dimeric and monomeric intermediates [17]. These differences suggest that extensive study of the folding mechanism of TIM from different sources may provide deep insight on the monomer's structural and energetic characteristics.

Unlike rabbit muscle TIM, chicken muscle TIM unfolding study was retarded after the initial report of its unfolding kinetics about 30 year ago [18]. Recently our group reported the equilibrium unfolding of chicken muscle TIM in GdnHCl using multiple spectral methods [19]. Our results indicate that chicken muscle TIM's unfolding in GdnHCl follows an apparent two-state process with no TIM concentration dependent. In this report, chicken muscle TIM's equilibrium unfolding process was monitored by both fluorescence and CD spectrum for isothermal denaturation and by DSC for thermal denaturation. The results indicate that the spectromonitored unfolding observed is most perhaps represent the

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folded monomer to unfolded monomer with an apparent two-state model, which is further supported by the results from DSC observation that chicken muscle TIM was cooperatively unfolded with each subunit independently.

MATERIALS AND METHODS

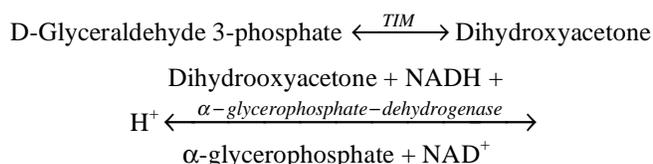
Chemical Reagents

D-Glyceraldehyde 3-phosphate, NADH sodium salt, Triethanolamine (Tris), 1,4-Dithiothreitol (DTT), urea and α -glycerophosphate dehydrogenase were from Sigma. Other reagents were from local chemical company with analytical grade.

Preparation and Activity Assay of Chicken Muscle TIM

Commercial fresh chicken breast muscle was used as the source of the enzyme. Previous published purification procedure [20-22] was followed with minor modification [19]. The fresh muscle was minced and stirred for 30 min with the buffer solution of 1.3 mM EDTA (pH7.0) containing 1 mM 2-mercaptoethanol. The homogenate is centrifuged (1200 \times g for 45 min) and the supernatant is filtered through muslin to remove any particles of fat. Ammonium sulfate is added to the supernatant with 65% saturation at 4 °C. The resulting precipitate is centrifuged (5000 \times g for 60 min) and discarded. Further amount of ammonium sulfate were then added to the supernatant to reach 90% saturation. The sediment was collected by centrifugation and was dissolved in minimum volume of 20 mM Tris-HCl buffer (pH 7.2). Then the crude TIM was further purified by DEAE-Sephacel fast Flow and Q-Sephacel ion-exchange chromatography. The sample was eluted with 30 mM NaCl buffer solution (20 mM Tris-HCl (pH 7.8)) with a single band in SDS-PAGE.

TIM activity was assayed using α -glycerophosphate dehydrogenase as a coupling enzyme (shown in scheme 1). The signal decrease of NADH was utilized to monitoring the catalysis progress using UV-spectrometer at 340 nm (30°C). The specific activity of the enzyme [21, 22] was calculated



Scheme 1.

according to

$$\text{Activity}(\text{units} / \text{mg}) = \frac{\Delta A \times V \times D}{C \times 6.3}$$

where ΔA is the absorbance decrease of the solution at 340 nm per min. V is the total reaction volume in the cuvette, D is the factor by which the stock enzyme has been diluted, and C is the protein concentration in milligrams per milliliter. TIM with activity not lower than 7200 units/mg was used throughout all the experiments.

Intrinsic Fluorescence Spectrum Measurements

For fluorescence measurement, the excitation wavelength of 282 nm was utilized and the emission spectra from 300

nm to 400 nm versus urea concentration were followed. The data was collected from a fluorescence spectrometer Hitachi F-4500 (Japan).

CD Spectrum Measurements

For CD measurement, the signal at 222 nm was recorded using an Applied Photophysics PiStar-180 spectrometer with a 1 mm pathlength quartz cuvette. The CD spectra data were taken at 1 nm intervals with an integration time of 1 s. The data represented the average of five scans after correction for the buffer baseline and were reported as mean residue ellipticity (θ).

Urea-Induced Equilibrium Denaturation

Urea-induced equilibrium denaturation of TIM was measured both by intrinsic fluorescence emission red shifts and CD spectrum change. Each sample contained certain amount of TIM in 50 mM phosphate buffer solution containing 1 mM EDTA, 1 mM DTT, 100 mM NaCl (pH 7.8) and a series concentration of urea was incubated at 25 °C overnight. The unfolding progress was determined by

$$f = \frac{y - y_N}{y_D - y_N} \quad (1)$$

where f is the fraction of protein in native state at a certain concentration of urea, y is the measured fluorescence signal or CD signal, y_N is the fraction of native protein in buffer solution. y_D is that of the extrapolated value for the unfolded state, and assuming that they are linearly dependent on urea concentration, i.e.

$$y_N = y_N^o + m_N \cdot [\text{urea}] \quad (2)$$

and

$$y_D = y_D^o + m_D \cdot [\text{urea}] \quad (3)$$

The isothermal transition curves were analyzed by a two-state model from either a folded dimer to unfolded monomer (model A), from a folded monomer to unfolded monomer (model B), or from folded dimer to partially unfolded dimeric intermediate then to unfolded monomer (model C) as following.

$$\text{Model A. } N_2 \rightleftharpoons 2U \quad (4)$$

according to the results given by Bowie and Sauer [23], the unfolding transition could be expressed as equation from (5) to (8)

$$y = f_N (f_N^o + m_N [D]) + f_U (f_U^o + m_U [D]) \quad (5)$$

$$f_D = \frac{K_D (\sqrt{1 + 8C/K_D} - 1)}{4C} \quad (6)$$

$$f_N = 1 - f_D \quad (7)$$

$$K_D = \exp(-(\Delta G_D(H_2O) - m_G [D]) / RT) \quad (8)$$

where C is the concentration of native TIM in monomer, and K_D is the unfolding equilibrium constant.

$$\text{Model B. } N_2 \rightleftharpoons 2N' \rightleftharpoons 2U \quad (9)$$

in which the dissociation of native dimeric to folded monomer is fluorescence and far UV CD signal silence. The unfolding transitions observed are only for the folded monomer to unfolded monomer transitions and the data could be analyzed according to the procedure of Santoro and Bolen [24].

$$f = \frac{f_N + m_N[\text{urea}] + (f_D + m_D[\text{urea}]) \cdot K_D}{1 + K_D} \quad (10)$$

The data were analyzed with the software of Sigmaplot 6.0.

$$\text{Model C: } N_2 \rightleftharpoons I_2 \rightleftharpoons 2U \quad (11)$$

in which native dimer dissociates into partially unfolded dimeric intermediate with tertiary and secondary structure destroys, and the dissociation of dimeric intermediate to fully unfolded monomer is fluorescence and far UV CD signal silence. The data analysis of the observed $N_2 \rightleftharpoons I_2$ unfolding step is similar as in model B.

DSC Studies

Differential scanning microcalorimetry studies have been performed with MicroCal VP-DSC in the temperature range from 20°C to 90°C by using different heating rates. Due to the irreversibility of the transitions, the heat capacity peaks exhibit a variation in the shape and transition temperature. Each sample (1.0 mg/ml) run was preceded by a baseline run with buffer-filled cells. Integration of the transition curve was done numerically to obtain the calorimetric enthalpies, ΔH_{cal} , after subtracting the baseline. ΔH_{vH} values were calculated from the heat capacity curves using the appropriate factor for a two-state dissociation reaction of the type $N_2 \rightleftharpoons 2U$ [25];

$$\Delta H_{vH} = 6RT_m^2 \frac{C_{p,max}}{\Delta H_{cal}} \quad (12)$$

The unfolding cooperative number is calculated as

$$n = \frac{\Delta H_{cal}}{\Delta H_{vH}} \quad (13)$$

RESULTS AND DISCUSSION

Native chicken muscle TIM is a homodimer with five Trps and 4 Tyr's in each subunit, but none of them is located at the subunit interface (Fig. 1). The maximum emission wavelength (λ_m) of fluorescence spectrum is located at 327 nm, consisting with the X-ray crystal structure that the Trp residues are buried in hydrophobic environment in certain extent. During denaturation the λ_m increased to 355 nm in 8 M urea, indicating that the aromatic residues have been exposed into solution environment and TIM has been denatured at that condition. Analysis the unfolding transition curve monitored by the relative change of λ_m by setting the native state as unity and the fully unfolded state in 8 M urea as zero, indicates that the unfolding is a two-state process, at least apparently (Fig. 2), and the process is fully reversible (data not shown). In a previous report, it has been shown that the unfolding of chicken muscle TIM is protein concentration independent in GdnHCl [19]. In order to know whether TIM denaturation in urea follows the same rule, 22 fold of

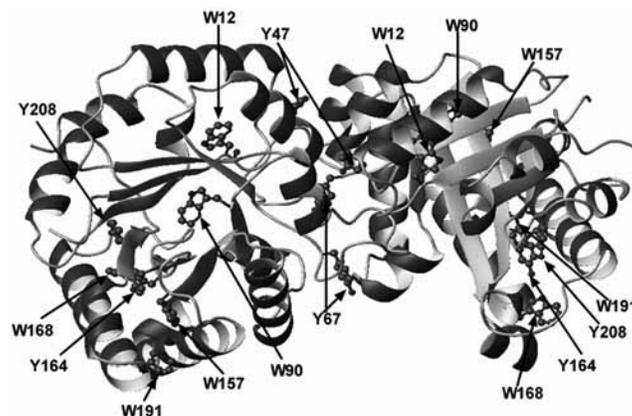


Figure 1. Crystal structure of chicken muscle triose phosphate isomerase. The graph was drawn using the software of MolMol (PDB entry: 1TIM). The side chains of Y47, Y67, Y164, Y208, W12, W90, W157, W168 and W191 in each subunit were shown as sticks and balls.

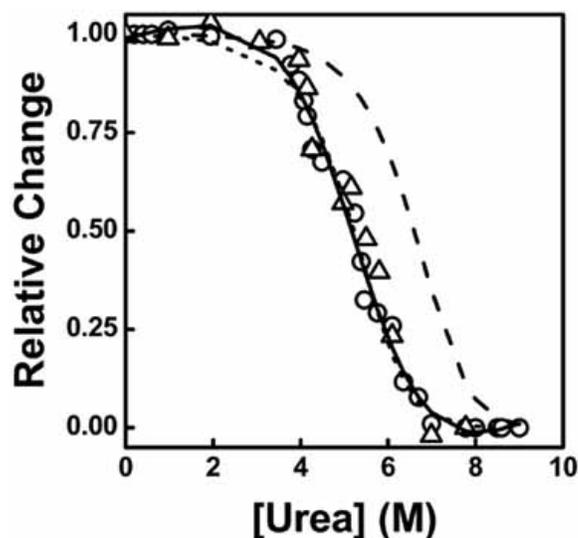


Figure 2. Urea-induced equilibrium unfolding transition curves of chicken muscle triose phosphate isomerase at 25°C (pH 7.8). The circles indicate the relative change of the intrinsic fluorescence wavelength at their emission maximum. Triose phosphate isomerase concentrations were 18 $\mu\text{g/ml}$. The triangles indicate the relative change of the far UV CD signal changes at 222 nm, and the enzyme concentrations were 400 $\mu\text{g/ml}$. The solid line refers to a non-linear best fit of the experimental data using a two state folded monomer to unfolded monomer transition model; the dot line represent the best fit of the experimental data monitored by fluorescence using a two-state folded dimer to unfolded monomer model, and the dashed line predicts the concentration dependent unfolding transition curve monitored by CD assuming the two state folded dimer to unfolded monomer model is rational.

TIM concentration was applied, and the same unfolding was monitored by CD signal at 222 nm. With the same scaling ruler, the unfolding transition curve monitored by CD was superimposed to that one by fluorescence emission wavelength changes very well, indicating that the tertiary structure and secondary structure unfold cooperatively and protein concentration change does not move the transition curve to

Table 1. Thermodynamic Parameters of Urea Induced Unfolding Using Difference Models

| Unfolding model | ΔG° (kcal mol ⁻¹) | m_G (kcal·mol ⁻¹ ·M ⁻¹) | $C_{1/2}$ (M) |
|-----------------|--|--|---------------|
| Model A* | 15.4±0.4 | 1.34±0.1 | 5.1 |
| Model B** | 3.54±0.67 | 0.67±0.14 | 5.2 |
| Model C** | 3.54±0.67 | 0.67±0.14 | 5.2 |

*In model A the parameters were calculated from fluorescence monitored unfolding transition curve. **In model B and C, the parameters were done from both fluorescence and CD monitored transition curves.

high urea concentration direction as expected from the law of mass action. The unfolding transition was both fitted with model A and model B respectively. In model A using TIM monomer concentration of 0.67 μ M, the thermodynamic parameters of urea induced unfolding were drawn with the standard Gibbs free energy, $\Delta G^\circ(N_2 \rightarrow 2U) = 15.4 \pm 0.38$ kcal·mol⁻¹, and $m_G = 1.34 \pm 0.07$ kcal·mol⁻¹·M⁻¹ (see Table 1). The urea concentration at the midpoint of the transition is about 5.1 M. If this model is correct, 22 folded increase of TIM concentration would predict the transition curve move to high urea concentration direction and could be discerned from experimental uncertainty. To test that hypothesis, the predicted unfolding transition curve for CD measurement was calculated and the result is showed in Fig. 2 dashed line using the same standard Gibbs free energy parameters. The calculated urea concentration at the midpoint of the transition is about 6.3 M. This difference of predicted moving is significant than the observed unmoved transition curves monitored by fluorescence and CD, suggesting that model A is inappropriate. With model B, the observed unfolding is a monomolecular reaction, the standard Gibbs free energy is $\Delta G^\circ(N' \rightarrow U) = 3.54 \pm 0.67$ kcal·mol⁻¹·M⁻¹, and $m_G(N' \rightarrow U) = 0.67 \pm 0.14$ kcal·mol⁻¹·M⁻¹ (see Table 1). This value is consistent with that ones obtained from GdnHCl from our group [19], and consistent with reported data that the Gibbs free energy of dimerization of rabbit muscle TIM and yeast TIM are dominant in these two TIMs' stabilization and the folded monomer showed low value of Gibbs free energies ([13] and reference therein). The third model, model C, i.e. native dimer unfolded into partially unfolded dimer with tertiary and secondary structure collapse, could also be applied for the data analysis. However, that model seems unfavorable as discussed in the later.

In order to get further information to distinguish the model B and model C, thermal unfolding of chicken muscle TIM was performed by DSC measurements. The advantage of this method is heat capacity change could reflect protein structure change without the need of certain structure probe at the appropriated location. Rational analysis of the thermal unfolding data needs to know whether its unfolding is reversible. Thus the protein sample in 1 mg/ml was first heated up to 90°C, and cooled to room temperature quickly, then a second heating scan was followed with the same sample as showed in Fig. 3A. Clearly there are no positive heat capacity transition occurred again, suggesting that the thermal unfolding under this condition is fully irreversible. However, unfolded TIM at 80°C with a low concentration of 18 μ g/ml could be partially refolded at 25°C as evidence by partial recovery (80%) of the intrinsic fluorescence intensity and the

blue shift of the maximum emission wavelength (data not shown). This difference indicates the unfolding of chicken muscle TIM could be treated as an apparent two state process, and the aggregation during DSC measurement is a slow process after TIM unfolding, normally described as Lumry-Eyring process [27]. Thus a series of DSC measurement were done under different heating rates. Fig. 3B showed the extra heat capacity transitions versus temperature with heating rate of 30°C/h, 45°C/h, 60°C/h, 75°C/h and 90°C/h respectively, after baseline subtraction. In all the cases the unfolding transitions are asymmetry and the transition temperature (T_m) is increased with increasing the heating rate (Fig. 3C and Table 2). The T_m value at the heating rate of 30°C/h was 65.4°C, at the heating rate of 90°C/h, it is 68.3°C and non-linearly. The transition temperatures have been fitted to a second-order polynomial in heating rate r

$$T_m = a + br + cr^2$$

with the following values of the coefficients: a=63.014, b=0.09138, c=-0.00035873. This function predicts that at a heating rate of 128°C/h, the transition temperature of TIM reaches its maximum of 68.8 °C, which is very close to that one obtained at the heating rate of 90°C/h. The chicken muscle TIM T_m obtained in this study is very close to that one from human TIM [3], and significantly higher than *Yeast* TIM (59 °C) [28] and *E. coli* TIM (55 °C) [11]. Standard numerical integration of the transition curves yields the measured enthalpy changes (ΔH_{cal}) presented in Table 2. *Van't Hoff* enthalpy (ΔH_{vH}) was calculated using a native dimer to unfolded monomer model. The value of ΔH_{vH} reaches to a constant of about 200 kcal·mol⁻¹. The cooperativity ratio of $\Delta H_{cal}/\Delta H_{vH}$ is close to 2.

The cooperativity ratio (n) could provide structural information of chicken TIM during its thermal denaturation. The value of 2 indicates that during thermal unfolding each subunit of the native dimeric TIM unfolded independently to the unfolded state [29], which is aggregated slowly. This means that interface interaction of each subunit is not strong enough to maintain the dimer after its tertiary and secondary structures are destroyed during denaturation, i.e. model C is inadequate. On the contrary, during thermal unfolding, the interface interaction is collapsed coupling with each subunit tertiary and secondary structure unfolding. This means that chicken muscle TIM is initiated from its dimer dissociation. In conjunction with denaturant induced equilibrium unfolding results, the equilibrium unfolding pathway of chicken muscle TIM in denaturants should experienced a weak, spectroscopic silent dimer dissociation step, followed by a coop-

erative unfolding of folded monomer into fully unfolded monomer, i.e. only model B is proper.

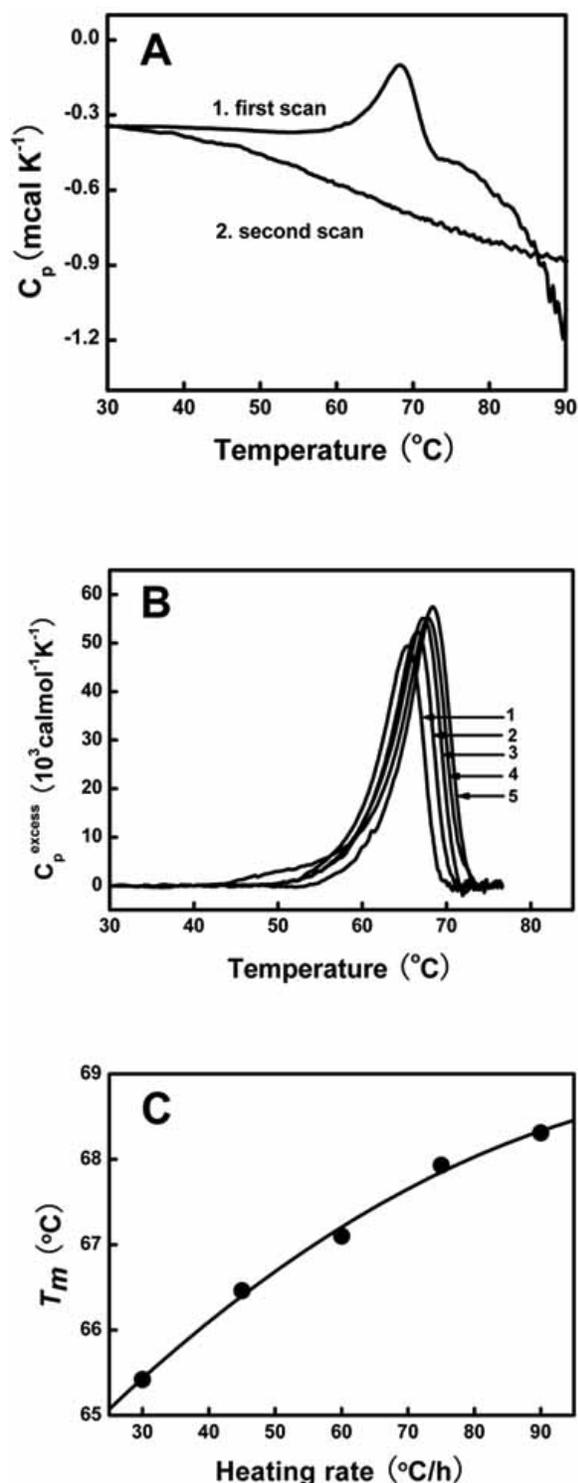


Figure 3. Differential scanning calorimetry measurement of chicken muscle triose phosphate isomerase. A, reversibility test of triose phosphate isomerase at the scanning rate of 90°C/h. B, excess heat capacity changes of triose phosphate isomerase at different scanning rates. C, the transition temperature versus the heat scanning rate relationship. In all the experiments, the protein concentrations were 1 mg/ml in 10 mM phosphate buffer at pH 7.8.

The characteristic of chicken muscle TIM thermal unfolding monitored by DSC is similar with the TIMs from other source with an irreversible single unfolding transition process. The difference is mainly on the thermal stability difference. Chicken muscle TIM has similar thermal unfolding transition temperature (T_m) to human TIM, but significantly higher T_m value than that from yeast TIM and *E. coli* TIM. Beyond that only in this studied the heating rate effect on chicken muscle TIM T_m movement and the cooperativity ratio (n) was determined, which provided the structural information on chicken muscle TIM unfolding reaction. However, the characteristic of chicken muscle TIM concentration independent unfolding induced by denaturants is significantly different from the TIMs from other sources [14]. Deviation of the mass action law was also observed from other oligomer proteins [26]. The association of subunits in such oligomers has been described as a deterministic process and has been attributed to long-lived conformational heterogeneity in the ensemble of molecules. Only rabbit muscle TIM has showed protein independent unfolding transition induced by hydrostatic pressure. Although GdnHCl induced rabbit muscle TIM showed TIM concentration dependent, the change don't fit the two-state unfolding transition of model A [14]. The existence of slow subunit exchange of rabbit muscle TIM has been tested by fluorescence resonance energy transfer changes [15]. In that case, rabbit muscle TIM solution were separately labeled with 1,5-IAEDANS or FITC as a fluorescence donor/acceptor pair, and were mixed in an equal molar ratio. This mixed TIM sample were applied to hydrostatic pressure just before rabbit muscle TIM unfolding, then released the pressure for several cycles, FRET between 1,5-IAEDANS labeled TIM and FITC labeled TIM was observed as indicated by the decrease of fluorescence intensity at 476 nm monitored by 1,5-IAEDANS and increase at 525 nm monitored by FITC fluorescence intensity. This change has been interpreted as the evidence of the existence of the heterologous conformations of rabbit muscle TIM in solution. The similar unfolding behavior of chicken muscle TIM in denaturants with rabbit muscle TIM by hydrostatic pressure reflects the similar intersubunit interaction between them. As predicted from the high sequence homologous between chicken and rabbit muscle TIM (86% identities and 92% positives), the differential residues are not located in the interface between the two subunits. It is rational to say that the equilibrium unfolding of chicken muscle TIM in denaturants should be considered as silent dimeric dissociation following tertiary and secondary structural cooperative unfolding mechanism. The advantage of DSC study in this work is that it provides confident information to exclude the model C, i.e. the unfolding of chicken TIM cannot precede its dimer dissociation.

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Table 2. Thermodynamic Parameters of TIM During its DSC Measurement at Difference Heating Rate

| Heating Rate (°C/h) | T_m (°C) | ΔH_{cal} (kcal·mol ⁻¹) | ΔH_{vh} (kcal·mol ⁻¹) | n |
|------------------------|------------|--|---|------|
| 30 | 65.42 | 336 | 201. | 1.67 |
| 45 | 66.46 | 357 | 201 | 1.78 |
| 60 | 67.1 | 397 | 190.5 | 2.08 |
| 75 | 67.93 | 377 | 201 | 1.88 |
| 90 | 68.31 | 387 | 205.5 | 1.88 |

ABBREVIATION

GdnHCl = Guanidine hydrochloride

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