铜离子对烟草多酚氧化酶变性和复性影响的热力学研究

肖厚荣 刘清亮* 徐小龙 施春华
(中国科学技术大学化学系,合肥 230026)

摘要: 在有无 5 mmol·L^{-1} $CuSO_4$ 存在的两种情况下,运用荧光光谱研究了烟草多酚氧化酶在盐酸胍诱导下的变性和复性平衡。烟草多酚氧化酶在 6.0 mol·L^{-1} 盐酸胍变性 30 min(25 ℃)即完全失活。荧光光谱结果表明:铜离子能够提高烟草多酚氧化酶的结构稳定性和抗盐酸胍变性的能力,进而影响烟草多酚氧化酶在盐酸胍诱导下的变性和复性过程。没有外源铜存在的情况下,盐酸胍诱导的烟草多酚氧化酶变性和复性是一个可逆的二态过程;在 5 mmol·L^{-1} $CuSO_4$ 存在的条件下,由于结合了 Cu^{2+}的酶的中间态稳定性增加,显示特征荧光,结果显示,外源铜存在时,盐酸胍诱导的烟草多酚氧化酶变性和复性是一个可逆的三态过程。根据相应模型,进行了热力学计算。上述实验结果得到酶活性测定的进一步证实,在 6 mol·L^{-1} 盐酸胍中放置 5,10,15,20,25,30 min,烟草多酚氧化酶的剩余活性分别为 18.7%,11.7%,8.9%,6.6%,3.6%,0.06%,但在 5 mmol·L^{-1} $CuSO_4$ 存在的条件下,剩余活性则分别为 60.3%,44.6%,42.5%,40.2%,25.6%,25.3%。

关键词: 铜离子; 多酚氧化酶; 荧光光谱; 变性; 复性
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Thermodynamic Studies on Cu^{2+}-induced Unfolding and/or Refolding of COI from Tobacco

XIAO Hou-Rong LIU Qing-Liang* XU Xiao-Long SHI Chun-Hua
(*Department of Chemistry, University of Science and Technology of China, Hefei* 230026)

Abstract: The equilibrium between unfolding and refolding of catechol oxidase I (COI) from tobacco in guanidine hydrochloride (GdnHCl) solutions both in the absence and in the presence of 5 mmol·L^{-1} $CuSO_4$ was studied by using the fluorescence spectroscopy. Incubating COI at 6 mol·L^{-1} GdnHCl for 30 min (25 ℃) deactivated the enzymatic activity completely. The results obtained from the analysis of the intrinsic fluorescence of Trp residues in COI show that Cu^{2+} could increase the structural stability of COI against GdnHCl and, further, influence its unfolding/refolding procedure. The GdnHCl-induced unfolding/refolding of COI in the absence of 5 mmol·L^{-1} $CuSO_4$ follows a two-state process with no detectable intermediate state, however, the GdnHCl-induced unfolding/refolding of COI is realized through a three-state process with an intermediate state in the presence of 5 mmol·L^{-1} $CuSO_4$. In addition, the presence of Cu^{2+} shifts both the initial zone and the ending zone of the denaturation curve toward higher concentration. The fact that the unfolding curve and refolding curve are nearly superimposable also indicates that the unfolding/refolding is reversible both in the absence and in the presence of 5 mmol·L^{-1} $CuSO_4$.

Key words: tobacco; catechol oxidase; fluorescence; unfolding; refolding

0 Introduction

Catechol oxidase (CO) or *o*-diphenol: oxygen-oxidoreductase (EC 1.10.3.1), which catalyses oxidation of *o*-diphenol and its derivatives to diquinones, is a member of the copper-containing polyphenol oxidase (PPO) family, which consist of Catechol oxidase, Laccase or *p*-diphenol: oxygen-oxidoreductase (EC 1.10.3.2) and

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*通讯联系人。E-mail:qliu@ustc.edu.cn;Tel:0551-3603214(O)
第一作者:肖厚荣,男,40 岁,博士,副教授,现工作单位为合肥学院生物与环境工程系;研究方向:生物无机化学及生物化学。

Cresolase or monophenol monooxygenase (EC 1.18.14.1). PPO is widely distributed in higher plants, animals, fungi and bacteria, but its biological function has not been defined clearly, although many possible roles have been proposed[1-3]. At present, the most likely functions for PPO are its involvement in plant resistance against diseases and insect herbivory[4,5]. For most plant tissues, PPO is compartmentalized in plastids, whereas its phenolic substrates located in vacuoles[6]. Action of PPO only occurs when this compartmentation is disrupted after tissues have been wounded, as observed in diseased tissues or those damaged by insect attack. An increase in PPO mRNA has been detected in artificially wounded tissues[7,8]. In some plants, the increase in PPO activity and PPO mRNR have been related to the production of compounds (jasmonic acid and methyl jasmonate) of the octadecanoid signal transaction pathway[9,10], but this does not appear to be universal, as several plants had little or no induction of PPO by wounding or treatment with methyl jasmonate[11].

PPO is a nuclear encoded protein, located in the thylakoïd lumen[12,13]. Studies on the routing of PPO to chloroplasts have demonstrated a typical thylakoïd lumen targeting[13]. However, inhibition of PPO import into plastids by low concentrations of Cu^{2+} tended to suggest that the intermediate forms have to be unfolded for protein import. Cu^{2+} was suspected to bind to the precursor, preventing complete unfolding[13]. Indeed, many plastidal proteins are maintained partially unfolding *in vivo* by chaperonins during the targeting into the thylakoïds[14,15]. Copper salts have already been used in purification protocols of PPO to lower the loss of activity during dialysis, which is a key step during PPO purification[16].

Protein folding/unfolding is a highly cooperative process and it can be affected by factors such as pressure[17], temperature[18], pH[19], and disulfide bond[20]. Recently, the effects of cations/anions and metal ions on protein folding/unfolding have received considerable attention. It has been demonstrated that metal ion-induced conformation changes in several enzymes lead to stabilization of the proteins during protein folding/unfolding[21,22]. In addition, without changing the concentration of the denaturant, the refolding of several proteins can be induced simply by adding anions[23] or cations[24].

1 Experimental

1.1 Materials and methods

1.1.1 Materials

The fresh tobacco leaves (*N. tobaccum*) with light yellow color, were harvested directly from the test field belonged to University of Science and Technology of China (USTC), washed, weighed, packed, and stored at −18 ℃ in a refrigerator. DEAE-Sephadex A-50, DEAE-Celluse DE-52, and Sephadex G-75 were purchased from Pharmacia Biotech Corporation (Sweden), all of them were pretreated before use; guanidine hydrochloride (GdnHCl, ultrapure) was obtained from Sigma-Aldrich Co. (St. Louis, MO, U.S.A). Chelex-100 was purchased from Bio-Rad Laboratories (Richmond, CA, U.S.A). All other chemical reagents were analytical grades. Tris-HCl buffer used for refolding/unfolding studies was freed from any possible contamination of multivalent cations by passage through a column (25 × 3 cm) of Chelex-100. GdnHCl was determined to be metal-free by extraction with dithizone (6 mg·L^{-1}) in carbon tetrachloride. All utensils used during the experiments were made metal-free by soaking in 2 mol·L^{-1} HNO_3 for 24 h and then by extensively rinsing with Milli-Q purified water[25].

1.1.2 Methods

1.1.2.1 Assay of protein content and enzymatic activity

Protein content was determined according to Bradford method[26] using bovine serum albumin (BSA) as the standard.

COI activity was determined by spectrophotometric method in a 3-mL cuvette at 25 ℃. 15 μL of the enzyme solution was added to 3 mL of 50 mmol·L^{-1} catechol in 50 mmol·L^{-1} phosphate buffer (pH 7.0). After standing for 5 minutes, the reaction mixture was determined for its absorbance at 420 nm. One unit of enzyme activity is defined as the amount of enzyme that causes an absorbance increase of 0.01 per minute under the described assay conditions.

1.2.2.2 Steady-state fluorescence measurements

All fluorescence measurements were performed on a Shimadzu RF-5301 spectrofluorometer using a 10 mm quartz cuvette. The sample temperature was kept at 25 ℃ with a circulating water bath. In all experi-

ments, the samples were excited at 282 nm, and the slit bandwidths for excitation and emission were both set to 5 nm. Each spectrum was the average of the three consecutively acquired spectra. All spectra were corrected by subtracting the spectrum of the blank i.e. sample without protein.

1.2.2.3 Denaturation experiments

COI denaturation was performed by adding GdnHCl to purified COI (1.5×10^{-4} g·mL^{-1}) prepared in 25 mmol·L^{-1} Tris-HCl buffer (pH 7.0), to a final concentration of 6.0 mol·L^{-1} [27] and incubated 30 min at 25 ℃. For the studies of the effect of $CuSO_4$ on the denaturation, the conditions were the same as the above except for $CuSO_4$ being present in the buffer solution. The enzymatic activity and fluorescence spectra were determined at intervals during unfolding.

1.2.2.4 Renaturation experiments

Renaturation was realized by dialysis of denatured solutions according to reference[28] with the following modifications: the dialysis lasted 12 h at 25 ℃ against 25 mmol·L^{-1} Tris-HCl (pH 7.0) containing 1.5 mol·L^{-1} GdnHCl, and the dialysis buffer volume was large enough so that the changes in GdnHCl concentration could be ignored after dialyzing. The effect of Cu^{2+} on COI refolding was tested by adding $CuSO_4$ solution to the buffer solution to a final concentration of 5 mmol·L^{-1}.

2 Results and discussion

2.1 Analysis of enzymatic activity

Fig.1 shows the time course effect of 6 mol·L^{-1} GdnHCl on COI activity in the presence and absence of 5 mmol·L^{-1} of Cu^{2+} at 25 ℃, respectively. It clearly implicates that Cu^{2+} can help COI resist to GdnHCl within the experimental time range, i.e., Cu^{2+} ions increase the structural stability of COI against GdnHCl denaturation. The results also indicates that incubating COI at 6 mol·L^{-1} GdnHCl for about 30 min (25 ℃) inactivated the enzymatic activity completely.

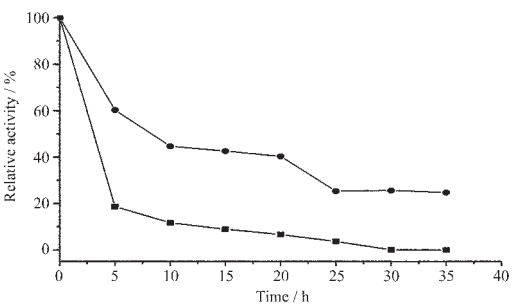

Fig.1 Enzymatic activity as a function of the deactivation time in the presence (●) and in the absence (■) of 5 mmol·L^{-1} of Cu^{2+}, both in 6 mol·L^{-1} guanidine hydrochloride, at 25 ℃

All the determination for enzymatic activity were performed by adding 15 μL of the enzyme solution (partial or total unfolding) to 3 mL of 50 mmol·L^{-1} phosphate buffer (pH 7.0) containing 50 mmol·L^{-1} catechol, at 25 ℃. The enzymatic activity was pre sented in relative activity (%).

2.2 Steady-state fluorescence of tryptophan

The intrinsic fluorescence of Trp residues in COI from tobacco was used for unfolding/refolding behavior study. As there are about 7 Trps in COI from tobacco (data not published), the overall changes in fluorescence reflect global changes in protein structure, and the mean microenvironments of all the Trp residues can be assessed. Fig.2 shows fluorescence emission spectra of COI at different concentrations of GdnHCl by exciting the protein at 282 nm. It is notable that the GdnHCl-induced unfolding causes the fluorescence emission intensity of COI to increase with the red shift of emission maximum λ_{max} (from 334 nm to 349 nm), indicating that COI assumes a compactly folded structure in which most Trps and quenchers, such as the charged carboxyl and/or amino groups in the interior protein, are adjacent as observed for bovine β-lactoglobulin[29]. As shown in Fig.3, GdnHCl-induced denaturation of COI was found to be a single-step process with no detectable intermediate state (s). The transition starts at about 1.25 mol·L^{-1} GdnHCl and slopes off at about 5.0 mol·L^{-1} GdnHCl. The refolding transition plots of COI almost superposes on its unfolding transition curve (also see Fig.3), which shows that the GdnHCl-induced denaturation of COI is reversible.

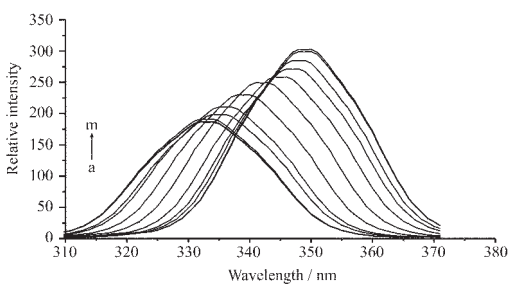

Fig.2 Effect of GdnHCl on the fluorescence emission spectra of COI

(The concentrations of GdnHCl for a~m are 0.0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0 mol·L^{-1}, respectively.)

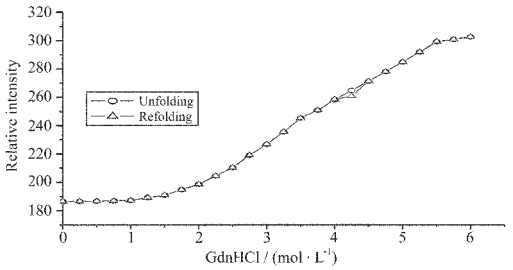

Fig.3 GdnHCl-induced unfolding and refolding of COI in 25 mmol·L^{-1} Tris-HCl buffer, pH 7.0, 25 ℃
The protein concentration was 1.5×10^{-4} g·mL^{-1}. Unfolding (○) and refolding (△) were monitored by measuring fluorescent intensity at λ_{max} of COI excited at 282 nm. The curves are the fitting curves based on experimental points by nonlinear least-squares analysis for the two-state transition according to Equation 9.

Fig.4 shows the relationship between GdnHCl concentrations and fluorescent intensity of COI in the presence of 5 mmol·L^{-1} of $CuSO_4$ by exciting the protein at 282 nm. The slight linear increase of fluorescence intensity with no obvious λ_{max} shift at low concentration of GdnHCl, less than 1.0 mol·L^{-1}, was thought to be the solvent perturbation effect on its fluorescence, which was therefore regarded as baseline. GdnHCl-induced denaturation of COI in the presence of 5 mmol·L^{-1} of Cu^{2+} was found to be a two-step process with accumulation of an intermediate state, and GdnHCl-induced denaturation of COI in this case was reversible, too (see Fig.5). The first transition of COI unfolding corresponding to the transformation of the N state to the I state starts at about 1.25 mol·L^{-1} GdnHCl and completes at 2.50 mol·L^{-1} with an increase in fluorescence intensity and a significant red shift of the λ_{max} from 334 nm to 340 nm, which shows that the unfolding I state of COI in the

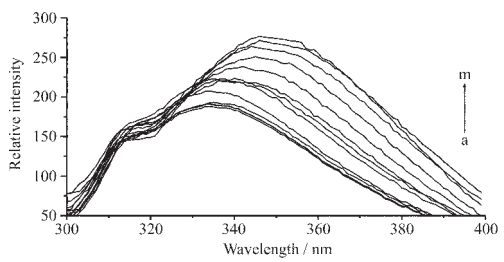

Fig.4 Effect of GdnHCl on the fluorescence of COI in the presence of 5 mmol·L^{-1} $CuSO_4$
(The concentrations of GdnHCl for a~m are 0.0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0 mol·L^{-1}, respectively.)

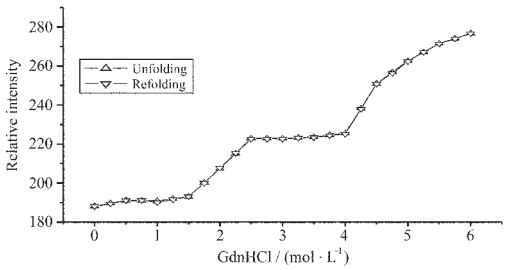

Fig.5 GdnHCl-induced unfolding and refolding of COI in 25 mmol·L^{-1} Tris-HCl buffer, pH 7.0, 25 ℃ in the presence of 5 mmol·L^{-1} of Cu^{2+}
The protein concentration was 1.5×10^{-4} g·mL^{-1}. Unfolding (△) and refolding (▽) were monitored by measuring fluorescent intensity at λ_{max} of COI excited at 282 nm. The curves are the fitting curves based on experimental points by the global analysis for the three-state transition according to Equation 10.

presence of 5 mmol·L^{-1} of $CuSO_4$ already has disordered structure. The I state is stable in the GdnHCl concentration range of 2.50~3.75 mol·L^{-1}. The second transition, which implicates the unfolding of I state, starts at around 3.75 mol·L^{-1} GdnHCl and gets to the D state at 6.0 mol·L^{-1} GdnHCl concentration with further increase of fluorescence intensity and further red shift of λ_{max} from 340 nm to 347 nm, suggesting that some folding conformations must exit in the I state which contains some local hydrophobic regions around some Trp residues.

Unfolding and refolding curves can be analyzed using either two-state or three-state mechanisms.

Two-state mechanism. The two-state model can be described as

$$N \longleftrightarrow D \quad (1)$$

where N and D are the native and denatured states, respectively. The equilibrium constant is defined as

$$K=[D]/[N] \quad (2)$$

The fraction of each species is

$$f_N=1/(1+K);\ f_D=K/(1+K) \quad (3)$$

The difference in free energy between the native and the unfolded states, ΔG, is then calculated by the following equation:

$$\Delta G=-RT\ln K \quad (4)$$

where R is the universal gas constant, and T is the absolute temperature in Kelvin. Note that the equilibrium constant in Eq.2 is for the unfolding reaction, so that a positive ΔG indicates that the native state is

more stable than the unfolding state.

Three-state mechanism. The three-state model used in the global analysis can be described as[30]

$$N \longleftrightarrow I \longleftrightarrow D \quad (5)$$

where N, I and D are the native, intermediate, and denatured states, respectively. The two equilibrium constants describing the system are defined as

$$K_{NI}=[I]/[N];\ K_{ID}=[D]/[I] \quad (6)$$

The fraction of each species is

$$f_N=1/(1 + K_{NI} + K_{NI}K_{ID})$$
$$f_I=K_{NI}/(1 + K_{NI} + K_{NI}K_{ID}) \quad (7)$$
$$f_N=K_{NI}K_{ID}/(1 + K_{NI} + K_{NI}K_{ID})$$

The free energy change corresponding to each equilibrium constant is

$$\Delta G_{NI}=-RT\ln K_{NI};\ \Delta G_{ID}=-RT\ln K_{ID} \quad (8)$$

The measured signal, Y, is assumed to contain contributions from each species. For the two-state model

$$Y=f_N Y_N + f_D Y_D \quad (9)$$

For the three-state model

$$Y=f_N Y_N + f_I Y_I + f_D Y_D \quad (10)$$

in which f_j represents the fraction of species j (as defined in Eq. 3 and Eq. 7) and Y_j is the molar signal of species j. The signals corresponding to the native and the unfolding state are considered as local fitting parameters that have a linear dependence on denaturant:

$$Y_j=Y_j^0 + \alpha_j[\text{GdnHCl}] \quad (11)$$

where Y_j^0 is the molar signal of species j at zero denaturant and α_j is a "slope" describing the dependence of signal Y_j on denaturant concentration. In the denaturant range where the unfolding process does not make a significant contribution to changes in the spectroscopy. Eq.11 describes the "baseline" of species j. The signal of the intermediate, Y_I, is described by the nondimensional parameter Z[30]:

$$Y_I=(1-Z)Y_N + ZY_D \quad (12)$$

in which Z is considered to be a global fitting parameter.

ΔG corresponding to each equilibrium constant is assumed to be linearly dependent on [GdnHCl] denoted by C, essentially as described in detail previously[31]:

$$\Delta G=\Delta G_0 - mC = m(C_m - C) \quad (13)$$

where ΔG_0 and ΔG represent the free energy of unfolding or refolding in the absence and presence of GdnHCl, respectively, C_m is the midpoint concentration of GdnHCl required for unfolding or refolding, and m stands for the slope of the unfolding or refolding curve at Cm and is a measure of the dependence of ΔG on denaturant concentration.

The thermodynamic and spectroscopic parameters were optimized in global fits by nonlinear least-squares analysis using the Marquardt-Levenburg algorithm in a software routine.

The thermodynamic and spectroscopic parameters were obtained by nonlinear least-squares analysis for the two-state transition according to Eq.9 and by the global analysis for the three-state transition of COI unfolding in the presence of 5 mmol·L^{-1} of $CuSO_4$ according to Eq.10. The ΔG^0, C_m and m values obtained for the GdnHCl-induced two-state unfolding transition are 26.46 ± 0.05 kJ·mol^{-1}, 3.12 ± 0.06 mol·L^{-1}, and 20.13 ± 0.03 kJ·(mol·L^{-1})$^{-1}$·mol^{-1}, respectively. The ΔG_{NI}^0, C_m^{NI} and m_{NI} values obtained for the first transition (N⟷I) of the three-state process are 20.47 ± 0.07 kJ·mol^{-1}, 1.87 ± 0.05 mol·L^{-1}, and 19.05 ± 0.09 kJ·(mol·L^{-1})$^{-1}$·mol^{-1}, respectively. The ΔG_{NI}^0 value is the difference in free energy between the native and the intermediate state of COI in the absence of GdnHCl. The ΔG_{ID}^0, C_m^{ID}, and m_{ID} values associated with the second transition (I⟷D) are 13.52 ± 0.05 kJ·mol^{-1}, 4.67 ± 0.05 mol·L^{-1}, and 13.86 ± 0.07 kJ·(mol·L^{-1})$^{-1}$·mol^{-1}, respectively. The ΔG_{total}^0 for the transition N⟷D (first N⟷I, then I⟷D) was calculated to be 34.00 ± 0.12 kJ·mol-1. A comparison of the free energy changes between the two-state process (N⟷D) and three-state process (N⟷I⟷D) during GdnHCl-induced unfolding evidently shows that the ΔG_{total}^0 for the three-state process is greater than the ΔG^0 for the two-state process, and the difference is calculated to be 7.53 ± 0.13 kJ·mol^{-1}. All the above results indicate that bound-Cu^{2+} ions obviously increase the conformational stability of COI.

Similar equilibrium parameters of refolding transitions are obtained for both two-state process and three-state process in GdnHCl solution compared with their unfolding transition. The ΔG_0, Cm and m values obtained for the GdnHCl-induced two-state unfolding transition are 25.58 ± 0.06 kJ·mol^{-1}, 3.21 ± 0.06 mol·L^{-1}, and 19.43 ± 0.05 kJ·(mol·L^{-1})$^{-1}$·mol^{-1}, respectively. The ΔG_{ID}^0, C_m^{ID}, and m_{ID} values obtained for the first refolding transition (I⟷D) of the two-step process are 14.28 ± 0.03 kJ·mol^{-1}, 4.63 ± 0.08 mol·

L^{-1}, and 14.23 ± 0.06 kJ·(mol·L^{-1})$^{-1}$·mol^{-1}, respectively, while the ΔG_{NI}^{0}, C_m^{NI} and mNI values obtained for the second refolding transition (N⟷I) are 20.77 ± 0.11 kJ·mol^{-1}, 1.86 ± 0.05 mol·L^{-1}, and 19.55 ± 0.06 kJ·(mol·L^{-1})$^{-1}$·mol^{-1}, respectively. The difference in refolding free energy between the two-state process in the absence of Cu^{2+} and three-state process in the presence of 5 mmol·L^{-1} of $CuSO_4$ is 9.46 ± 0.08 kJ·mol^{-1}. The results indicate that Cu^{2+} ions stabilize COI conformation to almost the same extent as observed from its unfolding transition.

3 Conclusions

One of the most intriguing observations in this study is that Cu^{2+} ions not only increase the structural stability of COI from tobacco, but also influence its unfolding and refolding behavior. The GdnHCl-induced unfolding/refolding of COI in the presence of 5 mmol·L^{-1} of Cu^{2+} follows a two-step process with a detectable intermediate state, while the GdnHCl-induced unfolding/refolding of COI in the absence of 5 mmol·L^{-1} of Cu^{2+} is a single-step process with no intermediate(s). The presence of 5 mmol·L^{-1} of Cu^{2+} shifts the initial zone of the unfolding curve and the ending zone of the refolding curve toward higher GdnHCl concentrations. It is plausible to deduce from all the above results, together with the enzymatic activity analysis, that Cu^{2+} ions play an important role in the stabilization of the structures of the native and I states. Fig.5 suggests that the intermediate state should be an on-pathway intermediate.

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