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The role of cupric in maintaining the structure of CopC

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The CopC protein from pseudomonas syringae pathovar tomato is expressed as one of four proteins encoded by the operon CopABCD that is responsible for copper resistance. And there are one tryptophan (83), one tyrosine (79), and three phenylalanines (35, 43, 99) in apoCopC. The fluorescence peak of apoCopC is located near 320 nm, and the peak shifts toward 353 nm in the presence of 10 mol·L^{-1} urea with excitation at 280 nm. Using urea as a chemical agent, the conformational stabilities of apoCopC and Cu_N^{2+}-CopC were monitored by fluorescence spectrum in 20 mmol·L^{-1} phosphate buffer and 100 mmol·L^{-1} sodium chloride at pH 6.0. The free energy of stabilization for apoCopC and Cu_N^{2+}-CopC is 16.29±0.65 kJ·mol^{-1} and 26.26±0.35 kJ·mol^{-1}, respectively. The distance between the tryptophan residue and the Cu^{2+} in Cu_N^{2+}-CopC has been studied by observing Förster type nonradiative energy transfer. And it is calculated to be 11.6 Å.

apoCopC, Cu_N^{2+}-CopC, urea, spectra

Copper is one of the most prevalent transition metals in living organisms. Cells use copper as a structural element in regulatory proteins and harness the chemistry of this element in single-electron-transfer reactions which are related to its biological function[1]. However, excess of copper is very toxic in the free form because of its ability to produce radicals by cycling between oxidized Cu^{2+} and reduced Cu^{+} and the binding with functional proteins which can cause imbalance in trace elements[2]. Copper's homeostasis in living systems is strictly controlled by subtle molecular mechanisms.

The CopC is a soluble protein present in the oxidizing environment of periplasm and is proposed to be a copper carrier. NMR, EXAFS and X-ray structural data revealed a β-barrel topology and the presence of two separated but interdependent binding sites, with high specific affinity, one for Cu^{+} and the other for Cu^{2+}. And the two binding sites are about 30 Å apart. The copper ion appears to exchange between the two sites upon a change of the oxidation state. This intriguing copper chemistry is consistent with the proposed role of CopC as a copper carrier in the oxidizing periplasmic space. These properties would allow it to exchange either Cu^{+} or Cu^{2+} with its putative partners CopA, CopB, and CopD, contrasting with the role of the Cu^{+} (only) chaperones found in the reducing cytoplasm[3−7]. In this paper, we use urea denaturation to explore the important role of Cu^{2+} in maintaining the protein conformational stability and use Förster-type nonradiative energy transfer principle to calculate the distance between the Cu^{2+} and tryptophan (83) in Cu_N^{2+}-CopC.

1 Experimental

1.1 Materials and instruments

Potassium dihydrogen phosphate, potassium hydrogen phosphate and phosphoric acid, urea, sodium chloride

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and cupric chloride were all analytical grade reagents.

The instruments used are F-2500 fluorescence spectrophotometer, Hewlett Packard 8453 spectrophotometer and pH meter.

1.2 Stock solution

Cupric solution was prepared by dissolving a certain weight of the cupric chloride in a small volume of distilled water.

1.3 Preparation of protein samples

ApoCopC was expressed and purified as previously described[5]. Protein concentration was measured at 280 nm (the molar extinction coefficient ε_{280} = 6970 $mol^{-1}\cdot L\cdot cm^{-1}$) by a Hewlett Packard 8453 spectrophotometer.

Cupric saturated apoCopC (Cu_N^{2+}-CopC) was prepared by adding one equivalent of cupric chloride to apoCopC in 20 $mmol\cdot L^{-1}$ phosphate buffer (PBS) and 100 $mmol\cdot L^{-1}$ sodium chloride at pH 6.0, and the cupric ion occupied N-terminal binding site of apoCopC.

1.4 Fluorescence spectra and UV-Vis spectra

An F-2500 fluorescence spectrophotometer was applied to recording the protein fluorescence spectra. After adding each urea solid, it was slowly dissolved and mixed. The emission spectrum was recorded until the solution temperature reached that of room. The curves (unfolded fraction at 400 nm vs. urea concentration) were normalized to give the fraction of unfolded molecules as a function of the denaturant concentration. To correct the dilution, the fluorescence intensity at 400 nm was converted to molar fluorescence intensity by dividing the fluorescence intensity via the analytical concentration of CopC.

The distance measurement apoCopC sample was excited at 280 nm while the protein spectrum was recorded from 300 to 450 nm. After the cupric stock solution was added to protein solution in 1/1 mole ratio, the sample was slowly and carefully mixed before the emission and difference UV-visible spectrum was recorded. Under the same condition the sample of tryptophan was prepared and recorded on fluorescence and UV-visible spectrophotomere.

In addition, distilled water was used to prepare 20 $mmol\cdot L^{-1}$ PBS, 100 $mmol\cdot L^{-1}$ sodium chloride and cupric chloride titrant. All the glasswares including cuvette were routinely washed in 1.0 $mol\cdot L^{-1}$ HNO_3 and then rinsed with distilled water.

2 Results and discussion

2.1 Urea denaturation

2.1.1 Fluorescence characterization of the protein. In Figure 1, curves a and c are the fluorescence spectra of apoCopC (C_{urea} = 0 $mol\cdot L^{-1}$) and the high urea denaturation form (C_{urea} = 9.4 $mol\cdot L^{-1}$) which is considered completely to be unfolding state, respectively, and curve b is a middle state (C_{urea} = 5 $mol\cdot L^{-1}$) which includes folding and unfolding proteins. It is obvious that the fluorescence maximum peak of apoCopC is located near 320 nm with excitation at 280 nm and the half bandwidth reaches to 48 nm (Figure 1a). Sequence analysis suggests that there are one tryptophan (83), one tyrosine (79), and three phenylalanines (35, 43, 99) in apoCopC[4]. Because of the little Stokes shift, it is hard to judge that the fluorescence of apoCopC stems from tryptophan or tyrosine residue. If the fluorescence of protein is caused absolutely by tryptophan residue, the denaturation of proteins by high urea concentration which causes practically complete unfolding of a protein, gives rise to the shifts of fluorescence spectral maxima toward 350—353 nm[8]. Thus, using urea as a chemical agent seems to be an optimal method for determining the tryptophan residue fluorescence and measuring the differences in conformational stability among proteins. As we know, many proteins have been found to closely approach a two-state mechanism[9]. If we fit the plots of curve b following this mechanism, it can be seen that the calculated value is nearly coincided with the experimental plots. Thus, we prone to think this protein denaturation obey the two-state mechanism.

From Figure 1, it can be seen that the maximum peak wavelength changed gradually (red shift) with the increase concentration of urea, and finally it was located at 353 nm. At the same time, the half bandwidth reached to 60 nm (Figure 1c), and this phenomenon consisted with that one of the tryptophan residue was completely exposed to polar environment[8,10]. It allows one to consider that the fluorescence of CopC is caused absolutely by the tryptophan residue which is surrounded by hydrophobic amino acids in apoCopC, and the addition of urea converts the environment of tryptophan residue from hydrophobicity to polarity. Adding urea to Cu_N^{2+}-CopC, the similar change in the maximum peak as Figure 1 was observed.

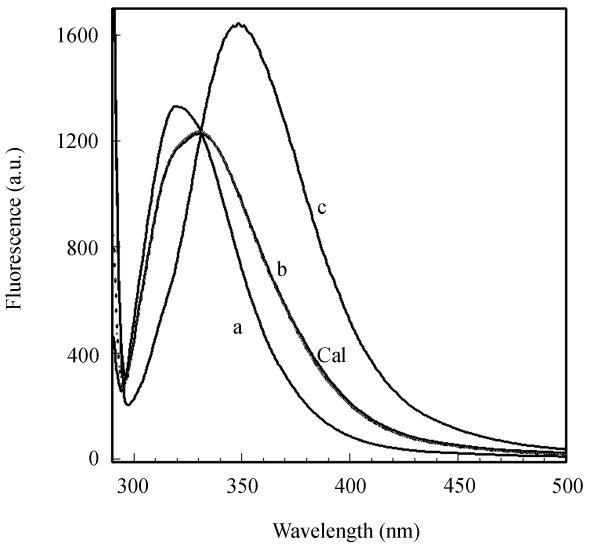

Figure 1 Fluorescence spectra for apoCopC in the absence or presence of urea, which were detected in 20 mmol·L^{-1} PBS and 100 mmol·L^{-1} sodium chloride, pH 6.0. Urea (mol·L^{-1}): a, 0; b, 5; c, 9.4. Cal represents the fitted curve according to two-state mechanism.

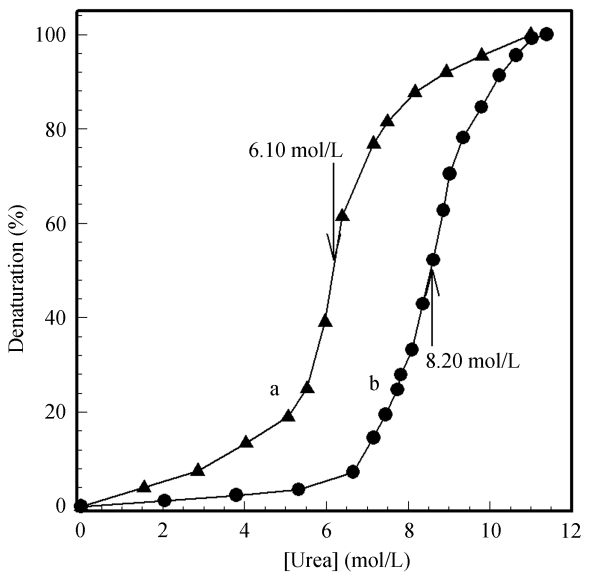

Figure 2 Urea unfolding curves. apoCopC (triangle), Cu_N^{2+}-CopC (dot) were monitored in 20 mmol·L^{-1} PBS and 100 mmol·L^{-1} sodium chloride, pH 6.0, at room temperature.

2.1.2 Free energy of the secondary structure. Based on these hypotheses that the increased volume of the solution in cuvette is linearly with the addition of urea, the protein denaturation obeys to the two-state mechanism and the protein is considered to be in unfolding state after the urea concentration reaches 10 mol·L^{-1}, the analytical concentration of protein and urea can be calculated. We analyzed the unfolding of apoCopC, Cu_N^{2+}-CopC with the increase of urea, as shown in Figure 2.

The unfolding profile of the intact protein showed the denaturant concentrations, C_m, which is at the midpoint of the transition yields, was 6.10 and 8.20 mol·L^{-1}, respectively. And C_m could be considered to reflect that whether or not the reaction of urea denaturation protein was easy. Comparing the two urea unfolding curves, it is inferred that cupric has the contribution to the protein conformational stability. This conclusion is further supported by the value of their free energy.

The Gibbs free energy of the protein stability at a certain urea concentration is given by the following relationship:

$$G_D = -RT \ln K, \quad (1)$$

where $K = F_u/(1-F_u)$ and F_u is the unfolded fraction of the protein. It is reasonably supposed that the Gibbs free energy is linearly dependent on the urea concentration[9,10].

$$G_D = G_D^{H_2O} - mC_{Urea}, \quad (2)$$

where m is a constant reflecting the strength of the dependence of the protein stability on denaturant concentration. $\Delta G_D^{H_2O}$ is the extrapolated value of ΔG_D at zero denaturant concentration. The best fit of the experimental data from Figure 2 following eq. (2) gave the intrinsic free energy of unfolding, as shown in Figure 3. The Gibbs free energy of apoCopC (a) and Cu^{2+}-CopC (b) was 16.29±0.65 kJ·mol^{-1} and 26.26±0.35 kJ·mol^{-1}, respectively.

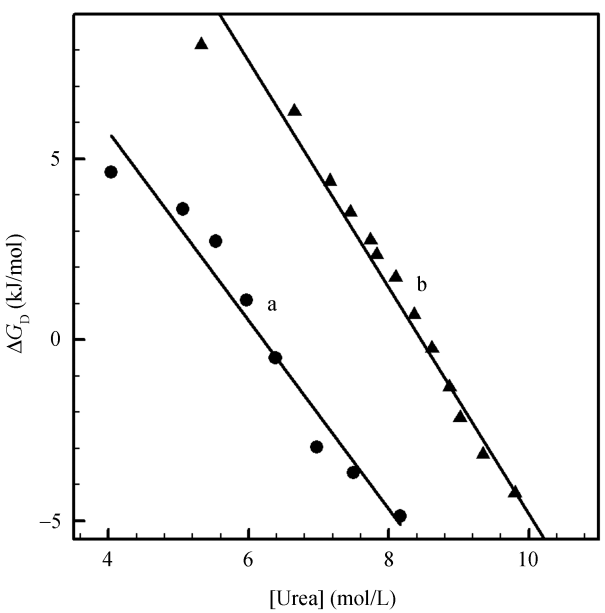

Figure 3 ΔG_D (apoCopC (a) or Cu_N^{2+}-CopC (b)) as a function of urea molarity. ΔG_D was calculated from the data in Figure 3 using eq. (2).

Urea unfolding of CopC reveals a distinctly higher structural stability (C_m = 8.20 mol·L^{-1}, $\Delta G_D^{H_2O}$ = 26.26 ±0.35 kJ·mol^{-1}) of Cu_N^{2+}-CopC than that (C_m = 6.10 mol·L^{-1}, $\Delta G_D^{H_2O}$ = 16.29±0.65 kJ·mol^{-1}) of apoCopC.

2.2 The distance between Cu^{2+} and tryptophan (83) residue in Cu_N^{2+}-CopC

The general feature of energy transfer from a donor to a distant acceptor was originally worked out by Förster[11]. Transfer of energy depends on the overlap of the emission spectrum of a donor, measured by its fluorescence $F(\bar{v})$ ($\bar{v}$ represents wave numbers), with the absorption spectrum of an acceptor, measured by the extinction coefficient $\varepsilon(\bar{v})$. The spectrum overlap integral J is determined by eq. (3).

$$J = \frac{\int F(\bar{v})\varepsilon(\bar{v})\bar{v}^{-4}d\bar{v}}{\int F(\bar{v})d\bar{v}}. \quad (3)$$

Figure 4 illustrates the spectral overlap between tryptophan residue of apoCopC emission and Cu_N^{2+}-CopC absorption.

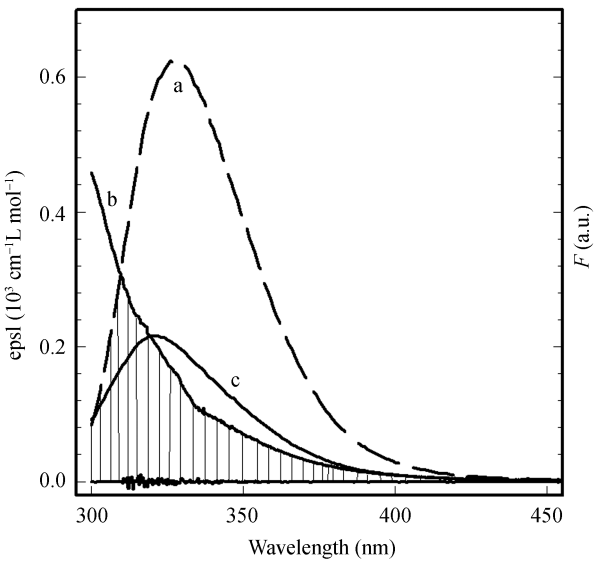

Figure 4 Spectral overlap between the emission spectrum of apoCopC (a) and Cu_N^{2+}-CopC absorption spectrum of (b) in 20 mmol·L^{-1} PBS and 100 mmol·L^{-1} sodium chloride, pH 6.0, at room temperature. c, the emission spectrum of Cu_N^{2+}-CopC

R_0, the distance at which 50% energy transfer is achieved, can be calculated from eq. (4).

$$R_0^6 = 8.78\times10^{-25}k^2Q_2n^{-4}J. \quad (4)$$

The orientation factor k^2 measures the angular orientation of one dipole with respect to the other. Here, k^2 is given its average value of 2/3 due to the effective isotropic symmetry of the electronic transition of both donor and acceptor. The quantum yield, Q_2, is calculated by eq. (5).

$$Q_2/Q_1=(F_2/F_1)\cdot(A_1/A_2). \quad (5)$$

A_1 and A_2 represent tryptophan and apoCopC absorbance at 280 nm under the same condition in 20 mmol/L PBS and 100 mmol/L sodium chloride at pH 6.0, respectively. F_1 and F_2 represent tryptophan and apoCopC fluorescence intensity at 350 and 320 nm excitation at 280 nm. Q_1 is the quantum yield of tryptophan in water phase. Here, Q_1 is given 0.14[12]. The index of refraction, n, of the intervening medium is given 1.33[12]. By use of the values above mentioned, R_0 for tryptophan residue→ cupric-CopC transfer was calculated to be 1.28 nm. The efficiency of the energy transfer, E, can be measured by eq. (6).

$$E = 1-\frac{F}{F_0}. \quad (6)$$

F_0 and F represent fluorescence intensity of apoCopC and Cu^{2+}-CopC, respectively. The distance is calculated to be 11.6 Å following eq. (7). The consistence of this value with the X-ray crystal data provided strong evidence of the reasonable in using energy transfer to calculate this distance[7].

$$E = \frac{1}{1+(r/R_0)^6}. \quad (7)$$

It is not inconceivable that under a certain condition, the distance between Cu^{2+} and tryptophan (83) residue in Cu_N^{2+}-CopC is a constant. If the protein binds to small molecules, its conformation may be changed and produce a new r. As a consequence, we can use the change of r to conjecture the interaction between the protein and small molecules.

3 Conclusion

ApoCopC structure can be stabilized by being saturated with cupric, the free energy of stabilization $\Delta G_D^{H_2O}$ is changed from 16.29±0.65 kJ·mol^{-1} to 26.26±0.35 kJ·mol^{-1}. In addition, the distance between the Cu^{2+} and the tryptophan (83) residue in Cu_N^{2+}-CopC is calculated to be 11.6Å.

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