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Investigation on the Inclusion Behavior of ApoCopC with Vitamin B_6

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In neutral phosphate buffer solution of pH 7.0, the interaction between apoCopC and Vitamin B_6 has been investigated in detail by means of fluorescence spectroscopy. According to the change of Vitamin B_6 fluorescence spectra and fluorescence polarization, we can conclude that a novel supramolecular system is generated. ApoCopC can form a 1:5 host-guest inclusion supramolecular complex with Vitamin B_6, and the formation constant has been calculated to be $(2.24 \pm 0.08) \times 10^4\,M^{-1}$. It suggests the strong inclusion ability of apoCopC to the guest molecules. In addition, the mechanism of the apoCopC protein fluorescence quenching by Vitamin B_6 was also discussed. And based on the Stern-Volmer equation, the apparent quenching constant was estimated to be $(2.75 \pm 0.05) \times 10^4\,M^{-1}$.

Keywords: ApoCopC; Vitamin B_6; Fluorescence; Inclusion

INTRODUCTION

Investigations of the intermolecular assembly have attracted much attention in supramolecular chemistry. Macromolecular inclusion behavior is of great interest due to a cavity or analog to encapsulate a guest molecule. It has been demonstrated that several weak forces, including Van der Waals, hydrophobic, electrostatic, dipole–dipole, and hydrogen-bonding interactions, cooperatively govern the inclusion complex. The noncovalent interactions are weaker than covalent-bonds, however, they can provide a sufficient thermodynamic driving force to form stable molecular capsules [1–5].

The CopC protein, a small soluble molecule (10.5 kDa), from *Pseudomonas syringae pathovar tomato* is expressed as one of four proteins encoded by the operon CopABCD that is responsible for copper resistance [6]. This protein has two completely distinct copper binding sites which are about 30 Å apart. They seem specifically designed to selectively bind two copper ions, one in the reduced state and the other in the oxidized one. The suggested ligand environments are $Cu^{I}(His)(Met)_x$ ($x = 2$ or 3) and $Cu^{II}(His)_2(Asp)(Glu)(OH_2)$ [7,8], respectively.

The CopC protein has a β-clam structure composed of nine β-strands that enclose an internal binding cavity and form a classical hydrophobic core [9]. The aromatic ring of tryptophan residue, sandwiched between the two β sheets, locates in a rather hydrophobic microenvironment and has numerous contacts with residues in strand β2 and strand β7 [7,9]. Based on apoCopC protein special construction, we are prone to think that apoCopC can be treated as a substrate to form stable host–guest inclusion complexes with molecules of suitable dimensions in supramolecular chemistry. Small molecules are expected to enter the cavity. It is obvious that the inclusion complex formation often results in remarkable variations in photophysical and photochemical properties of guest molecules because of the microenvironmental difference between the apoCopC interior and the aqueous medium.

In this paper, Vitamin B_6 as a guest molecule was studied in detail based on fluorescence measurements. The remarkable distinctness of its fluorescence spectra between hydrophobic and aqueous medium allowed us to directly probe Vitamin B_6 interactions with apoCopC across the formation of inclusion complex. And it was confirmed that the

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small molecule Vitamin B_6 entered the cavity of apoCopC and formed a supramolecular system.

EXPERIMENTAL

Apparatus

The absorption and the fluorescence measurements were performed with a Hewlett Packard 8453 spectrophotometer, and a Hitachi F-2500 spectrofluorometer. Excitation and emission bandwidths were both set at 5 nm. All experiments were carried out at room temperature. Molecular modeling was performed by Insight II 2000 (Accelrys) software platform and on a O2 workstation (Silicon Graphics).

Reagents

Tryptone, yeast extract, ampicillin, chloramphenicol, isopropyl-β-D-thiogalactopyranoside (IPTG) and sodium chloride were from Sangon Ltd. CM-sephadex (CM-25) was obtained from Pharmacia and cellulose (DE-52) was obtained from Whatman.

Potassium hydrogen phosphate, potassium dihydrogen phosphate, and phosphoric acid which were used to prepare for 20 mM phosphate buffer solution (PBS), were all analytical grade reagents.

ApoCopC was expressed and purified as previously described [10]. Protein concentration was measured at 280 nm (the molar extinction coefficient $\varepsilon_{280} = 6970\,\mathrm{mol}^{-1} \times \mathrm{L} \times \mathrm{cm}^{-1}$) by a Hewlett Packard 8453 spectrophotometer.

The solution of Vitamin B_6 was prepared by directly dissolving the solid in water.

Fluorescence Spectra

A Hitachi F-2500 fluorescence spectrophotometer was applied to record the fluorescence spectra of the interaction between Vitamin B_6 and apoCopC protein. The emission spectra were recorded until the solution reached room temperature. To correct the dilution, the fluorescence intensities were converted to molar fluorescence intensity by dividing the fluorescence intensity via the analytical concentration of substrate.

In addition, doubly distilled water was used to prepare for 20 mM PBS buffer (pH 7.0) and the solution of Vitamin B_6. All the glasswares including cuvette were routinely washed in 1.0 M HNO_3 and then rinsed with double distilled water.

Molecular Modeling

Initial coordinates for apoCopC were taken from its X-ray structure (PDB entry 1NM4). The structure of Vitamin B_6 was generated and optimized by the Builder module of Insight II 2000. The potential binding sites for Vitamin B_6 were obtained by binding sites searching procedure. Then Vitamin B_6 was docked one by one into the binding sites.

RESULTS AND DISCUSSION

Formation of the Inclusion Complex of Vitamin B_6 and ApoCopC

Figure 1 shows the fluorescence properties of Vitamin B_6 in the hydrophobic and aqueous medium. As can be seen, the fluorescence spectral maxima shift from 390 nm to 350 nm when the polarity of the surroundings of Vitamin B_6 is changed from polarity to hydrophobicity. The fluorescence maximum peak of apoCopC is located near 320 nm with excitation at 280 nm. Sequence analysis suggests that there should be one tryptophan (83), one tyrosine (79), and three phenylalanines (35, 43, 99) in apoCopC [9,10]. Based on the studies by Burstein *et al.* on various proteins intrinsic fluorescence, it can be concluded that the fluorescence of apoCopC stems from tryptophan residue absolutely, and its microenvironment in protein is hydrophobic [11]. Figure 2 shows the addition of apoCopC protein to the solution of Vitamin B_6. It can be seen that the fluorescence intensity of Vitamin B_6 at 390 nm is weakened and of the protein at 320 nm is gradually increased. At the same time, a new peak at 350 nm was generated. Comparing Figs. 1 and 2, we are prone to think that with the increasing concentration of apoCopC protein, the peak of Vitamin B_6 shifts gradually from 390 nm to 350 nm. It indicated that the microenvironment of Vitamin B_6 is changed from aqueous to hydrophobic.

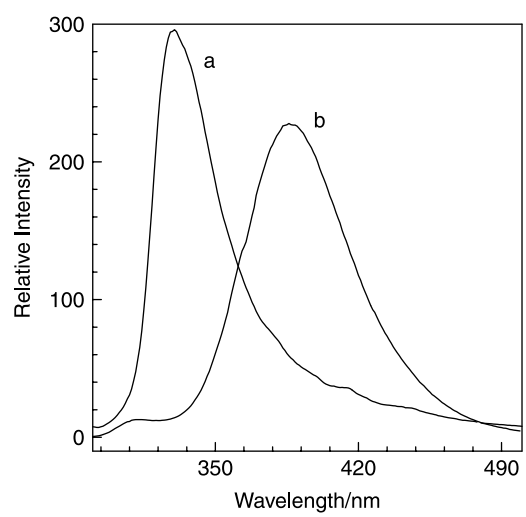

FIGURE 1 The fluorescence spectra of Vitamin B_6/20 μM in different solvent. a: chloroform; b: 20 mM PBS buffer, at pH 7.0, and the excitation wavelength $\lambda_{ex} = 280$ nm.

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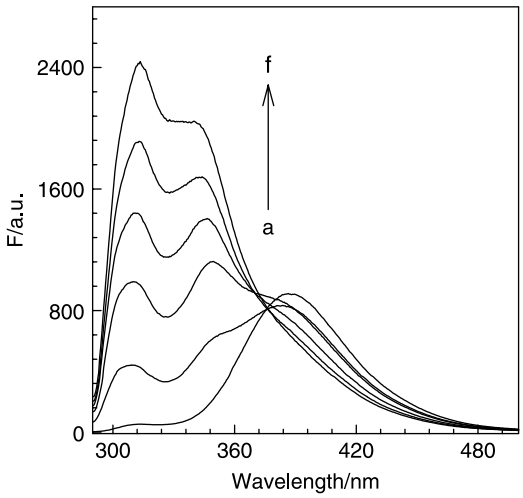

FIGURE 2 The fluorescence spectra of Vitamin B_6/200 μM at different concentrations of apoCopC in 20 mM PBS buffer, at pH 7.0. The volume of apoCopC/214 μM from a to f is 0, 80, 160, 240, 320, 400 μL, respectively, and the excitation wavelength $\lambda_{ex} = 280$ nm.

This phenomenon allowed one to consider that Vitamin B_6 entered the hydrophobic cavity of apoCopC and formed an inclusion complex.

The Mechanism of the Protein Fluorescence Quenching by Vitamin B_6

Figure 3 shows the fluorescence spectra of apoCopC in the absence and presence of Vitamin B_6 in PBS buffer at pH 7.0. With the increased concentration of Vitamin B_6, the fluorescence intensity of apoCopC at 320 nm gradually decreased, indicating effective quenching of the fluorescence by Vitamin B_6, whereas it increased at 350 nm, giving further evidence of the supramolecular assembly formation between molecules. Based on the Stern-Volmer equation:

$$F_0/F = 1 + K_{SV}[Q] \tag{1}$$

where F_0 and F refer to the fluorescence intensities of apoCopC in the absence and presence of Vitamin B_6, respectively; $[Q]$ represents the concentration of quenching reagent; K_{SV} is the apparent quenching constant. It is obvious that a plot of F_0/F against $[Q]$ should give a straight line with a slope equal to K_{SV}. Figure 4 shows the Stern-Volmer plots based on the data in Fig. 3. K_{SV} was calculated to be $(2.75 \pm 0.05) \times 10^4\,M^{-1}$. As we know, the maximum collisional quenching constant of biological macromolecule is $200\,M^{-1}$ [12]. The markedly large constant, K_{SV}, indicated that the mechanism of the protein fluorescence quenching by Vitamin B_6 was steady-state quenching.

Formation Constant of Supramolecular Assembly

The formation constant is an important parameter, which represents the inclusion capacity. The stoichiometry and binding strength of the interaction between apoCopC and Vitamin B_6 are determined in this experiment. According to Gauthier *et al.* [13] and Patterson *et al.* [14], we suppose an inclusion complex formation between apoCopC protein (P) and Vitamin B_6 (L) as follows:

$$P + L = P - L_{\text{Complex}} \tag{2}$$

Based on theoretical assumption that the interaction between small molecular Vitamin B_6 and apoCopC is consistent with Langmuir monomolecular adsorption mechanism, and the following

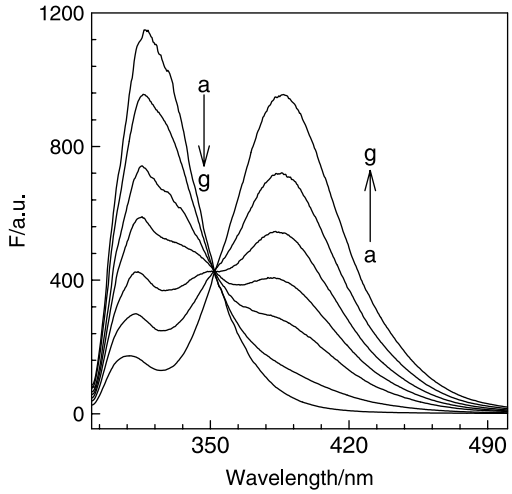

FIGURE 3 The fluorescence spectra of apoCopC/10 μM at different concentrations of Vitamin B_6 in 20 mM PBS buffer, at pH 7.0. The volume of Vitamin B_6/10 mM from a to g is 0, 10, 15, 20, 25, 30, 40 μL, respectively, and the excitation wavelength $\lambda_{ex} = 280$ nm.

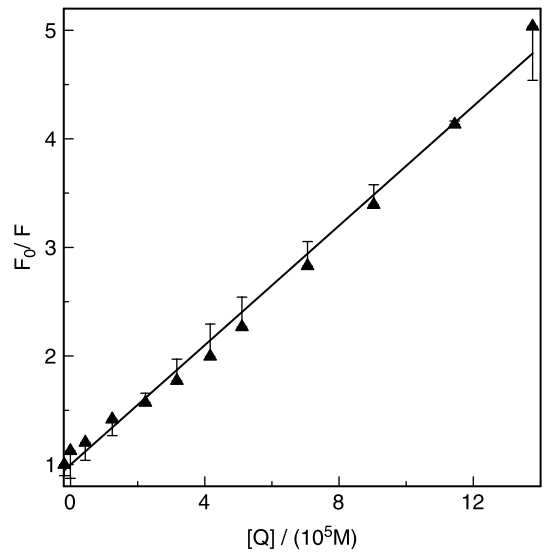

FIGURE 4 Stern-volmer plots of fluorescence quenching of apoCopC by Vitamin B_6.

equation is deduced [15].

$$K_{P-L} = \frac{n \cdot P_b}{P_f \cdot L_f} \tag{3}$$

where n is the binding ratio of Vitamin B_6 to apoCopC; K_{P-L} is the formation constant of the inclusion complex.

If we express a total concentration of P with P_t, L with L_t, the total mass balance equations become

$$P_t = P_f + P_b \tag{4}$$

$$L_t = L_f + nP_b \tag{5}$$

where P_f, and P_b, denote the concentrations of the free and bound forms of the apoCopC protein, respectively; L_f is the concentration of the free forms of Vitamin B_6.

If it is supposed that the change of apoCopC protein fluorescence intensity is due to the formation of supramolecular assembly. Then the following equation can be deduced.

$$\frac{P_b}{P_t} = \frac{F_0 - F}{F_0 - F_\infty} \tag{6}$$

By substituting Eqs. (4)–(6) into Eq. (3) we obtain Eq. (7).

$$\frac{L_t}{P_b} = \frac{n(F_0 - F_\infty)}{K_{P-L} \cdot (F - F_\infty) P_t} + n \tag{7}$$

Here, F_0 and F refer to the fluorescence intensities of apoCopC in the absence and presence of Vitamin B_6, respectively. F_∞ is the fluorescence intensity when all the apoCopC protein formed inclusion supramolecular. Following the data of Fig. 3, a plot of L_t/P_b vs. $1/P_t$ gave a straight line with a slope equal to n/K_{P-L}, and an interception equal to n, shown in Fig. 5. This implied the formation of an inclusion complex between Vitamin B_6 and apoCopC with a stoichiometry 5:1. The inclusion formation constant, K_{P-L}, is calculated to be $(2.24 \pm 0.08) \times 10^4\,\mathrm{M}^{-1}$. The result indicates that the apoCopC protein, owning a large cavity, has a strong supramolecular capacity. And this will provide a new substrate for supramolecular chemistry, and it also supplies a new path to investigate the apoCopC protein.

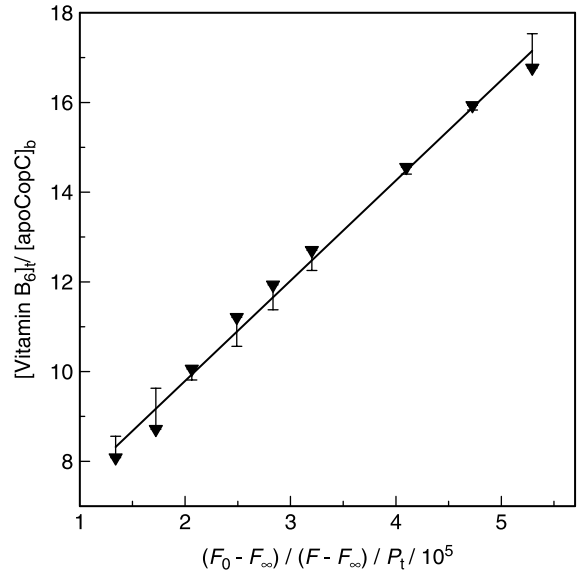

FIGURE 5 $[\mathrm{VitaminB_6}]_t/[\mathrm{apoCopC}]_b$ vs. $(F_0 - F_\infty)/(F - F_\infty)/P_t/10^5$ curve of apoCopC-Vitamin B_6 system in PBS buffer, at pH 7.0.

The Polarization of Guest Molecular Emitted Fluorescence

As we know, molecules undergo Brownian motion and are distributed randomly in solution. But an anisotropic distribution of excited molecules will be created if such an isotropic solution is illuminated by linearly polarized light. Because of that the fluorophores with absorption dipole moments parallel to the electric field of the light beam will be excited preferentially. It has been reported that P depends on the mobility of the fluorophore, thus information can be obtained about the local viscosity, size, shape and flexibility of the molecule under investigation [16]. In other words, P is a mass dependent parameter. It is conceivable that the small guest molecule Vitamin B_6 features a low polarization, but if it binds to a large receptor (like 10.5 kDa apoCopC), a maximal polarization will be observed. Using fluorescence polarization (P) seems to be an appropriate method for monitoring the binding of Vitamin B_6 to apoCopC protein. Based on the study by Shinitzky *et al.* [17], the steady-state fluorescence polarization P can be obtained following the formula (8) by measuring the vertical and horizontal components of the emitted light in a typical experiment.

$$P = \frac{I_{II} - I_\perp}{I_{II} + I_\perp} \tag{8}$$

where I_{II} and $I_\perp$ are the emission intensities parallel and perpendicular, respectively, to the plane of the excitation light. Following Eq. (8), the change of fluorescence polarization of Vitamin B_6 can be obtained with the addition of apoCopC, shown in Fig. 6. It is obvious that the fluorescence polarization of Vitamin B_6 was increased tremendously until the mole ratio of apoCopC to Vitamin B_6 is close to 0.2. The dramatically enhanced of Vitamin B_6 fluorescence polarization allows one to consider that the small molecular Vitamin B_6 is binding to apoCopC to form a heavy supramolecular inclusion complex with a binding ratio of five. This conclusion is according to the calculated value above.

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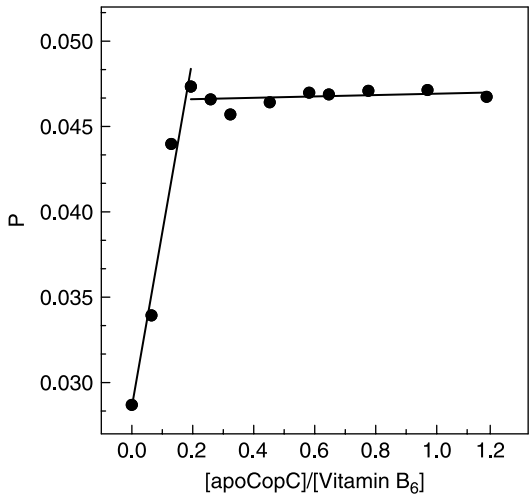

FIGURE 6 Fluorescence polarization of Vitamin B_6 with the increase concentration of apoCopC in 20 mM PBS buffer at pH 7.0 and the excitation wavelength $\lambda_{ex} = 320$ nm.

Molecular Modeling for the Supramolecular System

From the structure of apoCopC, the potential binding sites for Vitamin B_6 were obtained by a binding sites searching procedure. Then Vitamin B_6 was docked one by one into the binding sites. The optimization results by monitoring the total energy were shown in Fig. 7a. Meanwhile, the change of hydrogen bond energy with a gradually increasing number of Vitamin B_6 was also monitored (see Fig. 7b) and the convergent phenomenon with the change of total energy can be found. From Fig. 7, it is obvious that there are five binding sites which can keep the complex in the lower energy structure, when Vitamin B_6 was docked in the sixth or more, a higher value of total or hydrogen bond energy can be seen. It is consistent with the experiment result, the formation of a 1:5 inclusion complex.

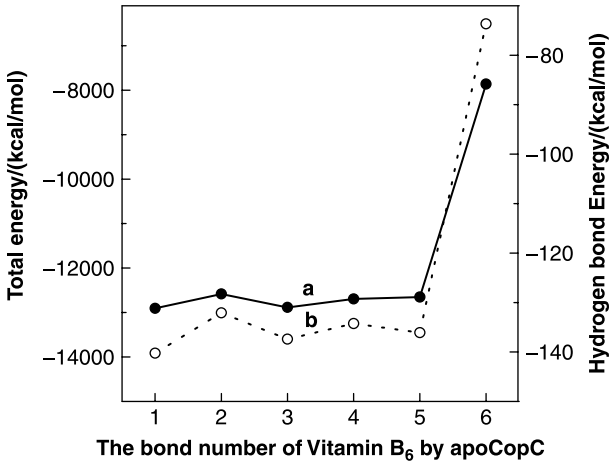

FIGURE 7 Molecular modeling energy values of the complex; a) the total energy; b) the hydrogen bond energy.

CONCLUSION

The formation and properties of novel supramolecular systems between apoCopC protein and Vitamin B_6 was studied by the methods of molecular fluorescence and fluorescence polarization. In neutral solution, apoCopC formed 1:5 host-guest inclusion complexes with Vitamin B_6 and the large value of formation constant, $(2.24 \pm 0.08) \times 10^4\,M^{-1}$, suggests that apoCopC has strong inclusive ability with small guest molecules. This paper not only supplied a new substrate for supramolecular chemistry but also established a brand new domain for the studies of apoCopC. The mechanism of apoCopC protein fluorescence quenching by Vitamin B_6 was also studied and the apparent quenching constant was calculated to be $(2.75 \pm 0.05) \times 10^4\,M^{-1}$.

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