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# Uranyl-cytochrome $b_5$ interaction regulated by surface mutations and cytochrome $c$*

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Understanding uranium-protein interaction is important for revealing the mechanism of uranyl ion ($UO_2^{2+}$) toxicity. In this study, we investigated the interaction between $UO_2^{2+}$ and a quadruple mutant of cytochrome $b_5$ (E44/48/56A/D60A cyt $b_5$, namely 4A cyt $b_5$) by spectroscopic approaches. The four mutated negatively-charged surface residues of cyt $b_5$ have been considered to be the interactive sites with cytochrome $c$ (cyt $c$). Also, we studied the interaction between $UO_2^{2+}$ and the protein-protein complex of 4A cyt $b_5$-cyt $c$. The results were compared to the interaction between $UO_2^{2+}$ and cyt $b_5$, and the interaction between cyt $c$ and cyt $b_5$-cyt $c$ complex, from previous studies. It was found that the interaction of $UO_2^{2+}$-cyt $b_5$, i.e., uranyl ion binding to cyt $b_5$ surface at Glu37 and Glu43 as previously proposed by molecular modeling, is regulated by both surface mutations of cyt $b_5$ and its interacting protein partner cyt $c$. These provide valuable information on metal-protein-protein interactions and clues for understanding the mechanism of uranyl toxicity.

Keywords: Uranium, Cytochrome, Heme protein, Protein-protein interaction, Fluorescence

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## I. INTRODUCTION

Developing nuclear energy is of social and economic importance, while this arises a major concern over environmental pollution by long-lived radioactive wastes, such as uranyl ion ($UO_2^{2+}$), the most stable form of uranium under physiological conditions [1]. In addition to its radiation hazard, $UO_2^{2+}$ is of high toxicity because it interacts with both DNA [2] and proteins [3–6] and disrupts their biological functions. To date, plentiful proteins have been found to be the targets of $UO_2^{2+}$, such as transferrin, ferritin and albumin [3–6]. A protein data bank (PDB) survey shows that $UO_2^{2+}$ binds to proteins mainly through carboxylic acid groups such as those of aspartate (Asp) and glutamate (Glu), as well as other coordinating amino acids such as histidine (His) and tyrosine (Tyr) [7]. In previous papers [8, 9], we studied the structural and functional consequences of $UO_2^{2+}$ binding to cytochrome $b_5$ (cyt $b_5$), cytochrome $c$ (cyt $c$), and the protein-protein complex of cyt $b_5$-cyt $c$, by both experimental and theoretical approaches.

Cyt $b_5$ is a small membrane heme protein, which is characterized by a highly negatively charged surface in presence of a series of acidic residues surrounding the heme group, known as an acidic cluster (Fig. 1) [10]. It has been shown that the acidic cluster participates in formation of cyt $b_5$-cyt $c$ complex [11–17], and is crucial in mediating cyt $c$ signaling in apoptosis [18]. With a positive charge, $UO_2^{2+}$ has a strong tendency to be absorbed at negatively charged surface of membrane [19], where it has a large possibility of interacting with membrane proteins such as cyt $b_5$. In previous study [8], we proposed a $UO_2^{2+}$ binding site for cyt $b_5$ at surface residues of Glu37 and Glu43 (Fig. 1), based on molecular modeling and dynamics simulation.

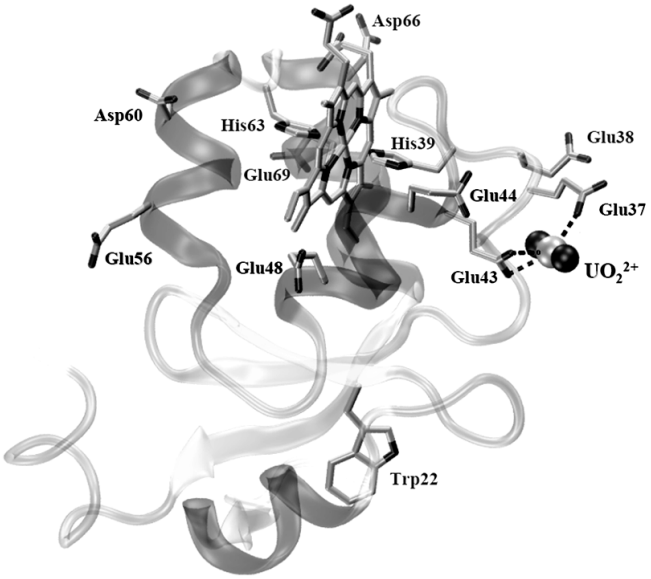


Fig. 1. Modeling structure of $UO_2^{2+}$-cyt $b_5$ complex, showing the $UO_2^{2+}$ ion binding to Glu37 and Glu43, the residues in acidic cluster, the heme group and Trp22 in the hydrophobic pocket.

To further probe the role of acidic cluster in uranyl-cyt $b_5$ interaction and to reveal the effect of surface mutations of cyt $b_5$ on $UO_2^{2+}$ binding, we herein choose a quadruple mutant of cyt $b_5$ as a target, in which four acidic residues were replaced with alanine, E44/48/56A/D60A cyt $b_5$, namely 4A cyt $b_5$. This mutant was designed previously for studying the interface between cyt $b_5$ and cyt $c$, where the replaced residues were considered to be interactive sites [16]. It keeps the uranyl-binding site of Glu37 and Glu43 but has a low binding affinity for cyt $c$, and therefore is an ideal model protein for investigating the regulation effect of cyt $c$ by a comparison

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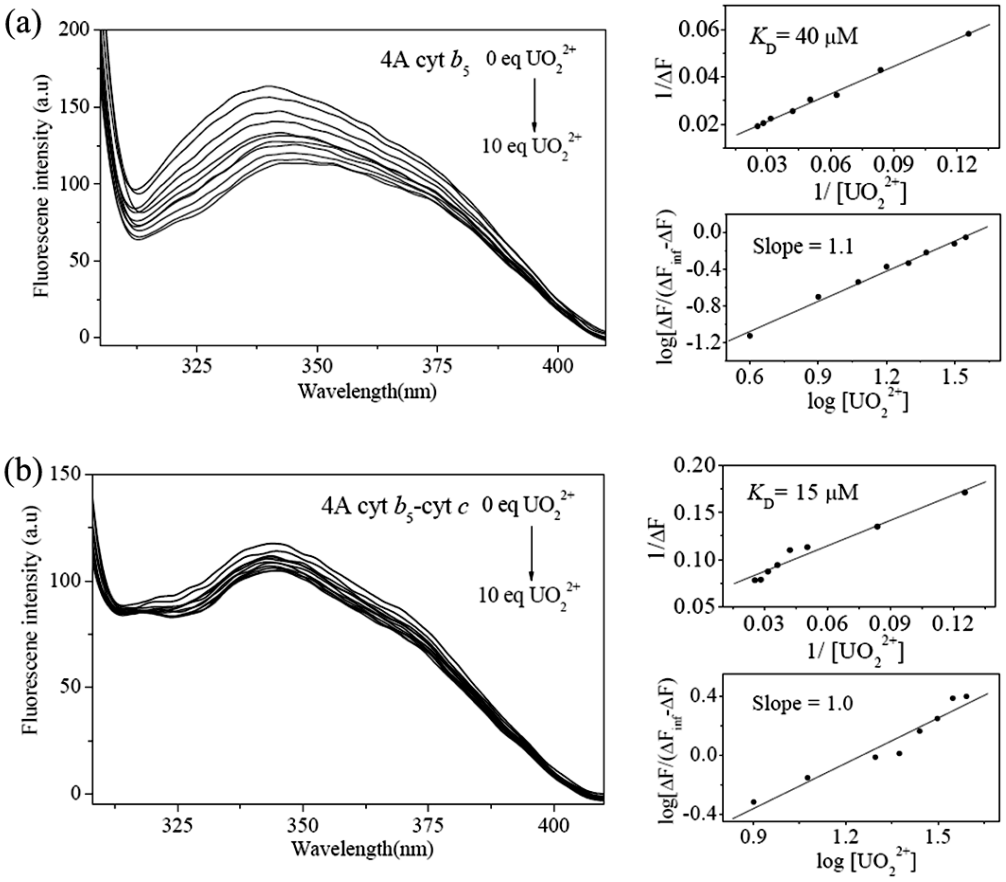


Fig. 2. Fluorescence titration of 4A cyt $b_5$ (4 μM) (a) and 4A cyt $b_5$-cyt $c$ complex (4 μM) (b), with $UO_2^{2+}$ in 50 mM Tris·HCl buffer (pH 7.0) at 25 °C. Double reciprocal plot and Hill plot are shown in right top and right down, respectively.

with that of cyt $b_5$-cyt $c$ complex.

## II. MATERIALS AND METHODS

4A cyt $b_5$ was expressed and purified as described previously [16]. Horse heart cyt $c$ (Type VI) was purchased from Sigma Chemical Co. Uranyl nitrate and other chemicals were commercial products and of analytical grade. Double distilled water was used throughout the experiments.

Fluorescence spectra of 4A cyt $b_5$ and 4A cyt $b_5$-cyt $c$ complex (4 μM in 50 mM Tris·HCl buffer, pH 7.0) with titration of $UO_2^{2+}$ up to 10 equivalents, were collected at 25 °C on a LS45 fluorescence spectrometer (Perkin Elmer, USA). The excitation wavelength was 295 nm and emission spectra were recorded from 310 nm to 410 nm. The apparent dissociation constant ($K_D$) was calculated from double reciprocal plot by fitting to Eq. (1) [20],

$$1/\Delta F = (K_D/\Delta F_{inf})(1/[UO_2^{2+}]) + 1/\Delta F_{inf}, \quad (1)$$

where $\Delta F$ is the difference between the maximum fluorescence in the absence and presence of $UO_2^{2+}$ and $\Delta F_{inf}$ is the fluorescence change for the complete binding of $UO_2^{2+}$. The number of $UO_2^{2+}$ binding site was calculated from $\Delta F$ of fluorescence spectra using the Hill plot [21], i.e. the yielded slope.

$$\log[\Delta F/(\Delta F_{inf} - \Delta F)] = slope \log[UO_2^{2+}] \quad (2)$$

Circular dichroism (CD) spectra of 4A cyt $b_5$ and 4A cyt $b_5$-cyt $c$ (20 μM in 50 mM Tris·HCl buffer, pH 7.0), in the absence or presence of 5 equivalents of $UO_2^{2+}$, were collected at 25 °C from 300 nm to 600 nm (1.0 cm path length), with a Jasco J720 spectrometer (Japan). Dilution effect of addition of $UO_2^{2+}$ was corrected by adding the same volume of buffer solution into the protein solution.

## III. RESULTS AND DISCUSSION

The single tryptophan residue (Trp22) in cyt $b_5$ (Fig. 1) serves as a convenient and sensitive reporter for studying the interaction of $UO_2^{2+}$-cyt $b_5$ by fluorescence spectroscopy. Figure 2(a) shows that the fluorescence intensity of 4A cyt $b_5$ decreases gradually upon titration of $UO_2^{2+}$ ions, suggesting a fluorescence quenching as observed for uranyl titration of transferrin, ferritin and albumin [3–6]. This observation is similar to that of wild-type (WT) cyt $b_5$ [9]. When the changes of the maximum fluorescence were fitted to a double

reciprocal plot (Fig. 2(a), right up) and a Hill plot (Fig. 2(a), right down), its binding affinity ($K_D$ = 40 μM) is 4 times lower than that of cyt $b_5$ (Table 1), though 4A cyt $b_5$ remains a single binding site of $UO_2^{2+}$ (slope = 1.1). It is likely due to a conformational change of residues Glu37 and Glu43 as a result of the four mutations in the acidic cluster, especially for Glu44 close to the proposed uranyl-binding site, as observed in the crystal structure of 4A cyt $b_5$ (PDB entry 1M2M) [16]. These observations indicate that the negative charges on the acidic cluster of cyt $b_5$ play important roles in uranyl-protein interaction.

TABLE 1. Binding affinity constants ($K_D$) for uranyl binding to cyt $b_5$, 4A cyt $b_5$, cyt $c$ and the complexes

| Uranyl-complex | $K_D$ (μM) | Refs. |
|---|---|---|
| $UO_2^{2+}$-cyt $b_5$ | 10 | [9] |
| $UO_2^{2+}$-4A cyt $b_5$ | 40 | This work |
| $UO_2^{2+}$-cyt $c$ | 87 | [9] |
| $UO_2^{2+}$-cyt $b_5$-cyt $c$ | 30 | [9] |
| $UO_2^{2+}$-4A cyt $b_5$-cyt $c$ | 15 | This work |

On the other hand, in titration of 4A cyt $b_5$-cyt $c$ complex with $UO_2^{2+}$ (Fig. 2(b)), the single uranyl-binding site on 4A cyt $b_5$ surface in the complex has a higher affinity of $UO_2^{2+}$ ($K_D$ = 15 μM) than that on the surface of isolated 4A cyt $b_5$ ($K_D$ = 40 μM). This observation suggests that the conformation of surface residues, Glu37 and Glu43, are tuned to be suitable for uranyl-binding as a result of cyt $c$ interacting with 4A cyt $b_5$, though the binding constant decreases from cyt $b_5$-cyt $c$ ($2.2 \times 10^4\ M^{-1}$) to 4 A cyt $b_5$-cyt $c$ ($5.5 \times 10^3\ M^{-1}$), as determined by NMR technique in a previous study [15].

In Table 1, the uranyl-binding factor ($K_D$ =15 μM) of 4A cyt $b_5$-cyt $c$ complex is two times lower than that of cyt $b_5$-cyt $c$ complex (30 μM), suggesting that a different protein-protein interface was adopted for 4 A cyt $b_5$-cyt $c$ complex as a result of mutation of four acidic residues on the same heme-exposed edge side of cyt $b_5$ (Fig. 1). In an earlier study, Huang and co-workers [17] showed that the charge neutralization of 4 A cyt $b_5$ greatly increased the relative contribution of the heme propionate and other charged residues surrounding the heme edge to contact with cyt $c$. Thus, our observations indicate that $UO_2^{2+}$-cyt $b_5$ interaction is regulated by both surface mutations of cyt $b_5$ and its partner cyt $c$.

The structural perturbations of 4A cyt $b_5$ and its complex with cyt $c$ upon uranyl binding were studied by circular dichroism (CD) spectroscopy (Fig. 3). It shows that $UO_2^{2+}$-4A cyt $b_5$ complex has a slightly decreased negative Cotton effect at 419 nm, whereas the Cotton effect in 300–350 nm is more positive than that of 4A cyt $b_5$ (Fig. 3(a)), which indicates that, similar to the observation for $UO_2^{2+}$-cyt $b_5$ [9], $UO_2^{2+}$ binding to the surface of 4A cyt $b_5$ slightly alters the conformation of heme-binding domain and aromatic amino acids such as Phe35 and Trp22 in the protein hydrophobic core. The CD spectra also show that upon uranyl binding, the 4A cyt $b_5$-cyt $c$ complex has an altered positive Cotton effect and a decreased negative Cotton effect compared to that in the absence of $UO_2^{2+}$ ions, with decreased intensity in 300–350 nm (Fig. 3(b)). These spectral changes are similar to the case of $UO_2^{2+}$ binding to cyt $b_5$-cyt $c$ complex, whereas both cases are less obvious than that for $UO_2^{2+}$ binding to cyt $c$ [9]. These observations suggest that $UO_2^{2+}$ ions alter the heme active site in both 4A cyt $b_5$-cyt $c$ and cyt $b_5$-cyt $c$ complexes, which are regulated by cyt $c$, likely through forming dynamic protein-protein complexes and competing with $UO_2^{2+}$ ions.

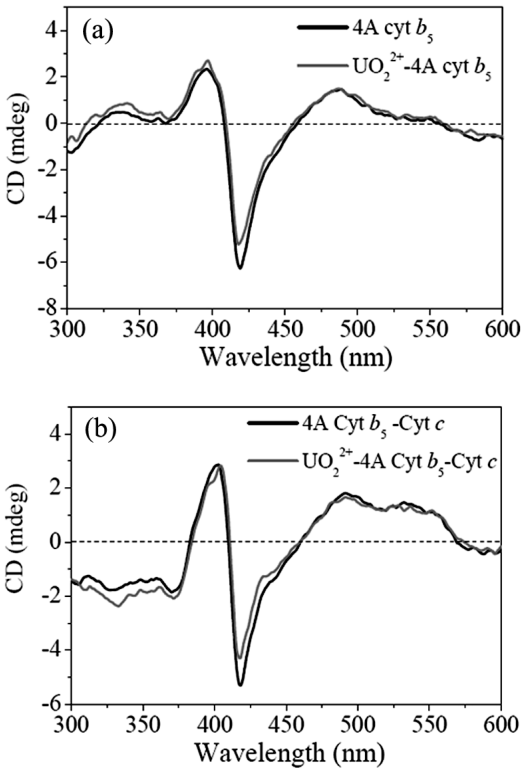


Fig. 3. CD spectra of 4A cyt $b_5$ (20 μM) (a) and 4A cyt $b_5$-cyt $c$ complex (20 μM) (b) in the absence (black lines) and presence (grey lines) of $UO_2^{2+}$ (100 μM) in 50 mM Tris · HCl buffer (pH 7.0) at 25 °C.

## IV. CONCLUSION

In summary, we investigated the interactions of $UO_2^{2+}$-4A cyt $b_5$ and $UO_2^{2+}$-4A cyt $b_5$-cyt $c$ complex, and compared to that of $UO_2^{2+}$-cyt $b_5$, $UO_2^{2+}$-cyt $c$ and $UO_2^{2+}$-cyt $b_5$-cyt $c$ from our previous studies [8, 9]. With four acidic residues on protein surface replaced by alanine, 4A cyt $b_5$ exhibits a lower affinity for $UO_2^{2+}$ compared to that for WT cyt $b_5$, though it remains the proposed uranyl-binding site at Glu37 and Glu43, which suggests a key role of the acidic cluster of cyt $b_5$ in tuning uranyl-protein interaction. On the other hand, $UO_2^{2+}$ binds more tightly to 4A cyt $b_5$-cyt $c$ complex than to the cyt $b_5$-cyt $c$ complex, presumably due to the conformational changes of Glu37 and Glu43 by a protein-protein interface different from that of cyt $b_5$-cyt $c$ complex. These observations indicate that $UO_2^{2+}$-cyt $b_5$ interaction is regulated by both the surface acidic cluster of cyt $b_5$ and its interacting protein partner cyt $c$, which

provides valuable information on metal-protein-protein interactions, and clues for understanding the mechanism of uranyl toxicity.

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