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Crystal structure of the CueO mutants at Glu506, the key amino acid located in the proton transfer pathway for dioxygen reduction

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ABSTRACT

Glu506 involved in the hydrogen bond network leading from solvent waters to the trinuclear copper center in a multicopper oxidase, CueO plays a crucial role to transport protons in the four-electron reduction of dioxygen to water. We performed X-ray crystal structure analyses of the Glu506Ala and Glu506Ile mutants, showing the formation of a compensatory proton transport pathway with only water molecules and a disruption of the hydrogen bond network due to the bulky side chain, respectively. We discuss the efficiency of proton transport through the hydrogen bond network based on the present results and our previous modification of the proton transport pathway by the Glu506 to Gln mutation, which have allowed us to trap and characterize the reaction intermediates.

1. Introduction

CueO [1,2] in the copper efflux system of *Escherichia coli* has received special attention because of its potential use as the cathodic enzyme of biofuel cell in addition to its biological role to maintain copper homeostasis. CueO has a type I (T1) copper and a trinuclear copper center (TNC) comprised of a type II (T2) copper and a pair of type III (T3) coppers to oxidize substrate and to reduce O_2 as the final electron accepter, respectively [3,4]. In the conversion of O_2 into two H_2O molecules, four protons are transported from solvent waters to TNC via a hydrogen bond network constructed within a CueO molecule.

It has been shown that acidic amino acids adjacent to TNC play crucial roles in the four-electron reduction of O_2 by CueO [5–7] and other multicopper oxidases (MCOs) such as laccase [8,9], bilirubin oxidase (BO) [10], Fet3p [11] and CotA [12]. Asp112 located at the interface of domain 1 and domain 3 of CueO is hydrogen-bonded with the OH^- ion coordinated to T2 copper via a water molecule (Fig. 1A). This amino acid residue has been reported to concern in the binding of O_2 at TNC and proton transport to it [5,13]. On the other hand, Glu506, which is hydrogen-bonded with the OH^- ion bridged between the T3 coppers via a water molecule (Fig. 1A), has also been considered to concern in the supply of protons to O_2. These acidic amino acids in CueO are conserved in other 3-domain MCOs such as BO [7], Fet3p [14], and CotA [12] judged from homologies in amino acid sequence (Fig. 1B) and spatial arrangements of them directed to TNC, while contribution from them remains unclear in the 2-domain MCOs such as SLAC [15] and mgLAC [16] and the 6-domain MCO, ceruloplasmin [17] because of diversity in the molecular architecture of MCO family enzymes (vide infra).

From a Fourier-transform infrared spectroscopy study [7], we showed that Glu506 in CueO and the parallel Glu463 in BO cycle between the protonated and deprotonated forms coupled with the redox state change in copper centers. Mutations of these Glu residues by Gln led to practical losses in enzymatic activities. Furthermore, the intermediate II [6] (the native intermediate [11]) has been trapped in the single turnover process of the Glu506Gln CueO mutant, because inability to relay proton(s) to this intermediate prevents the formation of water molecules. Although the life-time was much shorter, the intermediate II has also been trapped from the reactions of *Rhus vernicifera* laccase and BO by modifying them not at the corresponding Glu residue [8,11]. In the previous studies [7,18] we performed the mutations at Glu506 in CueO with Asp, Ala, and Ile, showing ca. 75% oxidizing activities to 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) in the Asp and Ala mutants and no activity in the Ile and Gln mutants. Based on the amino acids changed and enzymatic activities we supposed

Abbreviations: T1, type I; TNC, trinuclear copper center; T2, type II; MCO, multicopper oxidase; T3, type III.

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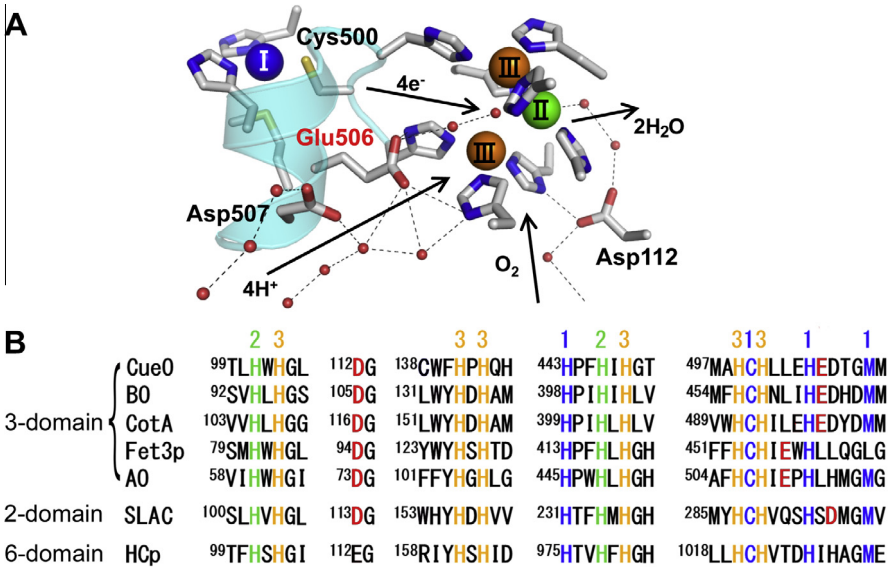

Fig. 1. The active site of CueO (A) and amino acid sequences of several MCOs (B). In (A) blue sphere, green sphere, and orange spheres represent T1, T2 and T3 coppers, respectively. In (B) AO, SLAC, and HCp represent ascorbate oxidase, small laccase, and human ceruloplasmin, respectively. Arabic and roman numbers represent types of copper centers. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

that a compensatory hydrogen bond network was constructed in the Ala mutant with only water molecules.

In the present study, we performed crystal structure analyses of the Glu506Ala and Glu506Ile mutants of CueO to display how the hydrogen bond network was modified in these mutants from those in the wild type enzyme and the Glu506Gln mutant (the double mutant also modified at Cys500 for a T1 copper ligand by Ser [19]) leading to the peculiar changes in enzymatic activities.

2. Methods

Crystals of the Glu506Ala and Glu506Ile mutants were prepared as previously [18]. For data collection, CueO mutant crystals were soaked in a cryo-protectant solution (10% glycerol) for a few minutes prior to freezing under a cold stream of nitrogen. X-ray diffraction data sets were collected at 100 K on beamline BL38B1 (λ = 1.0000) at SPring-8, using an ADSC Quantum315 CCD detector. The unit-cell parameters and integration of reflections were determined using HKL2000 [20] and the CCP4 program package [21]. Using the structures of the recombinant CueO (PDB code: 4E9T), the molecular replacement method was carried out. Further model building and structure refinements were performed using the programs COOT [22], REFMAC [23]. The progress and validity of the refinement process were checked by monitoring the R_{free} value for 5% of the total reflections [24]. The data collection and refinement statistics are summarized in Table 1. Model geometry was analyzed using MOLPROBITY [25] and no residue was found in the disallowed region of the Ramachandran plot. The figures were prepared using PyMOL (http://pymol.sourceforge.net). Occupancies of the four copper atoms were ~1, being in accordance with the four-copper content in a protein molecule and spectral properties.

Table 1
Summary of X-ray crystallographic data.

Crystallograhic data	Glu506Ala	Glu506Ile
Space group	$P2_1$	$P2_1$
Unit-cell parameters		
a, *b*, *c* (Å)	50.44, 90.87, 53.25	50.48, 90.91, 53.35
β(°)	102.7	102.7
Resolution (Å)	50.00–1.40 (1.45–1.40)	50.00–1.40 (1.45–1.40)
No. of unique reflections	91,858	91,458
Multiplicity	3.7 (3.7)	3.7 (3.7)
Completeness (%)	100.0 (100.0)	99.2 (98.4)
$\langle I/\sigma(I)\rangle$	27.5 (2.4)	26.7 (2.1)
R_{merge} (%)	5.4 (56.3)	6.0 (61.8)
R-value (%)	15.9 (23.6)	16.1 (24.9)
R_{free} (%)	18.4 (27.7)	18.3 (29.3)
No. of non-hydrogen atoms	4225	4216
Root mean square deviations		
Bond length (Å)	0.010	0.010
Bond angles (Å)	1.387	1.389
Ramachandran plot		
Favored residues (%)	97.3	97.8
Allowed regions (%)	2.7	2.2

3. Results and discussion

3.1. Structure and enzymatic activities of the Glu506 mutants of CueO

Both crystals of the Glu506Ala and Glu506Ile mutants have been X-ray analyzed at 1.4 Å resolution (Table 1). Overall structures of them (not shown) are practically superimposable with those of the wild type CueO [26] and double mutant Cys500-Ser/Glu506Gln [19]. Therefore, it is apparent that only the amino acid for the 506 position and its immediate vicinity are modified.

The structures of TNC in the Glu506Ala and Glu506Ile mutants of CueO are in Fig. 2, in which water molecules are shown as omit maps. In the wild type CueO and the Gln mutant the OH^- ion bridged between T3 coppers was hydrogen-bonded with the carboxyl group and the amide carbonyl group in the side chain of Glu and Gln, respectively, via a water molecule. Thus, the hydrogen bond network leading from the exterior of protein molecule to TNC was completed. In the Ala mutant with the less bulky methyl group in the side chain, however, an extra water molecule occupies the space produced by the mutation as expected from crystal structures of the wild type CueO and the Cys500Ser/Glu506Gln mutant (Fig. 2A). Thus this modified hydrogen bond network is comprised of only water molecules. Although some of them show low occupancy and double conformation, they are actually located

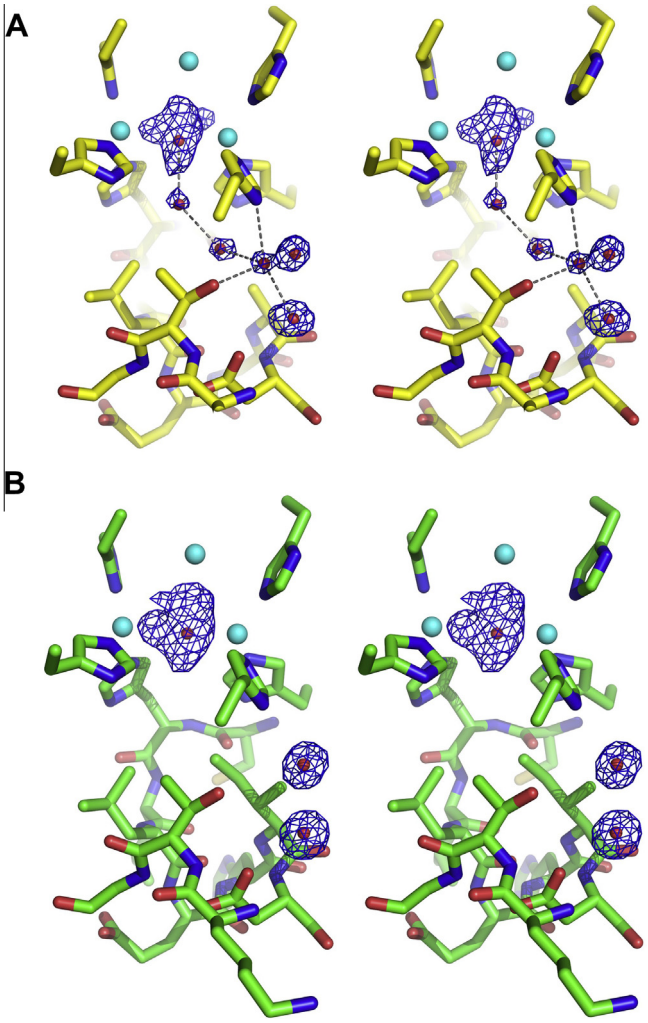

Fig. 2. X-ray crystal structures around TNC of the CueO mutants, Glu506Ala (PDB code: 4HAK) (A) and Glu506Ile (PDB code: 4HAL) (B). The omit maps of the water molecules are shown as blue mesh around TNC and contoured at 3.0 sigma. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

there. In contrast, the hydrogen bond network is completely shutdown in the Ile mutant with the bulky secondary butyl group (Fig. 2B). Further, the water molecule, which has been present between Glu506 and the OH^- bridged between T3 coppers in the wild type CueO and other mutants, is not seen in the Ile mutant.

3.2. Involvement of the Glu506 residue in the proton transport through the hydrogen bond network

These structural features of the hydrogen bond network in CueO and mutants accounts for their peculiar differences in enzymatic activity in regards to the facility of proton transfer for dioxygen reduction. The Ile mutant does not show enzymatic activity because protons are not transported from solvent waters to TNC in the conversion steps from the intermediate II to the resting form. Differing from the Ile mutant, the hydrogen bond network is completed in the Gln mutant, but the Gln residue is not able to relay protons because the amide group in the side chain does not cycle protonation and deprotonation coupled with the redox change in the copper centers. Thus, the conversion of dioxygen to water is also inhibited in the Gln mutant. On the other hand, the compensatory hydrogen bond network is constructed in the Ala mutant by allowing an extra water molecule to occupy the space left by the mutation. Differing from our expectation the Ala mutant exhibited considerably high enzymatic activity comparable to that shown by the Asp mutant. Formation of the compensatory hydrogen bond network is evidenced by the present X-ray crystallography, but a new question arises as to why the hydrogen bond network comprised of only water molecules exhibits such a high efficiency in the transport of protons in CueO.

In the study of attenuated total reflectance Fourier-transform infrared spectroscopy [7] we indicated that Glu506 plays a role as a pump to transport protons from solvent waters to TNC by cycling protonation and deprotonation states coupled with the change in the redox state of the copper centers. The present results show unequivocally that the hydrogen bond network leading from solvent waters to T3 coppers should be completed for the effective relay of protons to the intermediate II, but it appears that the presence of an amino acid with an exchangeable proton in the side chain is not necessarily essential. Amino acid sequences (Fig. 1B) indicate that MCOs comprised of three domains are divided into two groups as for the location of the Glu residue, although the location of the side chain carboxyl group directed to TNC is similar according to their crystal structures except *Trametes versicolor* laccase (amino acid sequence not shown), in which the Glu residue is replaced by Asp. In the 2-domain SLAC with a trimer structure, Asp295 might play as the proton donor to dioxygen [15,16]. However, a water molecule located between the OH^- bridged between T3 coppers and the Asp residue is not seen in the crystal structures of SLAC, although it does not necessarily mean that a water molecule is absent throughout its catalytic cycle. On the other hand, it seems to be difficult to discuss whether or not an acidic amino acid participates in the case of human ceruloplasmin (HCp) [17] (Fig. 1A) since this 6-domain MCO has a molecular architecture considerably different from those of the 2- and 3-domain MCOs.

In the conversion of dioxygen to water by cytochrome oxidases the proton relay pathway leading from the matrix to the heme-Cu center is constructed using some amino acids with exchangeable protons in the side chain together with a propionate group of heme a_3 and water molecules. The Ala mutations at Glu242 and Asp132 located in the proton relay pathway resulted in significant changes in the enzymatic activity of cytochrome oxidase [27,28]. Proton transfer coupled with electron transfer has been considered to take place across the electric field created between donor and acceptor. Compared to the proton relay pathway in the membrane-bound cytochrome oxidases, that in CueO is considerably short because of difference in molecular size and location to be present in the periplasm. Therefore, the absence of acidic amino acid in the hydrogen bond network might not be necessarily fatal in the production of the driving force to transport protons from solvent waters to TNC coupled with the redox changes in copper centers. Further, Fig. 1A shows that Glu506 is also hydrogen bonded with His143 coordinated to one of T3 coppers. Our previous studies to delete the hydrogen bond towards the coordinating groups to copper centers resulted in the positive or negative shift in the redox potential of the copper centers depending on the changes in the donating ability of the coordinating group [18,29,30]. The redox potential of the T3 copper coordinated to His143 might shift towards positive direction due to a reduction in the donating ability of His143, leading to an increase in the enzymatic activities because the driving force of electron transfer from T1 copper to TNC is increased. On the contrary, the occupation of water molecules in the space left may increase polarity around T3 coppers in the Ala mutant. Nevertheless, the former effect to positively shift the redox potential of one of T3 coppers might play a more significant role in the transport of protons via the alternative proton relay pathway comprised of only water

molecules due to an increase in the driving force of electron transfer coupled with proton transfer.

Analogous mutations have been performed on the corresponding Glu487 residue in Fet3p [14] and the Glu498 in CotA [12]. The Asp and Ala mutants of Fe3p exhibited 116% and 61% V_{max} values, respectively, compared to that of the wild type enzyme for the oxidation of Fe^{2+} ions. On the other hand, it has been reported that the Thr and Leu mutants of CotA resulted in a severe impairment, higher than 99%, for the phenolic and non-phenolic substrates. Although crystal structures of these CotA mutants have been determined, we will not discuss how the hydrogen bond network had been modified because of low occupancy at copper centers in TNC, which might have led to the production of mutant in a mixed form as for presence or absence of the intrinsic water molecules in the hydrogen bond network.

3.3. Roles of Glu506 and Asp112 in the four-electron reduction of dioxygen

Asp112 is located in the hypothetical outlet of water molecules constructed at the interface of domain 1 and domain 3 (Fig. 1B). Mutations at this amino acid by Glu, Asn and Ala exhibited considerably different enzymatic activities from those shown by the mutations at Glu506 [18]. The Asp112 to Glu mutant showed ~60% activity from that of the wild type enzyme, but Asn and Ala mutants showed ~10% activity [5]. The affinity of TNC in this Glu mutant for dioxygen was the same with that of the wild type CueO, but was decreased to ~50% in the Asn and Ala mutants. Based on these results on enzymatic activity and structural aspect of Asp112, being hydrogen-bonded with the His101 ligand to T2 copper, the His448 ligand to one of T3 coppers, and the hydroxyl group coordinated to T2 copper (Fig. 1), roles of this acidic amino acid are supposed to tune the redox potential of TNC and to construct the outlet of water molecules and/or to function as proton donor. The analogous mutation performed on CotA concluded that Asp116 plays a role to supply protons to dioxygen [13]. Since it is possible to trap the intermediate II, in which two oxygen atoms derived from dioxygen are present as the OH^- bridged between T3 coppers and O^{2-} at the center of TNC, in the reaction of the Glu506Gln mutant, it is considered that the first proton towards peroxide is supplied from a water molecule located near TNC. In the decay process of the intermediate II to the fully reduced form, waters are formed and eliminated from copper centers coupled with the stepwise reduction of TNC [19]. The hydrogen bond networks involving Glu506 or Asp112 and water molecules indirectly play as proton donors in the formation and excretion of water molecules, although a question is left whether locating Glu506 in the hydrogen bond network is prerequisite for the prompt transport of protons in the four-electron reduction of dioxygen in CueO.

It seems difficult to replace Glu506 with His or Tyr judged from the size of the channel to accommodate the hydrogen bond network. On the other hand, it might be possible to replace Glu506 by Lys, if the disposition of a positive change in the channel does not interfere the protein folding. In order to understand the function of the hydrogen bond network constructed with an acidic amino acid and water molecules we will perform further modifications on the proton relay system of CueO and related enzymes.

In summary, the present X-ray crystal structures of the CueO mutants evidence that the hydrogen bond network constructed with Glu506 and water molecules functions as the pathway of proton relay from solvent waters to TNC for dioxygen reduction. Further studies are needed to clarify the effective proton transport via the compensatory pathway constructed with only water molecules in the Glu506Ala mutant.

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