In crystallo thermodynamic analysis of catalytic reaction in bacterial copper amine oxidase

Currently, protein X-ray structures are usually determined from diffraction data of cryocooled crystals at around 100 K. It is believed that the structures at such cryogenic temperatures are basically identical to those at room temperatures where proteins actually function. However, thermodynamic analyses of conformational changes occurring in protein crystals have been very difficult by cryogenic X-ray crystallography, because the proteins lose their dynamics properties and temperature-dependent information is lost after cryocooling in exchange for protection from X-ray damage. Therefore, there has recently been much interest in the use of X-ray diffraction measurements at non-cryogenic temperatures to obtain dynamic structural information. Copper amine oxidases catalyze the oxidative deamination of various primary amines to produce the corresponding aldehydes (Fig. 1(a)) [1]. The active site contains a Cu^{2+} ion and a protein-derived quinone cofactor, topaquinone (TPQ) (Fig. 1(b)). We have determined the crystal structures of a copper amine oxidase from *Arthrobacter globiformis* (AGAO) that had been reduced with substrate amines under anaerobic coenditions. The determined structures revealed that the reduced cofactor has two distinct states: a semiquinone radical form (TPQ_{sq}) and an aminoresorcinol form (TPQ_{amr}) having the copper-ligated "on-copper" and indirectly copper-contacting "off-copper" conformations, respectively [2]. Previous UV-vis spectral studies in solution revealed that the equilibrium between TPQ_{sq} and TPQ_{amr} is affected significantly by temperature and pH. The structural basis for the temperature- and pH-dependent equilibrium remains unresolved. To address this problem, we applied a temperature-controlled humid air and glue-coating (HAG) method to non-cryocooled crystals of AGAO at SPring-8 **BL38B1** and thermodynamically analyzed the conformational change of TPQ in the crystal [3]. The HAG method was originally developed by JASRI [4] and has recently been improved to allow strict temperature control of protein crystals under anaerobic conditions [5].

To investigate temperature-dependent equilibrium changes of the AGAO crystal structure, we collected

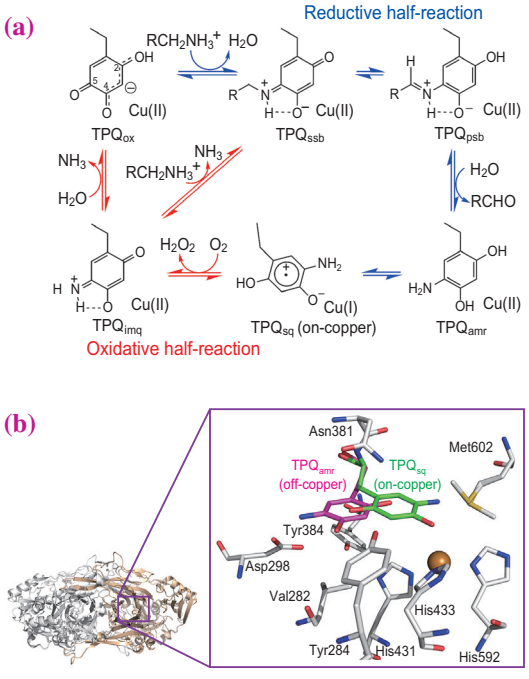

Fig. 1. **(a)** Presumed catalytic mechanism of AGAO. TPQ_{ox}, oxidative form of TPQ; TPQ_{ssb}, substrate Schiff base of TPQ; TPQ_{psb}, product Schiff base of TPQ; TPQ_{amr}, aminoresorcinol form of TPQ; TPQ_{sq}, semiquinone radical form of TPQ; TPQ_{imq}, iminoquinone form of TPQ. **(b)** Overall structure of the AGAO dimer as a ribbon model. Enlarged view: stick model of the active site. The off-copper conformer (TPQ_{amr}) and on-copper conformer (TPQ_{sq}) are drawn in magenta and green, respectively.

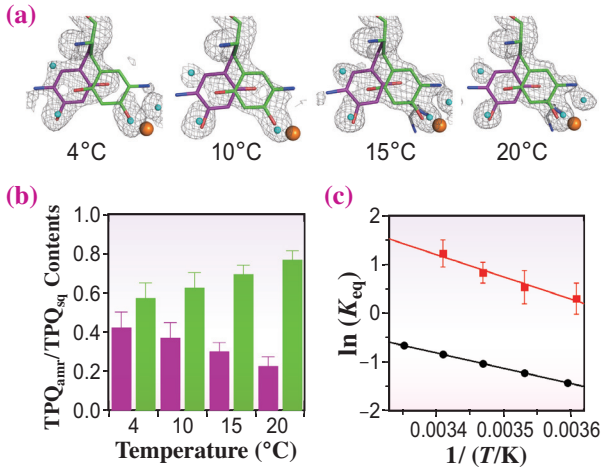

Fig. 2. Temperature dependence of TPQ_{sq}/TPQ_{amr} equilibrium. The TPQ conformation in the ethylamine-reduced AGAO crystal was examined at pH 6.0 by the temperature-controlled HAG method. **(a)** Assigned models of TPQ_{sq} (green sticks) and TPQ_{amr} (magenta sticks) are superimposed on the $F_o - F_c$ omit maps (gray mesh) for residue 382 (TPQ) contoured at 3.5 σ, determined at the indicated temperatures. **(b)** Average occupancies of TPQ_{amr} and TPQ_{sq} at various temperatures are shown by magenta and green bars, respectively, with S.E. ($n \geq 6$), assuming that the sum of occupancies of TPQ_{amr} and TPQ_{sq} is 1.0. **(c)** van't Hoff plots for the TPQ_{sq}/TPQ_{amr} equilibrium in solution (black) and crystal (red).

diffraction data of AGAO crystals anaerobically reduced with ethylamine at 4, 10, 15, and 20°C. Relative occupancies of each conformer provided K_{eq} values (equilibrium constant for TPQ_{sq}/TPQ_{amr}) that clearly indicate that the off-copper TPQ_{amr} shifts to the on-copper TPQ_{sq} as the temperature increases (Fig. 2(a,b)). Thermodynamic parameters obtained from a van't Hoff plot of K_{eq} values (Fig. 2(c)) for both crystalline ($\Delta H°$ = 38 kJ/mol, $\Delta S°$ = 139 J/mol/K) and solution ($\Delta H°$ = 26 kJ/mol, $\Delta S°$ = 83 J/mol/K) states indicated that the transition to TPQ_{sq} is entropy-driven in both crystal and solution states. The *in crystallo* increases of $\Delta H°$ and $\Delta S°$ can be ascribed to the packing effect in the crystal lattice and suggest the reason for an equilibrium shift of TPQ_{sq}/TPQ_{amr} that is directly linkied to structural changes. To clarify pH-dependent equilibrium changes, the structures of 2-phenylethylamine (2-PEA)-reduced crystals were determined at 15°C with various pHs under aerobic conditions. The $F_o - F_c$ omit maps for the TPQ moiety were considerably different depending on pH, indicating the formation of various intermediates (Fig. 3(a)). These differences suggest that the binding of the product aldehyde, phenylacetaldehyde (PAA), to the hydrophobic pocket facilitates the transition to TPQ_{sq} at high pH and the formation of TPQ_{psb} at low pH by the back reaction between TPQ_{amr} and PAA in the crystals. (Fig. 3(b)).

Our study revealed that the temperature-controlled HAG method has great advantages for detecting the equilibrium mixture of catalytic intermediates in protein crystals and will enable the elucidation of '*in crystallo*' thermodynamics of conformational changes. We expect that the temperature-controlled HAG method will provide an important addition to various X-ray crystallographic techniques currently being developed.

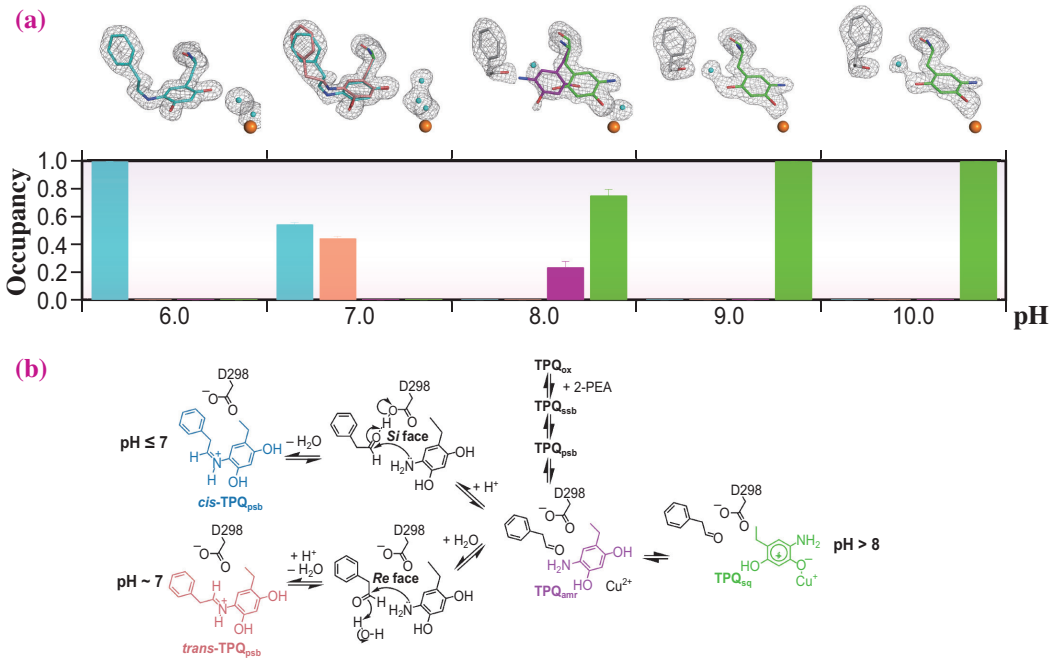

Fig. 3. pH dependence of equilibrium. **(a)** pH profile of 2-PEA-reduced AGAO crystal structures at 15°C at various pH determined by the temperature-controlled HAG method. The determined active-site structures are superimposed on the $F_o - F_c$ omit map (gray mesh) for residue 382 and the product aldehyde contoured at 3.5 σ. Residue 382 is represented by a stick model with the same color scheme as in the bar graph. Average occupancies of the intermediates are shown by bars with S.E. ($n \geq 4$): cyan, *cis*-TPQ_{psb}; brown, *trans*-TPQ_{psb}; purple, TPQ_{amr}; green, TPQ_{sq}. **(b)** Possible mechanism of pH-dependent equilibrium changes in the 2-PEA-reduced AGAO crystal.

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